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Aleksandra Nall

University of Pennsylvania, nall@mail.med.upenn.edu

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Uppers and Downers: Understanding Sleep Regulation Using Small Molecules in *Drosophila*

Abstract

Sleep is an important physiological state, but its function and regulation remain elusive. In *Drosophila melanogaster*, a useful model organism for studying sleep, forward genetic screens have identified important sleep-modulating genes and pathways; however, the results of such screens may be limited by developmental abnormalities or lethality associated with mutation of certain genes. To circumvent these limitations, we screened 1280 small molecules for effects on sleep in adult *Drosophila*. We used genetic and molecular approaches to elucidate the mechanisms by which two of these drugs altered sleep behavior.

We found that administration of reserpine, a small molecule inhibitor of the vesicular monoamine transporter (VMAT) that repackages monoamines into presynaptic vesicles, resulted in an increase in sleep. We found that VMAT-null mutants, like reserpine-fed flies, have an increased sleep phenotype, as well as an increased arousal threshold and resistance to the effects of reserpine. However, although the VMAT mutants are consistently resistant to reserpine, other aspects of their sleep phenotype are dependent on genetic background. Thus, they may not have been detected in a classical forward genetic screen, further attesting to the utility of a small molecule screen. Mutations affecting single monoamine pathways did not affect reserpine sensitivity, suggesting that effects of VMAT/reserpine on sleep are mediated by multiple monoamines.

We also studied the mode of action of caffeine, a common wake-promoting compound. Caffeine is thought to promote wake by inhibiting adenosine receptors, however previous work demonstrated that the wake-promoting effects of caffeine are independent of the adenosine receptor in the fly. We show that dopamine is required for the wake-promoting effect of caffeine in the fly, and that caffeine likely acts presynaptically to increase dopamine signaling. We identify a cluster of neurons, the paired anterior medial (PAM) cluster of dopaminergic neurons, which are essential for the caffeine response and which show increased activity following caffeine administration.

Overall, we find that small molecule screens can be used effectively to identify regulators of adult behavior. The results of our screen and follow-up experiments demonstrate that presynaptic modulation of monoamine signaling may be a major source of sleep regulation.

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UPPERS AND DOWNERS: UNDERSTANDING SLEEP
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Aleksandra Nall

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Supervisor of Dissertation

Amita Sehgal, Ph.D.

John Herr Musser Professor of Neuroscience

Graduate Group Chairperson

Daniel S. Kessler, Ph.D.

Associate Professor of Cell and Developmental Biology

Dissertation Committee

David Raizen, MD, Ph.D. , Assistant Professor of Neurology

Edwin Abel, Ph.D., Brush Family Professor of Biology

Julie Blendy, Ph.D., Professor of Pharmacology

Thomas Jongens, Ph.D., Associate Professor of Genetics

UPPERS AND DOWNERS: UNDERSTANDING SLEEP REGULATION USING SMALL
MOLECULES IN *DROSOPHILA*

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ABSTRACT

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Aleksandra Nall

Amita Sehgal, Ph.D.

Sleep is an important physiological state, but its function and regulation remain elusive. In *Drosophila melanogaster*, a useful model organism for studying sleep, forward genetic screens have identified important sleep-modulating genes and pathways; however, the results of such screens may be limited by developmental abnormalities or lethality associated with mutation of certain genes. To circumvent these limitations, we screened 1280 small molecules for effects on sleep in adult *Drosophila*. We used genetic and molecular approaches to elucidate the mechanisms by which two of these drugs altered sleep behavior.

We found that administration of reserpine, a small molecule inhibitor of the vesicular monoamine transporter (VMAT) that repackages monoamines into presynaptic vesicles, resulted in an increase in sleep. We found that VMAT-null mutants, like reserpine-fed flies, have an increased sleep phenotype, as well as an increased arousal threshold and resistance to the effects of reserpine. However, although the VMAT mutants are consistently resistant to reserpine, other aspects of their sleep phenotype are dependent on genetic background. Thus, they may not have been detected in a classical forward genetic screen, further attesting to the utility of a small molecule screen. Mutations affecting single monoamine pathways did not affect reserpine sensitivity, suggesting that effects of VMAT/reserpine on sleep are mediated by multiple monoamines.

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Overall, we find that small molecule screens can be used effectively to identify regulators of adult behavior. The results of our screen and follow-up experiments demonstrate that presynaptic modulation of monoamine signaling may be a major source of sleep regulation.

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INTRODUCTION

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Sleep is an important physiological state that has been observed in most well-studied animals. Many such animals have been used as models to study the genetic and molecular mechanisms underlying sleep regulation in an attempt to understand how and why we sleep. From all of these studies, it has become clear that sleep is controlled by the circadian clock and by a homeostatic mechanism (Borbély, 1982). The circadian clock communicates time-of-day information inferred from light and temperature cues to ensure that sleep occurs during the right times of day. The sleep homeostat keeps track of the duration of sleep and wake to ensure that daily sleep need is met.

Much of our understanding of circadian clock mechanisms has come from the fruit fly, *Drosophila melanogaster*. Clock genes and mechanisms discovered in *Drosophila* were found to be conserved in humans, underscoring how much we can learn about ourselves from these tiny insects (Cirelli, 2009; Crocker & Sehgal, 2010; Sehgal & Mignot, 2011). A *Drosophila* model for the homeostatic regulation of sleep has been established to exploit the genetic tractability and ease of high-throughput behavioral testing that these animals provide (Hendricks et al., 2000; Shaw et al., 2000). *Drosophila* sleep shares many similarities with human sleep, including diurnal distribution of sleep/wake activity, a homeostatic reaction to sleep deprivation, and an increased arousal threshold (i.e., reduced responsiveness to sensory stimulation) during sleep. It follows, then, that these behavioral states are likely regulated similarly, and the fly will be an invaluable tool in understanding our own drive to sleep.

Like other behaviors, including learning, courtship, aggression, and social behaviors, sleep is modulated by experience and environment in addition to being driven by intrinsic mechanisms. Signals in the brain integrate the various inputs to generate a coordinated output that governs successful behavior. These signals take the form of hormones, neuropeptides,

neurotransmitters, and neuromodulators, which can respond to external and physiological cues. Given that sleep involves widespread changes in brain activity, it is easy to see how modulators of neural activity are important in its regulation. For example, one of the canonical characteristics of sleep is an increased arousal threshold, which refers to lack of a response to a stimulus that elicits a response in an awake animal. One way that circuit-wide changes in neuronal excitability can be achieved is by neuromodulation, in which biogenic amines and other neuromodulators diffuse through the brain and affect general brain activity. My thesis work has demonstrated that two drugs that alter sleep behavior do so by modulating the signaling of one class of neuromodulators, monoamines. Therefore, this introduction focuses on what we currently know about the regulation of sleep and circadian rhythms by monoamine signaling, with a focus on knowledge gained from the *Drosophila* model.

Sleep and Monoamines

Neuromodulators involved in fine-tuning neuronal excitability, as well as direct synaptic transmission, are the monoamines. This class of neuromodulators includes dopamine, serotonin, norepinephrine and its invertebrate analog octopamine, histamine, and the trace amine tyramine. The most abundant monoamines in the *Drosophila* nervous system are dopamine, octopamine, and serotonin. These monoamines have conserved biosynthetic pathways (Livingstone & Tempel, 1983) and conserved effects on many behaviors. Recently, all of these monoamines have also been found to regulate sleep behavior at the circadian and homeostatic level. Dopamine, octopamine, and serotonin function in disparate and overlapping anatomical brain regions to integrate environmental information (e.g., light, social cues, and food abundance) to ensure appropriate timing and quantity of sleep. In addition, histamine acts in *Drosophila* to inhibit sleep, much as it is known to do in mammals.

Dopamine

One of the earliest sleep mutants identified in the *Drosophila* model implicated dopamine signaling in sleep regulation. This mutant, called *fumin*, sleeps far less than wild-type flies despite having normal waking activity and circadian rhythms (Kume et al., 2005). *fumin* flies contain a

defective copy of the dopamine transporter (DAT), which results in an augmentation of dopamine signaling presumably from retention of dopamine in the synaptic cleft. A forward genetic screen for short-sleeping mutants identified a different mutant allele of DAT, which causes a similar phenotype to *fumin* (Wu et al., 2008). This finding supports shared mechanisms of sleep/wake regulation across species, as previous studies found that DAT mutant mice experience shorter, more fragmented sleep and increased time awake (Wisor et al., 2001). DAT is also the molecular target of arousal-promoting drugs such as methamphetamine and cocaine (Eshleman et al., 1994; Kilty et al., 1991). Agonists of dopamine receptors also promote wake (Monti and Monti, 2007). An increase in extracellular dopamine is associated with natural waking states in mice, and a single population of dopaminergic neurons in the mouse ventral periaqueductal gray matter shows increased activity during periods of wakefulness (Feenstra et al., 2000; Léna et al., 2005; Lu et al., 2006).

Additional studies have corroborated the wake-promoting effect of dopamine signaling in *Drosophila*. Drugs that increase dopamine signaling (e.g., methamphetamine and cocaine) decrease sleep, and drugs that decrease dopamine signaling (e.g., the tyrosine hydroxylase [TH] inhibitor 3IY) increase sleep (Andretic et al., 2005). Mutating TH in the central nervous system, which prevents dopamine synthesis altogether, also causes a dramatic increase in sleep (Riemensperger et al., 2011). These flies have an increased arousal threshold, which means that they are less able to wake up in response to stimuli. This points to a role for dopamine in promoting an awake and attentive brain state, which could be due to the general neuromodulatory action of dopamine in increasing neuronal excitability in wake-promoting brain regions. Reducing synaptic transmission from dopaminergic neurons reduces activity in the fly brain in response to visual stimulation (Andretic et al., 2005), which supports a role for dopamine in maintaining arousal and attention.

In addition to *fumin*, other low-sleeping mutants identified in *Drosophila* have been linked to dopamine signaling. Mutations in the BTB-domain-containing protein insomniac (Inc) and its associated E3-ubiquitin ligase Cullin-3 (Cul3) were recently both shown to decrease sleep

(Stavropoulos & Young, 2011). Inc and Cul3 were also identified as wake-promoting genes in a reverse genetic screen performed by Pfeiffenberger and Allada (2012), which targeted genes with either sleep- or wake-dependent expression or with essential roles in neuronal function. This study showed that normal amounts of sleep could be induced in Cul-3 and Inc mutants by reducing dopamine levels with TH inhibitors 3-iodotyrosine and alpha-methyl-p-tyrosine.

Supporting the hypothesis of increased dopamine signaling in these mutants, they are resistant to the additional wake-promoting effects of increased dopamine synthesis produced by L-DOPA feeding. However, the link to dopaminergic signaling seems to be non-cell-autonomous because Inc and Cul3 are

required in cholinergic cells, not dopaminergic cells, for normal sleep behavior (see Figure 1; Pfeiffenberger & Allada, 2012). This finding underscores the importance of taking a circuit-wide view of sleep regulation because many different neurons and signaling systems are likely contributing to this complex behavior.

Effects of dopamine on sleep and circadian behavior are mediated in different structures of the *Drosophila* brain (see Figure 1). On a molecular level, dopamine acts through specific

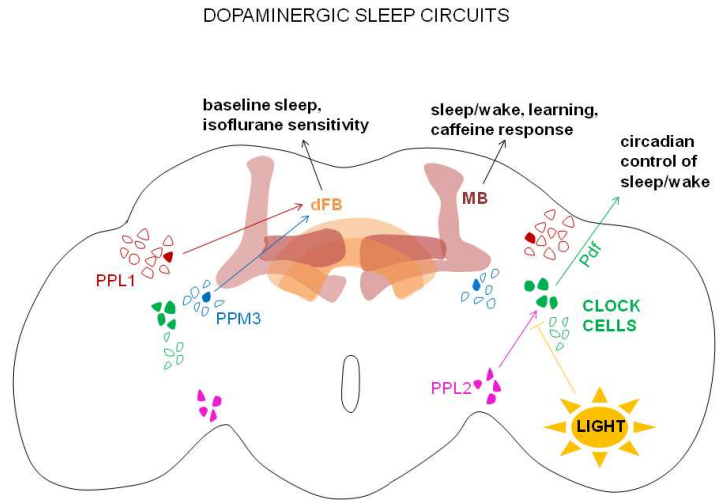


Figure 1 – Dopaminergic sleep circuits

Multiple dopaminergic cell groups projecting to different anatomical structures have been implicated in the regulation of sleep. Individual PPL1 (red) and PPM3 (blue) neurons projecting to the FB control baseline sleep and isoflurane sensitivity. Dopamine receptors in the MB are required for decreased sleep after caffeine feeding as well as the interaction of sleep with learning and memory. Dopamine receptors in the ILNvs are sufficient to confer normal sleep amount and may be involved in the circadian modulation of dopamine receptor sensitivity and the inhibition of dopaminergic arousal cues by light (yellow). The PPL2 dopaminergic cluster (magenta) projects to the ILNvs.

receptors, which are coupled to different intracellular signaling cascades. In *Drosophila*, there are two type I excitatory dopamine receptors (dDopR and dDopR2), which are coupled to G_s and activate adenylate cyclase, and one inhibitory dopamine receptor (dD2R), which couples to G_i and inhibits adenylate cyclase (Gotzes et al., 1994; Han et al., 1996; Hearn et al., 2002). Despite their categorization as type I and type II, these receptors may not always conform to their predicted excitatory or inhibitory activity. Knockdown of dD2R causes decreased locomotor activity (Draper et al., 2007), and feeding with a dD2R-specific agonist increases locomotor activity (Lee et al., 2013). That a receptor categorized as inhibitory promotes dopamine-dependent locomotor activity is surprising; however, it is possible that dD2R acts as an excitatory receptor in this context or that it disinhibits excitatory neurons controlling locomotion.

Although dD2R regulates locomotor activity, the sleep-relevant dopamine receptor seems to be dDopR. Null mutation of this receptor causes increased sleep, with longer sleep bouts, in addition to hypoactivity (Lebestky et al., 2009). It is interesting to note that dDopR mutants are also resistant to the effects of the wake-promoting drug caffeine (Andretic et al., 2008). The caffeine response phenotype of these mutants can be rescued by restoring dDopR to the mushroom bodies (MBs), a region of the fly brain involved in learning and memory (see Figure 1; Andretic et al., 2008; Kahsai & Zars, 2011). Previous studies have also demonstrated a role for the MBs in sleep regulation; ablating the MBs in their entirety causes a sleep decrease, as does silencing a subset of MB neurons (Pitman, 2006). However, promoting activation of a different, non-overlapping group of neurons in the MB also causes a sleep decrease, indicating that the MBs likely contain both sleep-promoting and wake-promoting cell groups (Joiner et al., 2006). Determining the sleep-relevant function of dDopR in these distinct MB cell groups will help to elucidate the processing of dopaminergic inputs to this region.

Although the MBs are involved in sleep regulation and express dopamine receptors that may modulate the response to caffeine, dopaminergic circuits establishing baseline sleep behavior have mapped elsewhere. The high sleep phenotype of dDopR mutants cannot be reversed by restoring receptor expression to MBs (Lebestky et al., 2009). Two recent studies

identified specific dopaminergic neurons, the activation of which is sufficient to induce wakefulness (Liu et al., 2012; Ueno et al., 2012). Despite disagreement on the exact anatomical location of the implicated cell bodies, both studies demonstrated the sufficiency of a single pair of dopaminergic neurons to promote arousal via projections to the dorsal fan-shaped body (FB; see Figure 1).

The FB is a sleep-promoting region that causes increased sleep when activated (Donlea et al., 2011). Because dopaminergic neurons promote wake, it follows that they likely suppress the activity of the FB. Indeed, the FB is responsive to sleep-suppressing dopamine signals in a dDopR-dependent manner (Liu et al., 2012; Ueno et al., 2012). It is surprising that the suppression of sleep-promoting signals from the FB is dependent on a type I dopamine receptor, which is typically thought of as excitatory. It has been suggested that dDopR has an inhibitory action in these cells in this context. However, it is also possible that the FB, like the MB, contains sleep- and wake-promoting neurons, and the relevant dopaminergic projections synapse onto wake-promoting cells. It is interesting to note that this dopaminergic sleep circuit is targeted by the common volatile anesthetic isoflurane, which induces a sleep-like state. Flies with increased dopamine signaling are resistant to isoflurane whereas flies with reduced dopamine signaling are hypersensitive, and the ability of dopamine to suppress the activity of isoflurane activity maps to the FB (see Figure 1; Kottler et al., 2013). It makes sense that effective and safe anesthetics should target the brain's natural sleep circuits to create a sleep-like unconscious state.

The high sleep phenotype of dDopR mutants is rescued by expression of dDopR in the FB and peptidergic neurons, as demonstrated by Liu et al. (2012) and Ueno et al. (2012), but it can apparently also be rescued by restoring dDopR expression in circadian clock neurons (Lebestky et al., 2009). Pigment dispersing factor (PDF) is a neuropeptide expressed in the small and large ventral lateral neurons (sLNvs and lLNvs, respectively), which are regarded as the central clock; although of these, it is only the sLNvs that are critical for self-sustained circadian behavior. The lLNvs are wake-promoting cells; they express dDopR and therefore may be subject to dopaminergic modulation (Kula-Eversole et al., 2010). Indeed, GRASP analysis (green

fluorescent protein [GFP] reconstitution across synaptic partners) has shown that projections from the protocerebral posterior lateral 2 (PPL2) cluster of dopaminergic neurons contact clock cell dendrites, and these connections are likely functional because ILNvs increase intracellular calcium in response to direct dopamine application (see Figure 1; Shang et al., 2011; Wegener et al., 2004). How the dDopR mutant phenotype can be rescued by expression in either the FB neurons or clock cells is not clear yet; it is possible that these neurons converge on shared downstream targets, and therefore correction of the circuit upstream can occur at one or the other location.

Clock cell involvement in sleep and arousal circuits is important to integrate environmental cues and cellular and molecular context to coordinate sleep and wake behavior at appropriate times and in appropriate situations. The ILNvs promote wake in the light phase (Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008) whereas dopamine only promotes wake during dark conditions (i.e., at night in cycling light/dark conditions; Kumar et al., 2012). Light is able to suppress dopamine's wake-promoting effects by upregulating inhibitory dopamine receptors (dD2R) in the ILNvs (see Figure 1; Shang et al., 2011). dD2Rs are also regulated in a circadian manner in the peripheral control of locomotion. In the peripheral nervous system and neuromuscular junction, the sensitivity of these receptors cycles across the circadian day. The cycling of receptor sensitivity is controlled by light-sensitive body clocks, which are entrained to light cues by the photosensitive clock protein cryptochrome (CRY; Andretic & Hirsh, 2000). dD2R agonist-induced hyperactivity is highest in the dark phase and low in the light phase, which is consistent with the other studies showing that dopaminergic signaling is repressed by light. This study is also consistent with previous findings that the dD2R paradoxically promotes hyperactivity despite belonging to the canonically inhibitory class of type II dopamine receptors (Lee et al., 2013).

In the absence of the circadian clock gene *Clock* (*Clk*), dopamine signaling is increased, which results in increased arousal at night, thereby producing nocturnal behavior. The nocturnal behavior of *Clk* mutants can be suppressed by silencing dopaminergic inputs to the ILNvs or by

pharmacologically antagonizing dopamine receptors (Kumar et al., 2012). Nighttime activity in these mutants also depends on increased expression of the circadian photoreceptor CRY in the central clock cells in the brain, suggesting links between dopamine signaling and CRY. Daytime light inhibits wake-promoting dopaminergic signaling, but dopamine is essential for the behavioral response to dim light. Mutants lacking dopamine in the nervous system are defective in circadian entrainment and phase shifts triggered by low-intensity light cues (Hirsh et al., 2010).

Dopaminergic modulation of clock cells, and possibly CRY levels, offers some insight into how it plays a role in entrainment, but more work must be done to understand the specificity of low-light sensitivity. In addition, understanding the role of the dopaminergic arousal cues during the nighttime hours and determining sleep-relevant clock outputs will help us understand the plastic and interconnected circuits that allow for normal cycles of sleep/wake behavior in wild-type flies. This multiplicity of wake-promoting signals to the clock that can trigger suppression of other signals is an excellent example of how sleep circuits can receive and prioritize multiple contextual inputs to result in the most advantageous behavior in a particular situation.

In summary, dopaminergic cell groups project to different anatomical regions of the *Drosophila* brain to modulate arousal in response to various cues. Having several parallel neuromodulatory circuits that each communicates different aspects of environmental and physiological context to sleep centers in the brain provides flexibility in adapting behavior to a changing environment. The ability to prioritize conflicting environmental cues by strengthening one circuit and suppressing others may be an evolutionary advantage to having multiple sites of neuromodulatory input.

Octopamine

Octopamine in invertebrates is an analog of adrenergic neurotransmitters in vertebrates. It acts similarly to epinephrine and norepinephrine and controls some of the same behaviors, including memory formation and aggression (Roeder, 2005; Sara, 2009; Yanowitch & Coccaro, 2011). Norepinephrine has been shown to regulate mammalian sleep (Aston-Jones & Bloom,

1981), and we now know that octopamine regulates sleep in *Drosophila*. Decreasing octopamine levels by mutating biosynthetic enzymes causes an increase in total sleep amount as well as changes in other indicators of increased sleep drive such as increased arousal threshold and decreased latency to sleep (Crocker & Sehgal, 2008). These phenotypes can be rescued by pharmacologic administration of octopamine. Electrically exciting or silencing octopaminergic neurons causes decreased or increased sleep, respectively. Ectopically expressing a sodium channel to excite only the anterior superior medial (ASM) cluster of octopaminergic neurons decreases sleep, identifying these as the sleep-relevant octopaminergic cells (Crocker et al., 2010). The adrenergic antagonist mianserin blocks the wake-promoting effect of octopamine, underscoring that these systems are homologous (Crocker & Sehgal, 2008).

The sleep-promoting effect of octopamine requires protein kinase A (PKA) signaling (Crocker & Sehgal, 2008). It is interesting to note that PKA is a signaling molecule already known to play a role in sleep regulation, especially in the MBs (Joiner et al., 2006). In mapping the sleep-relevant neuronal targets of octopamine signaling, Crocker et al. (2010) discovered that inhibiting PKA in the pars intercerebralis (PI)—not in the MBs—was able to block the wake-promoting effects of octopamine (see Figure 2). The PI is thought to be the *Drosophila* equivalent of the hypothalamus based on similarities of development and function, as well as expression of similar neuropeptides (de Velasco et al., 2007). It consists of a collection of neurosecretory cells in the far dorsomedial area of the brain with pronounced ventral projections. These cells express multiple octopamine receptors, one of which, octopamine receptor in the mushroom body (OAMB), is required for octopaminergic control of sleep.

The PI cells downstream of octopamine in the sleep circuit are marked by Dilp2-Gal4, which is expressed in insulin-producing cells. Despite insulin production being the major function of these cells, this does not seem to be the mechanism by which octopamine modulates sleep. Increasing or decreasing insulin signaling does not itself alter sleep behavior. On the other hand, increasing octopamine signaling does increase triglycerides—a measure of metabolism and energy storage—and this connection is mediated by insulin signaling. Although the control by

octopamine of sleep and metabolism seems to be unrelated, it is true that metabolic state can affect sleep behavior. For example, in periods of starvation, flies with low octopamine signaling will overcome their sleep drive to forage, and flies with high octopamine signaling will not increase foraging activity despite being more generally active. This is likely because flies with low octopamine have low energy stores and cannot withstand lack of food supply whereas

those with high octopamine have nutrient reserves and therefore have less need to forage (Erion et al., 2012).

Thus, in either scenario, metabolic needs dominate over sleep need. The mechanism by which octopamine modulates baseline

sleep behavior through the PI is still unknown, but the interplay and prioritization of arousal and metabolic signals through this brain region is another example of how numerous inputs can allow for behavioral plasticity on the basis of context.

Octopamine not only modulates sleep through the PI, but it may also communicate wake-promoting signals to the ILNvs, as does dopamine (see Figure 2). The ILNvs express octopamine

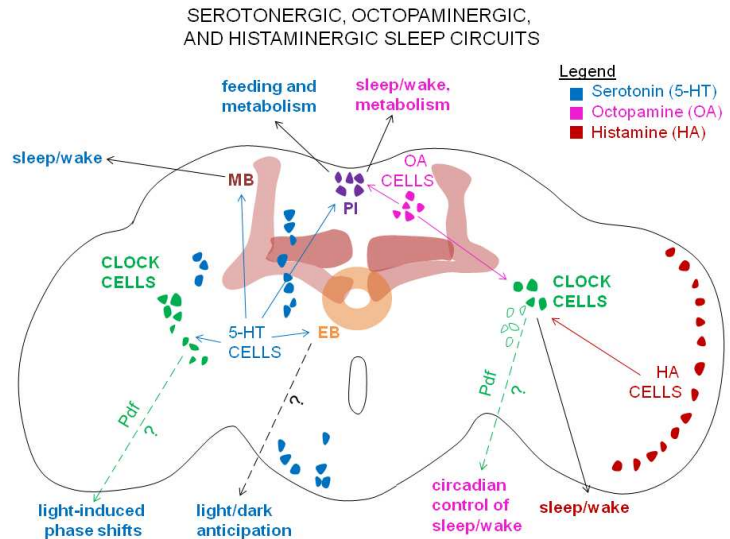


Figure 2 – Serotonergic, histaminergic, and octopaminergic sleep circuits

Serotonergic cells (blue) project to many brain regions, including the MB, where they promote sleep through the d5-HT1A receptor. They project to the PI, where they promote sleep and feeding. They also project to the ellipsoid body, where they may participate in some elements of circadian-dependent behavior through the d5-HT2 receptor. The 5HT-1B receptor inhibits sensitivity to light-induced phase shifts and may act through the LNvs to mediate this aspect of circadian entrainment. Octopaminergic neurons (magenta), specifically the ASM cluster, promote wake via their projections to the PI. They also signal to the ILNvs, which respond to octopamine specifically during the night phase of the circadian day. In addition, histaminergic neurons (red) seem to promote wakefulness via HisC11 receptors in the PDF clock neurons.

receptors and respond to octopamine application by increasing cyclic adenosine monophosphate (cAMP). The sensitivity of clock cells to octopamine is dependent on time of day: In light/dark conditions, ILNvs respond to octopamine in the dark phase, and in constant dark conditions octopamine sensitivity is high only during the subjective night. In addition, when the clock gene *period (per)* is mutated, octopamine sensitivity of the clock neurons decreases significantly (Shang et al., 2011). Therefore, the wake-promoting effects of octopamine are dependent on circadian and metabolic cues and may depend on other endogenous or environmental conditions. Octopaminergic cells also project to other brain loci previously reported to be involved in sleep regulation, such as the FB and the MBs; however, it is currently unknown whether these inputs can modulate sleep in different contexts (Busch et al., 2009).

Serotonin

Similar to dopamine and octopamine, serotonin is involved in modulating many behaviors, including learning, mating, and aggression, and it has been implicated in sleep and circadian behaviors (Becnel et al., 2011; Dierick & Greenspan, 2007; Sitaraman et al., 2008). In 2006, Yuan and colleagues tested for sleep phenotype in mutants of three different serotonin receptors and discovered that receptor d5-HT1A promotes sleep. Flies expressing a mutated form of the receptor showed reduced and fragmented sleep. In addition, these flies were unable to get wild-type levels of rebound sleep after a period of sleep deprivation. Pharmacological and genetic augmentation of serotonin production increases sleep, confirming that serotonin is sleep-promoting (Yuan, Joiner, & Sehgal, 2006).

Serotonergic neurons project to multiple brain regions that have been previously identified as part of sleep-regulatory circuitry, including the PI and the MB (Lee et al., 2011; Luo et al., 2012; Pech et al., 2013). In the d5-HT1A mutant flies, wild-type sleep levels can be restored by expressing d5-HT1A only in the MB, indicating that this is the structure that receives sleep-relevant serotonergic inputs (see Figure 2; Yuan et al., 2006). On the other hand, serotonin signaling to the PI seems to modulate feeding and metabolism (Luo et al., 2012).

In addition to regulating baseline sleep levels and homeostatic response to sleep deprivation, serotonin is also involved in modulating circadian control of sleep and activity. The serotonin receptor d5-HT2, which is expressed in the protocerebrum and ellipsoid body, may modulate circadian behavior. Flies fed a d5-HT2 agonist display increased activity in the early daytime and lose the anticipatory behavior that precedes light/dark transitions (Nichols, 2007). This anticipatory behavior is a typical sign of circadian clock entrainment, and loss of anticipation can indicate reduced rhythmicity or impaired entrainment. Increasing serotonin signaling in flies, either by feeding serotonin or serotonin reuptake inhibitors, decreases their sensitivity to light-induced phase shifts, which is another indicator of weakened circadian entrainment. This effect on phase shift sensitivity seems to act through the d5-HT1B receptor because overexpressing or knocking down this receptor decreases or increases circadian sensitivity to light pulses, respectively. d5-HT1B is expressed in clock cells in the brain, where it likely acts to modulate circadian entrainment (see Figure 2; Yuan et al., 2005). However, it is also expressed in the MBs and PI, where it could potentially modulate the sleep circuit on the basis of light and circadian cues.

Histamine

Histamine is a monoamine neurotransmitter with a well-established role in *Drosophila* photoreception and temperature sensing (Hong et al., 2006; Witte et al., 2002). However, until very recently, it was only known to regulate sleep and wake in mammalian systems (Parmentier et al., 2002). A wake-promoting role for histamine has now been demonstrated in *Drosophila* by knocking down histamine biosynthetic pathways and demonstrating an increase in sleep (Oh et al., 2013). In addition, mutation of a single histamine receptor, HisC11, causes a similar increase in sleep. It is interesting to note that this histaminergic sleep regulation maps to the clock neurons because expression of HisC11 in PDF⁺ cells is necessary and sufficient for normal sleep behavior (see Figure 2; Oh et al., 2013). An earlier anatomical study identified histaminergic projections from extraocular eyelet photoreceptors to the ventral lateral neurons, which could be the wake-promoting histaminergic circuit (Hamasaka & Nässel, 2006). Thus, histaminergic

neuromodulation may provide a novel mechanism for communicating light cues from the visual system to the central clock for regulation of behavior. The ability of the circadian clock to drive robust rhythmic locomotor behavior, even in the absence of light cues, would seem to preclude the necessity for this type of neuromodulatory input. However, it is possible that the wake-promoting cues from the ILNvs (as opposed to the circadian cues from the sLNvs) are more plastic and responsive to slight modulation. More research must be done to establish the role for histamine in controlling sleep/wake behavior and the type of environmental information it may be conveying.

Each of the major monoamine neurotransmitters discussed regulates broad and overlapping lists of complex behaviors, including sleep. Sleep regulation has been attributed to many different signaling pathways, brain structures, and circuits, but we still lack a cohesive understanding of how this essential behavior is established. We undertook a small molecule screen to determine whether modulating behavior specifically in adulthood would uncover new information that has been missed in genetic screens. Further work investigating two drugs from this screen found that both affect sleep/wake balance by presynaptically modulating monoamine signaling. This body of work underscores the power of pharmacological screens and utilizes a sleep-promoting and a wake-promoting drug to uncover novel presynaptic controls of sleep behavior.

CHAPTER 1 – Small-molecule screen in adult *Drosophila* identifies VMAT as a regulator of sleep

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ABSTRACT

Sleep is an important physiological state, but its function and regulation remain elusive. In *Drosophila melanogaster*, a useful model organism for studying sleep, forward genetic screens have identified important sleep-modulating genes and pathways; however, the results of such screens may be limited by developmental abnormalities or lethality associated with mutation of certain genes. To circumvent these limitations, we used a small-molecule screen to identify sleep-modulating genes and pathways. We administered 1280 pharmacologically active small molecules to adult flies and monitored their sleep. We found that administration of reserpine, a small molecule inhibitor of the vesicular monoamine transporter (VMAT) that repackages monoamines into presynaptic vesicles, resulted in an increase in sleep. Supporting the idea that VMAT is the sleep-relevant target of reserpine, we found that VMAT-null mutants have an increased sleep phenotype, as well as an increased arousal threshold and resistance to the effects of reserpine. However, although the VMAT mutants are consistently resistant to reserpine, other aspects of their sleep phenotype are dependent on genetic background.

These findings indicate that small-molecule screens can be used effectively to identify sleep-modulating genes whose phenotypes may be suppressed in traditional genetic screens. Mutations affecting single monoamine pathways did not affect reserpine sensitivity, suggesting that effects of VMAT/reserpine on sleep are mediated by multiple monoamines. Overall, we identify VMAT as an important regulator of sleep in *Drosophila* and demonstrate that small-molecule screens provide an effective approach to identify genes and pathways that impact adult *Drosophila* behavior.

INTRODUCTION

Sleep is an important physiological state, as evidenced by the fact that we spend a third of our lives in this state. Additionally, sleep deprivation causes cognitive and health deficits, indicating that it plays an essential role in physiological homeostasis. Sleep is not just required in humans; all well-studied animals exhibit sleep-like states. Among these animals are common model organisms, including mice, zebrafish, flies, and nematode worms (Allada and Siegel, 2008; Mackiewicz et al., 2008; Raizen et al., 2008; Bushey and Cirelli, 2011). These models and others are being used to investigate outstanding questions regarding the purpose of sleep and its regulation.

The rest state in the fly shares many commonalities with human sleep behavior (Shaw et al., 2000, Hendricks et al., 2000). For example, sleeping flies stop moving and assume a stereotyped posture. They also exhibit an increased arousal threshold, meaning that they require a stronger stimulus to reinitiate activity. Importantly, flies display a homeostatic need for sleep, such that they compensate for periods of sleep deprivation with subsequent rebound. *Drosophila melanogaster* follow a diurnal pattern, resting mostly during the night and taking a mid-afternoon “siesta.”

The simplicity of behavioral assays using *Drosophila*, combined with the ease of genetic screens, has led many researchers to turn to this model to elucidate the genetic and molecular mechanisms underlying sleep regulation. Genetic screens have uncovered a few low-sleeping mutants, but these studies have not yet led to a cohesive account of sleep regulation. Given that sleep appears to be an essential process (Rechtschaffen et al., 1983; Shaw et al., 2009), it is likely that many sleep-regulating genes are also essential. In other words, loss of these genes may cause lethality or gross developmental problems, precluding their detection in traditional genetic screens. To complement previous genetic screens and to find novel sleep-regulatory molecules and pathways, we conducted a small-molecule screen for sleep phenotypes in adult *Drosophila*. Here, we report the findings from this screen, which indicate a strong effect of

monoaminergic neurotransmission in regulating sleep quantity. Using both pharmacological and genetic approaches, we investigated the role of one of our hits in regulating sleep behavior.

MATERIALS AND METHODS

Flies

Wild-type iso31 flies (Ryder et al., 2004) were used for drug screen and subsequent experiments. *VMAT^{D1}* mutants were a kind gift of the Krantz laboratory (University of California–Los Angeles, Los Angeles). *TrH^{co1440}* (BSC10531), *Hdc^{MB07212}* (BSC25260) mutants were ordered from the Bloomington Stock Center (Bloomington, Indiana), and *Gad1^{f00602}* was ordered from the Exelixis collection at Harvard Medical School. The temperature-sensitive tyrosine hydroxylase mutant *ple^{ts}* (Pendleton et al., 2002) was a kind gift from Dr. Ralph Hillman (New York University, New York). The octopamine synthesis mutant *TbH^{nm18}* was previously published (Crocker and Sehgal, 2008).

Drug feeding

We used the LOPAC 1280 drug library (Sigma-Aldrich), which is made up of bioactive molecules with known molecular targets, of which approximately half are involved in neurotransmission. Four- to 6-day-old adult isogenic (iso31) flies were given access to drugs at 20 μ M, mixed into their 2% agar and 5% sucrose food, *ad libitum* for 1 week. Flies were kept in incubators at 25°C on a 12 h light/dark schedule. During this time, locomotor activity of flies was monitored using the Drosophila Activity Monitoring System (Trikinetics). Sleep behavior was calculated and averaged for four male flies and four female flies per drug treatment. Sleep graphs and calculations of sleep quantity for all experiments were generated using PySolo (Gilestro and Cirelli, 2009). For drugs that produced qualitative changes in sleep profile or quantitative changes in total minutes of sleep per day, four male and four female flies were tested again at 50 μ M drug. Drugs that showed a reproducible, dose-dependent effect on sleep quantity were considered screen hits. For reserpine (Sigma-Aldrich), the stock solution was made at 10 mM in DMSO. This stock solution was diluted

in 2% agar/5% sucrose food to final concentrations of 20 and 50 μM for the original screen and 10 μM for all subsequent experiments. A total of 0.2% DMSO vehicle controls were used as a comparison for 20 μM drug feeding during the screen, and 0.1% DMSO vehicle controls were used as a comparison for 10 μM reserpine feeding in subsequent experiments. For the *ple^{ts}* mutants, flies were kept at a restrictive temperature of 29°C for 24 h before placing on DMSO and reserpine and were kept at this temperature for the duration of behavioral monitoring.

Arousal threshold

Arousal threshold assay was conducted as previously published (Wu et al., 2008). Mechanical stimuli were applied manually by tapping a dowel on the behavior tubes containing the flies. Weak (one light tap), medium (one strong tap), and strong (six strong taps) stimuli were applied to behavior tubes at ZT16, ZT18, and ZT20, respectively. The percentage of spontaneously sleeping flies awoken was calculated for each genotype and stimulus.

Sleep deprivation

Flies were deprived during the final 6 h of the night (ZT18-ZT24) using a vortex to shake flies for 2 s of every 20 s, at random intervals (Huber et al., 2004). Amount of sleep lost was calculated by subtracting minutes of sleep during deprivation from the minutes of sleep during the same interval on the previous night. Sleep regained the following morning was calculated by subtracting the minutes of sleep during the first 3, 6, or 12 h the morning before deprivation from the same interval after deprivation.

PCR

The wild-type vesicular monoamine transporter (VMAT) allele was genotyped using VMATp1-F (5'-ATC GGG GGA TGC TTG ATA TT-3') and VMATp1-R (5'-ATC CGA ATC GGG AAC AGA T-3') primers, and the mutant VMAT^{P1} allele was genotyped using the Plac1 (5'-CAC CCA AGG CTC TGC TCC CAC AA-3') primer and VMATp1-R primers. PCR was conducted with GoTaq

Flexi (Promega), with the following cycling conditions: 95°C for 2 min, then 30 cycles of 95°C for 30 s, 52°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min.

Sleep latency

Latency to sleep was calculated by counting the number of minutes between lights off and the first stretch of 5 consecutive minutes with zero beam crosses, as recorded by the Drosophila Activity Monitoring System.

RESULTS

A small-molecule screen identifies sleep-modulating compounds

We screened 1280 small molecules for their effect on sleep:wake rhythms in the adult fly (Fig. 1.1). Observation of daily locomotor behavior allowed for quantitative comparisons of total sleep time, daytime and nighttime sleep, and qualitative assessment of sleep patterns, rhythm strength, and anticipation of light/dark transitions. Each drug was assayed in a limited number of flies to enhance throughput; therefore, only drugs with strong effects on sleep were identified as having an effect above individual variation. Additionally, all compounds were fed to flies at a relatively low dose that caused minimal lethality. Many of these drugs showed an effect in only one sex and were discarded from further testing. Even with these constraints, we were able to identify 38 compounds that affected sleep at the initial concentration. Only those drugs found to have a dose-dependent effect on sleep in both sexes when tested at a higher concentration were considered hits. With these stringent criteria, we initially found six compounds that qualified: five that decreased sleep and one that increased sleep. One of the sleep-reducing compounds, the cholinergic agonist carbachol, did not continue to have an effect in subsequent studies (data not shown). The four remaining sleep-reducing drugs caused a significant reproducible decrease in nighttime sleep at a 20 μ M concentration and a further reduction in sleep levels at 50 μ M. These sleep-promoting drugs are pergolide methanesulfonate (Fig. 1.2a), R(-)-2,10,11-

trihydroxyaporphine hydrobromide (Fig. 1.2*b*), paliperidone (Fig. 1.2*c*), and 1,3-dipropyl-7-methylxanthine (Fig. 1.2*d*). The screen revealed a single sleep-promoting drug, reserpine (Fig. 1.2*e*). Reserpine caused a significant increase in sleep during both the day and night, especially at light/dark transitions when flies are most active ($p=0.000161$ by one-way ANOVA with Tukey *post hoc* comparison for both the 20 μM and 50 μM reserpine-fed flies compared with DMSO controls). In female flies, sleep increased by 400 min at 20 μM reserpine and by 470 min at 50 μM reserpine (Fig. 1.3*a*). Male flies showed a similar behavioral response to drug treatment.

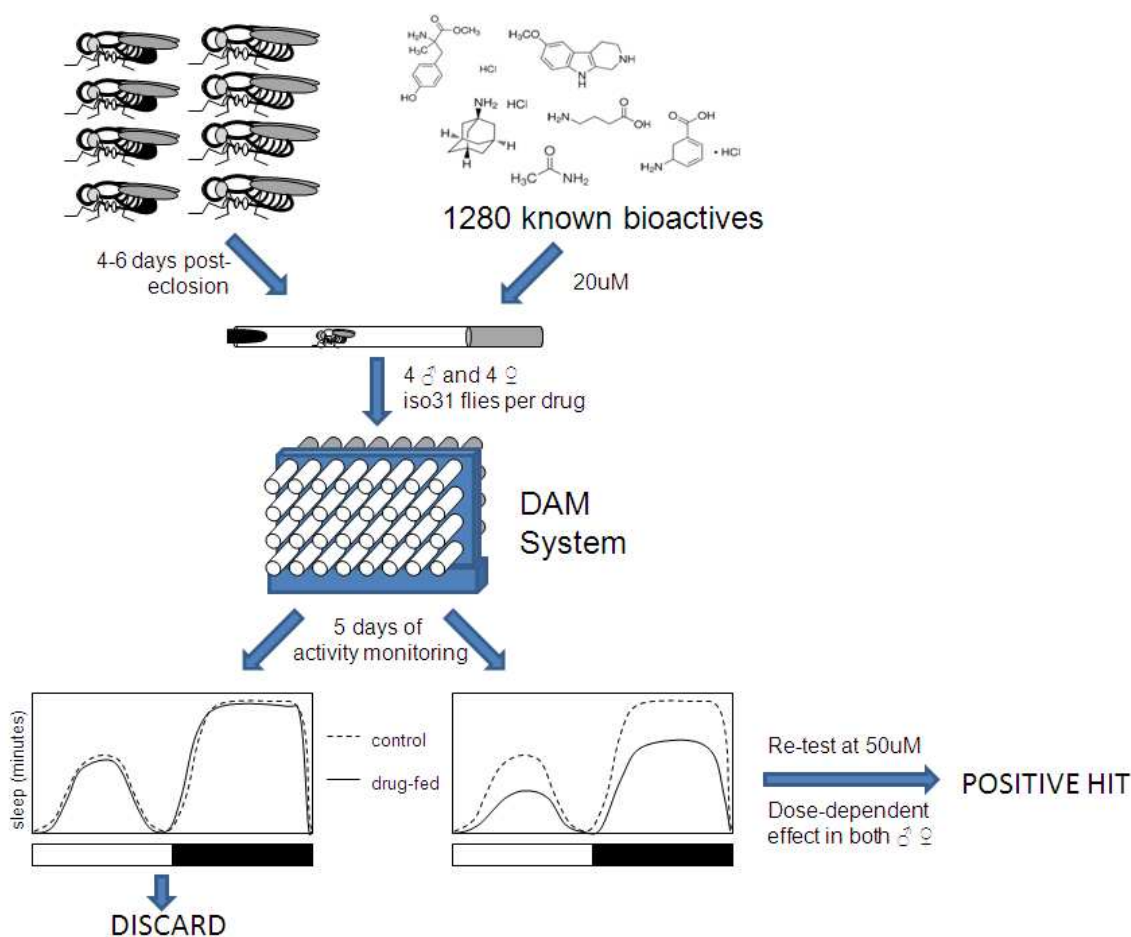


Figure 1.1 – Schematic of the small molecule screen

4 male and 4 female flies were screened for each of 1,280 known bioactive drugs. At 4-6 days post eclosion, adult flies were put in tubes with food containing 20 μM of drug. These tubes were placed in monitors, and locomotor activity was measured for 5 days using the *Drosophila* Activity Monitoring (DAM) System. Using pySolo, sleep profiles were generated for males and females for each drug and compared to flies fed 0.01% DMSO (control). If a drug altered sleep in both males and females, it was re-tested at 50 μM . If the drug had a dose-dependent effect, it was considered a positive hit.

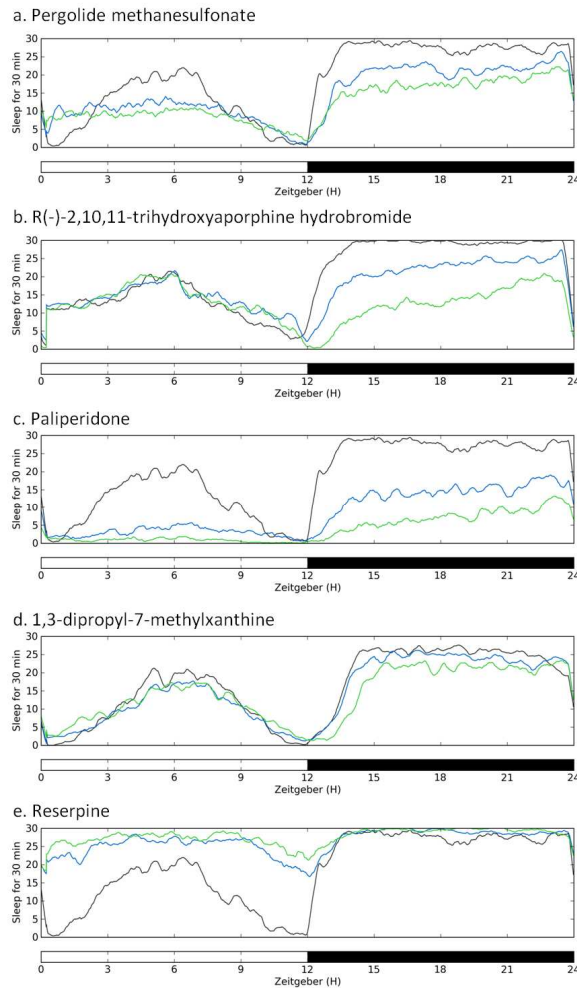


Figure 1.2 - Small molecule screen identifies sleep-modulating compounds

Sleep profiles for female flies fed 0.1% DMSO (black line), 20µM drug (blue line), and 50µM drug (green line) for each of the positive hits from the screen: pergolide methanesulfonate (a), R(-)-2,10,11-trihydroxyaporphine hydrobromide (b), paliperidone (c), 1,3-dipropyl-7-methylxanthine (d), and reserpine (e). Sleep is plotted as minutes of sleep per sliding 30 minute window across a 24 hour period (12 hours light (white bar) and 12 hours dark (black bar) with averaged data from 4 female flies over 5 days of recording.

Genetic ablation of VMAT alters sleep behavior

Reserpine is an inhibitor of the VMAT. To determine whether the effects of reserpine on sleep were mediated by its inhibition of VMAT,

we compared the sleep phenotype of reserpine-treated flies with that of a VMAT-null mutant, *VMAT^{p1}* (Simon et al., 2009). The *VMAT^{p1}* homozygous mutant has the same significantly increased sleep quantity as flies fed 50 µM reserpine ($p=0.000144$ for *VMAT^{p1}/VMAT^{p1}* compared with iso31 as shown by one-way ANOVA with Tukey *post hoc* comparison) (Fig. 1.3a).

Importantly, inhibition of VMAT by drug treatment or genetic mutation does not render flies hypoactive, as measured by activity index (activity per waking minute) (Fig. 1.3b), indicating that the effect is specific for sleep.

We next asked whether inhibition of VMAT causes increased sleep depth in addition to increased sleep quantity. To assess sleep depth, we measured arousal threshold, in other words,

the ability of the animal to wake up with sensory stimulation. We delivered weak, medium, and strong stimuli to flies at different times of the night and counted the number aroused in different fly lines. We found that a smaller percentage of the drug-treated iso31 and the *VMAT^{p1}* homozygous mutants awoken in response to these mechanical stimuli, compared with untreated iso31 flies (Fig. 1.3c). The contribution of genotype to arousability at all stimulus intensities was significant ($p < 0.0001$ by two-way ANOVA).

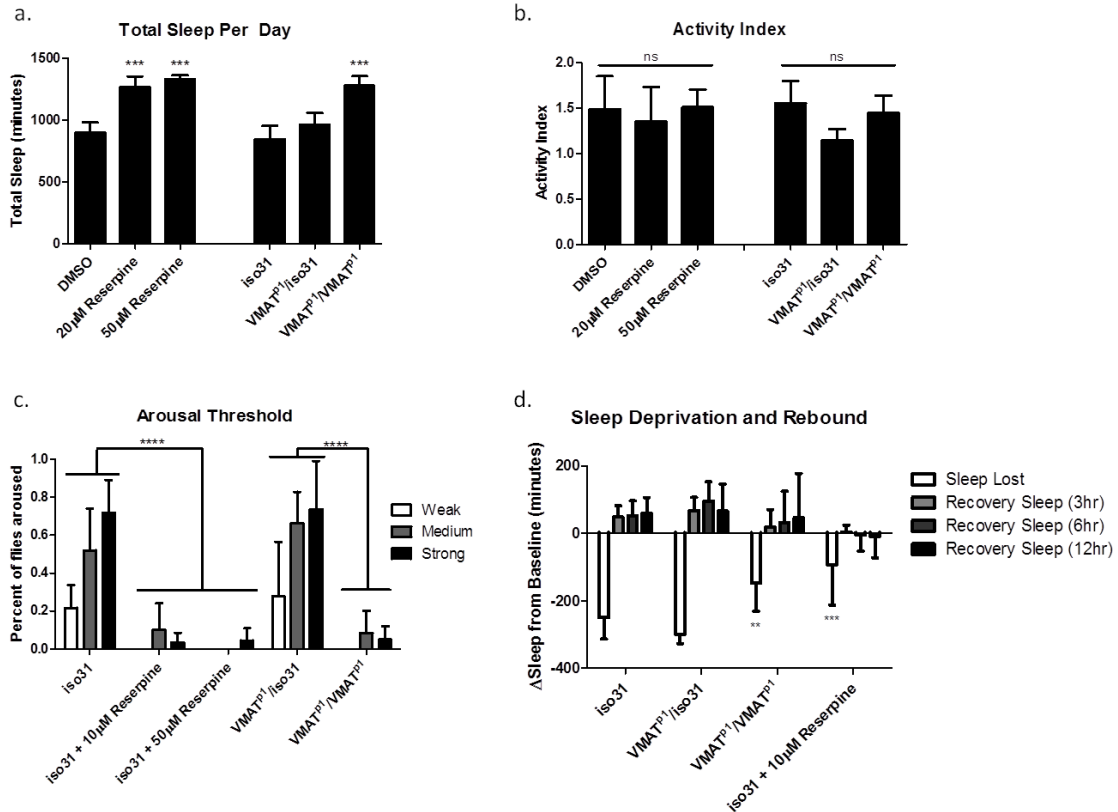


Figure 1.3 – Genetic ablation of VMAT Alters Sleep Behavior

(a) Total minutes of sleep per 24-hour day plotted for wild type (*iso31*) flies fed 0.1% DMSO (vehicle control), 20µM reserpine, and 50µM reserpine, as well as for *iso31*, *VMAT^{p1}/iso31* and *VMAT^{p1}/VMAT^{p1}* fed sucrose/agar food. $n=8$ for each genotype/treatment; *** indicates $p < 0.001$. (b) Activity index (infrared beam crosses per waking minute) for these reserpine-treated and *VMAT^{p1}* mutant flies shows these flies are not hypoactive. (c) The percent of sleeping flies that were aroused from sleep by a weak (white bars), medium (gray bars), or strong (black bars) stimulus at ZT16, ZT18, or ZT20, respectively. Data are averaged from three separate experiments; **** indicates $p < 0.0001$ for the effect of treatment/genotype on arousability. (d) Flies were deprived of sleep during the second half of the night (ZT18-ZT24). Minutes of sleep lost during this period (white bar) are plotted as a negative number, and rebound sleep was measured during the first 3 (light gray bar), 6 (gray bar), and 12 hours (black bar) the following morning. ** indicates $p < 0.01$ and *** indicates $p < 0.001$ for the amount of sleep lost compared to *iso31* controls, $n=16$ for each genotype/treatment.

We also measured the effect of VMAT inhibition on the homeostatic rebound that follows a period of sleep deprivation. We deprived flies of sleep during the second half of the night and assayed rebound sleep the following morning. Drug-treated and homozygous mutant flies were less effectively deprived than wild type and heterozygous flies ($p=0.00322$ and $p=0.000185$ for iso31 vs. $VMAT^{p1}/VMAT^{p1}$ and reserpine-treated flies, respectively, by one-way ANOVA with Tukey's *post hoc*), further supporting the idea that sleep depth is increased by inhibition of VMAT. Mutant and drug-treated flies experienced slightly less rebound sleep as well, although this difference did not reach significance because of large inter-individual variance (Fig. 1.3d). The apparent reduction in sleep rebound by these flies is likely the result of the relatively ineffective deprivation and already elevated baseline sleep levels.

Effects of reserpine on sleep map to the VMAT gene

In $VMAT^{p1}$ flies, the VMAT gene is disrupted by insertion of a PLacW transposon in the fifth exon of the *VMAT* gene (Bellen et al., 2011). To confirm the presence of this transposon, we genotyped $VMAT^{p1}$ flies by PCR. The wild-type VMAT allele, detected using primers specific for the VMAT genomic sequence, was amplified from iso31 flies and from flies heterozygous for the mutation, but not from homozygous mutants. Heterozygous and homozygous mutants were positive for the mutant *VMAT* allele, which was detected using one *VMAT* primer and one transposon-specific primer (Fig. 1.4a,b).

If effects of reserpine on sleep are mediated through inhibition of VMAT, then *VMAT* mutants should be resistant to reserpine. Indeed, the $VMAT^{p1}$ mutant does not show a further sleep increase after reserpine administration (Fig. 1.4c). Additionally, reserpine resistance of the $VMAT^{p1}$ mutant was not complemented by the deficiency Df(2R)BSC306, which spans the *VMAT* locus (Fig. 1.4c). The fact that the $VMAT^{p1}/Df(2R)BSC306$ flies had a phenotype comparable to homozygous mutants indicates the mutation is likely null. Together, these data indicate that *VMAT* mutants are resistant to reserpine, and the effect maps to the *VMAT* locus.

The sleep phenotype of VMAT mutants is background dependent

To determine whether the sleep phenotype of the *VMAT^{P1}* mutation is independent of genetic background, we outcrossed the mutation for five generations into an iso31 background. The presence of the transposon was confirmed by PCR (Fig. 1.4b). Surprisingly, outcrossed *VMAT* mutant flies (*VMAT^{P1}*(5x) flies) slept for approximately the same number of minutes per day as the iso31 control flies (846 min/d for iso31 females vs. 881 min/d for *VMAT^{P1}*(5x)/*VMAT^{P1}*(5x); Fig. 1.5a,b). Despite the largely normal baseline sleep behavior, however, the *VMAT^{P1}*(5x) flies consistently exhibited decreased sleep latency, which means that they fell asleep more quickly than wild-type or heterozygous flies after lights out ($p=0.035$ by unpaired t test with Welch's correction for unequal variances; Fig. 1.5c). Additionally, these mutant flies

retained an increased arousal threshold after outcrossing (Fig. 1.5d), similar to the original

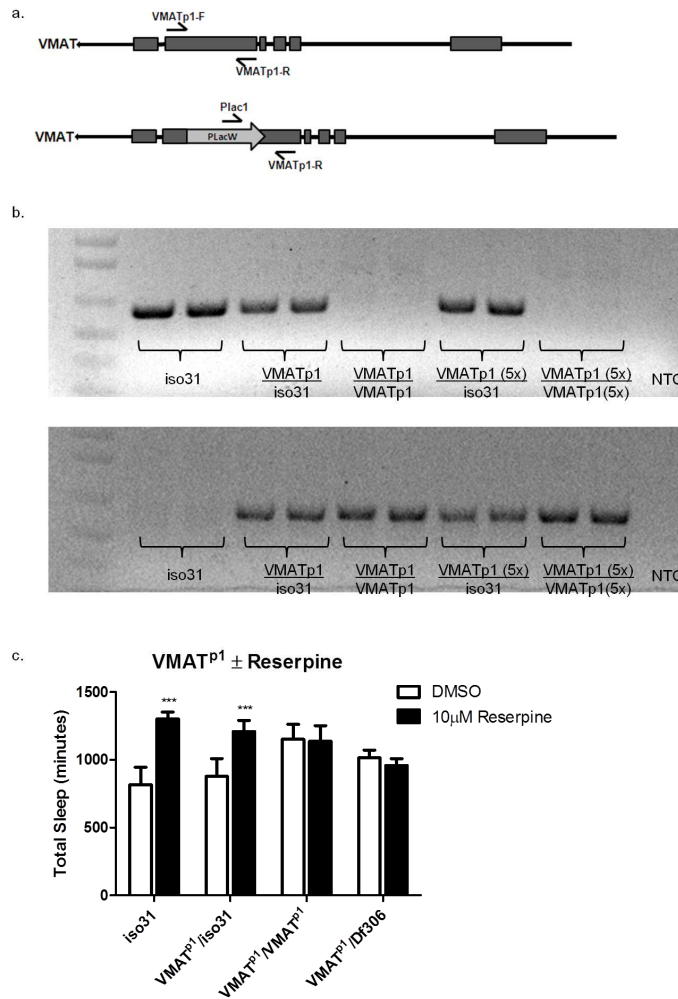


Figure 1.4 – Effects of reserpine on sleep map to the VMAT Gene

(a) The *VMAT* gene, with dark gray boxes representing exons, showing locations of PCR primers used to genotype the wild type *VMAT* (top) and P-element-containing mutant *VMATp1* (bottom) alleles. (b) PCR amplifies the wild type *VMAT* allele (top) in iso31 and *VMATp1*/iso31 heterozygous flies and the *VMATp1* mutant allele (bottom) in heterozygous and homozygous *VMATp1* mutant flies. (c) Total minutes of sleep per 24-hour-day for wild type, heterozygous mutant, homozygous mutant, and mutant/deficiency trans-heterozygotes fed 0.1% DMSO (white bars) or 10µM reserpine (black bars). *** indicates $p<0.001$, $n=16$ for each genotype/treatment.

mutant strain ($p=0.0100$ by two-way ANOVA for genotype contribution to arousability). Like the original mutant, the $VMAT^{P1}(5x)$ mutants were less efficiently sleep-deprived ($p=0.0003$ by one-way ANOVA) but showed a rebound the following morning proportional to sleep lost during deprivation (Fig. 1.5e).

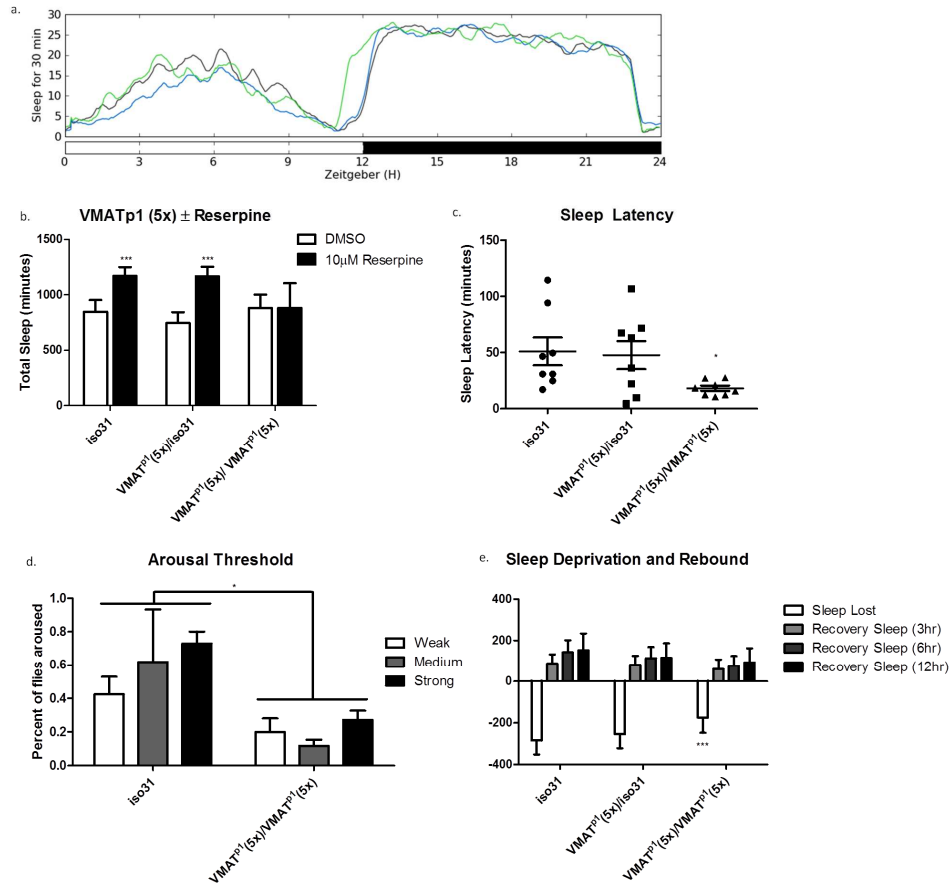


Figure 1.5 - The sleep phenotype of VMAT mutants is background dependent

(a) Sleep profile for iso31 (black line), VMATp1(5x)/iso31, and VMATp1(5x)/VMATp1(5x) outcrossed mutant flies. Sleep is plotted as minutes of sleep per sliding 30 minute window across a 24 hour period (12 hours light (white bar) and 12 hours dark (black bar) with averaged data from 16 female flies over 5 days of recording. (b) Total sleep per 24 hour period quantified for these flies fed 0.1% DMSO (white bars) or 10µM reserpine (black bars). *** indicates $p<0.001$. (c) Scatter plot of latency to sleep, or minutes between lights off and the first sleep bout. Horizontal line corresponds to group mean. * indicates $p<0.05$. (d) The percent of sleeping flies that were aroused from sleep by a weak (white bars), medium (gray bars), or strong (black bars) stimulus at ZT16, ZT18, or ZT20, respectively. Data are averaged from three independent experiments; * indicates $p<0.05$ for the effect of genotype on arousability. (e) Flies were deprived of sleep during the second half of the night (ZT18-ZT24). Minutes of sleep lost during this period (white bar) are plotted as negative numbers, and rebound sleep was measured during the first 3 (light gray bar), 6 (gray bar), and 12 hours (black bar) the following morning. *** indicates $p<0.001$ for the amount of sleep lost compared to iso31 controls, $n=16$ for each genotype.

Importantly, *VMAT^{P1}(5x)* mutants were still resistant to the sleep-promoting effects of reserpine (Fig. 1.5b). Because these mutants have normal baseline sleep, the lack of a response to reserpine cannot be the result of a ceiling effect. These data establish that VMAT is required for effects of reserpine on sleep.

Effects of reserpine on mutants of different monoaminergic systems

VMAT is a transporter protein that packages all monoaminergic neurotransmitters into presynaptic vesicles. To determine which of the monoamines is responsible for the sleep phenotype produced by reserpine, we fed reserpine to mutants deficient for the various monoamines. Mutants deficient in the synthesis of dopamine (*ple^{ts}*), serotonin (*TrH^{co1440}*), octopamine (*TbH^{nm18}*), histamine (*HdcM^{B07212}*), and the amino acid-derived neurotransmitter GABA (*Gad1^{f00602}*) all responded to reserpine (Fig. 1.6). Because dopamine synthesis is required during development, the only viable mutant (*ple^{ts}*) is temperature-sensitive, and so the drug treatment and sleep behavior for this mutant were measured at the restrictive temperature of 29°C. Reserpine increased sleep significantly for all of the neurotransmitter mutants (comparing total sleep of DMSO controls with 10 μM reserpine-treated flies of the following genotypes, $p=0.000139$ for iso31, $p=0.00355$ *ple^{ts}* at 29°C, $p=0.000144$ for *TrH^{co1440}*, $p=0.000137$ for *TbH^{nm18}*, $p=0.000137$ for *Hdc^{MB07212}*, and $p=0.000151$ for *Gad1^{f00602}* by two-way ANOVA with Bonferroni multiple comparisons), indicating that no single neurotransmitter system is required for sleep-promoting effects of reserpine. VMAT inhibition likely increases sleep by interfering with the signaling from more than one neurotransmitter system simultaneously.

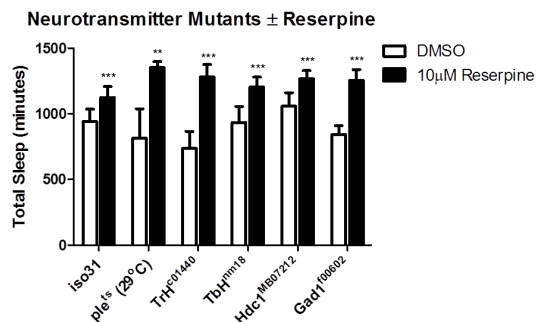


Figure 1.6 - Effects of reserpine on mutants of different monoaminergic systems

Total sleep per 24 hour period plotted for mutants defective in the synthesis of the neurotransmitters dopamine (plets at 29oC), serotonin (TrHco1440), octopamine (TbHnm18), histamine (HdcMB07212), and GABA (Gad1f00602), fed 0.1% DMSO (white bars) or 10μM reserpine (black bars). ** indicates $p<0.01$ and *** indicates $p<0.001$.

DISCUSSION

Genetic screens for sleep phenotypes have led to the isolation of a few mutants, including *Shaker* (Cirelli et al., 2005), *sleepless* (Koh et al., 2008), *insomniac* (Stavropoulos and Young, 2011), and *cyclinA1* (Rogulja and Young, 2012). Other mutations that cause reduced sleep were identified by chance, including *fumin* (Kume et al., 2005) and several mutations in the calcineurin signaling pathway (Nakai et al., 2011), or through assays of candidate genes (Yuan et al., 2006; Crocker and Sehgal, 2008, 2010; Sehgal and Mignot, 2011). Although these studies give valuable insight into molecular underpinnings of sleep behavior, they do not paint a complete picture of the molecular machinery of sleep regulation.

We note that traditional genetic screens may be limited in their ability to uncover molecules that regulate behavior because of factors, such as redundancy, lethality, developmental compensation, and developmental defects, which may mask or conflate adult phenotypes. The study of sleep is particularly susceptible to these limitations, as long-term sleep deprivation leads to death (Rechtschaffen et al., 1983; Shaw et al., 2009). In addition, sleep-regulating genes tend to also be required for other functions. One way to bypass the limitations intrinsic to traditional genetic screens is to use adult-specific manipulations. We asked whether we could use a small-molecule screen to discover new sleep-modulating proteins.

Small-molecule screens in whole animals are rare, especially when measuring a behavioral output. A small-molecule screen for aberrant sleep behavior in zebrafish assayed larvae, through automated methods, for effects of almost 4000 drugs (Rihel et al., 2010). Conducting a drug screen in *Drosophila*, although more labor-intensive, was important to find new sleep-modulating molecular targets in a well-established sleep model. Additionally, an enormous genetic toolkit is available in *Drosophila* for confirming and elaborating on drug screen findings.

In the screen reported here, we searched for drugs that dose-dependently and reproducibly affected sleep behavior in both male and female adult flies. Two of the compounds that met these strict criteria are dopamine receptor agonists. Other drugs that increase dopamine

signaling, such as methamphetamine and cocaine, are known to increase arousal in humans and model organisms, including *Drosophila*. Inhibition of dopamine biosynthesis biochemically (Andreatic et al., 2005) and genetically (Riemensperger et al., 2011) has the opposite effect, increasing sleep amount in the fly. Also, the dopamine type 1 receptor, dDopR, promotes arousal at appropriate times in the circadian sleep/wake cycle (Lebestky et al., 2009). The identification of small molecules targeting dopaminergic signaling validates the power of the drug screen to identify sleep-regulatory pathways.

Another sleep-inhibiting molecule identified in the screen was methylxanthine, which is a caffeine analog. Caffeine is well known as a robust wake-promoting stimulant. Although the target of its action in *Drosophila* is still unclear, its effects on behavior are similar to those in mammals/humans (Wu et al., 2008). As in the case of the molecules that affect dopaminergic signaling, the identification of a caffeine analog speaks to the efficacy of the small-molecule screen reported here. This screen also identified an atypical antipsychotic, paliperidone, as a sleep-inhibiting molecule. The target of this antipsychotic is not known, but its effect on sleep supports reports of links between sleep and affective disorders (Wulff et al., 2010).

Surprisingly, only one drug from the screen was found to increase sleep: reserpine. Although reserpine, which is typically used to treat hypertension and is also indicated as an antipsychotic, was shown many years ago to have a tranquilizing effect, it has not been mechanistically linked to sleep (Monroe et al., 1955; Steiner et al., 1963). We now have the genetic tools to understand the nature of this effect in a controlled and systematic manner and its implications for the normal regulation of sleep and wake. Reserpine inhibits the function of the VMAT, a transmembrane protein that transports monoaminergic neurotransmitters into presynaptic vesicles to prepare them for release. Vertebrates have two VMAT genes, VMAT1 and VMAT2, whereas flies have only one.

One common caveat in pharmacological studies is the possibility of off-target effects. We show that a *VMAT^{D1}* null mutant (Simon et al., 2009) has increased sleep. More importantly, this mutant is resistant to the effects of reserpine, indicating that the long-sleeping phenotype is not

the result of off-target effects. An apparent increase in sleep can sometimes result from sickness or physical impairment. However, despite sleeping significantly more, the reserpine-fed and *VMAT^{P1}* mutant flies do not have a decreased activity index. In addition, outcrossed *VMAT* mutants, which have normal levels of baseline sleep, are unresponsive to reserpine. Together, these data show that reserpine specifically increases sleep by inhibiting VMAT.

Alterations in sleep duration are often accompanied by changes in sleep depth, as measured by arousal threshold. Previous studies have shown that short-sleeping mutants tend to have decreased arousal thresholds during normal sleep (Koh et al., 2008). Interestingly, the long-sleeping *VMAT^{P1}* mutant has an increased arousal threshold, suggesting that they sleep more deeply (Andretic and Shaw, 2005). In general, loss of VMAT appears to increase sleep drive or decrease the ability to maintain wakefulness, as demonstrated also by the increased latency to sleep in flies carrying a five-generation outcrossed *VMAT^{P1}* allele. These outcrossed flies no longer have increased daily sleep, but they also display an increased arousal threshold. On the other hand, *VMAT^{P1}* flies have a normal rebound after deprivation, supporting the idea that the response to sleep deprivation is less tightly correlated with other measures of sleep.

The less severe phenotype of the outcrossed allele is consistent with other studies that have noted the importance of genetic background in animal behaviors, including sleep (Zimmerman et al., 2012). In an iso31 background, inhibition of VMAT throughout development with the *VMAT^{P1}* mutation does not alter daily sleep, but sleep is increased when VMAT is inhibited acutely in adults. Thus, developmental compensation mechanisms may account for the discrepancy between the sleep phenotype of the drug-fed and mutant flies in the iso31 genetic background. The original mutant background likely confers less developmental compensation, as these flies have the same long-sleeping phenotype as the drug-fed flies.

VMAT plays a presynaptic role in signaling by many different neurotransmitters, including the monoamine neurotransmitters dopamine, serotonin, histamine, and octopamine. Additionally, recent evidence suggests that VMAT transports the amino acid neurotransmitter GABA (Tritsch, 2012). Many of these neurotransmitters, including dopamine (Andretic et al., 2005; Kume et al.,

2005), octopamine (Crocker and Sehgal, 2008), serotonin (Yuan et al., 2006), and GABA (Agosto et al., 2008), have been independently implicated in regulating sleep behavior. We found that mutants deficient for each of these neurotransmitters displayed increased sleep after reserpine feeding, suggesting that no single neurotransmitter system accounts for the impact of VMAT inhibition on sleep. Similarly, Chen et al. (2013) found that circadian rhythms are perturbed in *VMAT^{D1}* mutants, and rescue of this phenotype requires VMAT in multiple neuronal populations. In mammals as well, Coulter et al. (1971) demonstrated that the effect of reserpine on sleep cannot be attributed to reductions in serotonin or norepinephrine. Our findings contribute to a picture of sleep regulation driven by a robust network of neurotransmission that requires VMAT in multiple neuronal populations.

Altered VMAT function has previously been studied in the context of many neuropsychiatric and neurological diseases, including depression, bipolar disorder, schizophrenia, and Parkinson's disease (Wimalasena, 2011). These diseases are accompanied by an increased prevalence of sleep perturbations, although these have not yet been linked to VMAT. Understanding the role of VMAT in sleep may elucidate the pathophysiology of sleep perturbations in the disorders noted here, as well as the natural regulation of sleep in healthy flies and humans.

The potential for screens in *Drosophila* to identify drugs for human use is high. Although numerous side effects make reserpine suboptimal as a treatment, more specific inhibitors of VMAT2 may be tolerated better by patients and improve their use as a sleep aid. The screen reported here identified a single sleep-promoting drug, but expanded screens could identify many more potential pharmacotherapies. We used a drug library with known biological targets, which may have biased the findings toward well-studied pathways. Additionally, a low concentration of drug was used to reduce lethality, meaning that only drugs with the strongest impacts on sleep were found. Now that the utility of these screens has been proven, larger screens can be used to identify other novel modulators of behavior.

In conclusion, we used a small-molecule screen to discover regulators of sleep phenotype. Using a genetic approach to confirm one of these drugs, we found that VMAT is required to establish normal sleep duration and arousal state, presumably by regulating transmission of several neurotransmitters. The role of genetic background in the expressivity of the VMAT phenotype highlights the strong effect of developmental compensation on behaviors, such as sleep, and the importance of targeting pathways acutely in adults to look at adult behavior. Small-molecule screens in live animals provide a powerful tool for dissecting molecular mechanisms of adult behavior.

CHAPTER 2 – Caffeine promotes wakefulness via dopamine signaling in *Drosophila*

ABSTRACT

Caffeine is the most widely-consumed psychoactive drug in the world, but our understanding of how caffeine affects our brains is relatively incomplete. Most studies focus on effects of caffeine on adenosine receptors, but there is evidence for other, more complex mechanisms. In the fruit fly *Drosophila melanogaster*, which shows a robust diurnal pattern of sleep/wake activity, caffeine reduces nighttime sleep behavior independently of the one known adenosine receptor. Here, we show that dopamine is required for the wake-promoting effect of caffeine in the fly, and that caffeine likely acts presynaptically to increase dopamine signaling. We identify a cluster of neurons, the paired anterior medial (PAM) cluster of dopaminergic neurons, which are essential for the caffeine response and which show increased activity following caffeine administration. While previous studies have demonstrated adenosine-mediated effects of caffeine on post-synaptic dopamine receptors, to our knowledge, this is the first set of studies implicating the synthesis of dopamine in the arousal-promoting effects of caffeine.

INTRODUCTION

Caffeine is the most widely consumed psychoactive drug in the world. Its popularity is likely due to its ability to fight drowsiness and promote arousal. In addition, caffeine can reverse the effects of sleep deprivation on alertness and cognition, as shown in both rats and humans (Penetar et al., 1993; Alhaider et al., 2010). Despite the ubiquity of caffeine in our food and drinks, our understanding of how caffeine affects our brains and bodies is relatively incomplete.

The most extensively-studied behavioral effect of caffeine is acute locomotor stimulation, which has been attributed to antagonism of adenosine receptors. There are four subtypes of adenosine receptor, and caffeine antagonizes both A2A and A1 receptors in vivo (Fredholm et al., 2001). Which of these two receptor subtypes is responsible for the motor-stimulating effect,

however, is a point of contention (Snyder et al., 1981; Spealman et al., 1988). The effect of caffeine on sleep has been relatively less well-studied. A2A receptors have been implicated in the acute wake-promoting effect of caffeine (Huang et al., 2005; Lazarus et al., 2011), but adenosine receptors do not have an essential role in driving baseline sleep behavior. A1 and A2A mutant mice, which should mimic receptor antagonism, have no baseline sleep defects (Stenberg et al., 2003; Huang et al., 2005). A brain-specific deletion of the A1 receptor causes reduced slow wave brain activity following sleep deprivation, but these mice do not exhibit a change in the time spent in sleep or wake states (Bjorness et al., 2009). In addition to adenosine receptors, caffeine has many other biological targets, including GABA_A receptors, ryanodine receptors, glycine receptors, and phosphodiesterases (reviewed in Mustard, 2014). Investigating the impact on sleep behavior of these other targets may further our knowledge of the effects of caffeine.

We turn to a powerful genetic model, the fruit fly *Drosophila melanogaster*, to further understand how caffeine promotes wakefulness. While *Drosophila* has proven to be a fruitful model for uncovering sleep regulatory mechanisms, the action of caffeine in this model is not yet understood. Flies have one known adenosine receptor, *dAdoR*. This receptor only shows 30% sequence similarity to the human adenosine receptors at the amino terminal, but the important ligand-binding residues are conserved (Dolezelova et al., 2007). Surprisingly, the *dAdoR* null mutant responds to caffeine identically to wild type flies, suggesting that caffeine promotes sleep in *Drosophila* via adenosine receptor-independent mechanisms (Wu et al., 2009). Because the behavioral effects of caffeine are similar between flies and humans, understanding the mode of action in the fly may elucidate novel actions of caffeine in mammals as well.

Here, we show that the wake-promoting effect of caffeine in *Drosophila* requires the synthesis of dopamine, a potent wake-promoting neurotransmitter. The modulation of dopaminergic signaling by caffeine likely occurs presynaptically. In addition, we identify a cluster of dopaminergic neurons which are essential for the caffeine response. We hypothesize that caffeine promotes wake by increasing activity of these neurons.

MATERIALS AND METHODS

Fly Lines

All flies were raised in vials containing molasses food. Wild type iso31 flies (Ryder et al., 2004) were used as controls for all experiments. *DTHg* and *DTHg*^{FS±} flies were shared with us by the Hirsh lab (UVA, Charlottesville, VA). *VMAT*^{P1} mutants were a kind gift of the Krantz lab (UCLA, Los Angeles, CA). *Ebony*¹ (BSC1658) *Dat*^{lo} (BSC3193) and *TH-Gal4* (BSC8848) were ordered from the Bloomington Stock Center (Bloomington, IL). The restricted dopaminergic drivers *TH-C1-Gal4*, *TH-D1-Gal4*, *TH-D4-Gal4*, *TH-F1-Gal4*, *TH-F2-Gal4*, *TH-G1-Gal4* were all generously shared with us by the Wu lab (Johns Hopkins University, Baltimore, MD), and the other dopaminergic drivers *InSite0104-Ga4* and *InSite0273-Gal4* were shared by the Clandinin lab (Stanford University, Stanford, CA). *UAS-Shibire*^{ts} flies were a gift of the David Anderson lab (CalTech, Pasadena, CA). *UAS-CaLexA* flies were a gift of Dr. Jing Wang's lab (UCSD, La Jolla, CA).

Behavioral Assays

To assay sleep behavior, we used the *Drosophila* Activity Monitoring System (DAMS, TriKinetics). 5- to 10-day-old flies were individually monitored in 5 mm glass tubes containing food composed of 5% sucrose and 2% agar (sucrose/agar food). Activity was monitored for five consecutive days in incubators kept on a 12 hour light/dark schedule at 25°C. Sleep behavior was analyzed using PySolo software, and sleep bouts were defined at 5 or more minutes of inactivity (Gilestro and Cirelli, 2009). For all experiments, 10-16 flies were used per treatment group, sex, and genotype.

For circadian experiments, flies were entrained in DAMS monitors for two days in 12 hour light-dark cycles and then moved to constant darkness for five days. Circadian rhythms of activity were determined using ClockLab software (Pfeiffenberger et al., 2010).

For Shibire^{ts} temperature shift experiments, fly crosses were set and raised at 18°C to avoid prematurely silencing the neurons. Flies were then loaded into DAMS monitors in incubators set 12 hours light-dark cycles with a temperature of 21°C, which is permissive for the Shibire^{ts} mutation. The next day, the temperature increased to 30°C, the restrictive temperature, at lights-on. Sleep was assayed during three days at 30°C and averaged across all days.

Drug Feeding

Caffeine (Sigma-Aldrich) was mixed into melted sucrose/agar food at a concentration of 0.5 mg/mL for all experiments except for the dose-response experiment where 0.2, 0.5, and 1 mg/ml were used. L-DOPA (Tocris) was mixed into melted sucrose/agar food at a concentration of 3mg/ml.

Confocal Microscopy

5- to 10-day-old CaLexA flies were moved from vials containing molasses food to vials containing either sucrose/agar food or sucrose/agar food with 0.5 mg/ml caffeine. After 24 hours, flies were anesthetized on ice, and brains were dissected in 1x phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PBS-T). Ten brains were dissected per genotype, and all brains were fixed for 1 hour in 4% paraformaldehyde (PFA; Electron Microscopy Sciences). Brains were washed in PBS-T and blocked for one hour in PBS-T containing 5% normal donkey serum (NDS; Jackson ImmunoRes). Brains were incubated at 4°C overnight in primary antibody in PBS-T with 5% NDS. CaLexA signal was labeled with 1:1000 dilution of rabbit α -GFP (Life Technologies) and neuropil was stained with a 1:1000 dilution of mouse α -nc82 (Developmental Studies Hybridoma Bank). Brains were washed three times with PBS-T and stained for 2 hours with secondary antibodies in PBS-T with 5% NDS. 1:1000 dilutions were used for α -rabbit AlexaFluor 488 (Invitrogen) and α -mouse AlexaFluor 633 (Invitrogen). Brains were washed three times in PBS-T and mounted on slides using VectaShield (Vector Laboratories, Inc.). Slides were imaged on a Leica SP5 confocal microscope with 20x objective and 0.5 μ m step size. GFP intensities

were quantified post-hoc on a cell-by-cell basis from individual Z-planes using ImageJ software (Abramoff et al., 2004).

RESULTS

The behavioral response to caffeine requires dopamine synthesis in *Drosophila*

Similarly to mammals, *Drosophila* experience reduced sleep following caffeine feeding. This reduction of sleep can be seen in both male and female flies across a 24-hour light/dark cycle, but the effect is most robust and reproducible in the dark (nighttime) phase (Figure 2.1). Wild type (iso31; Ryder et al., 2004) flies exhibit a dose-dependent decrease in nighttime sleep when fed increasing concentrations of caffeine (Figure 2.2a, d). The 0.5 mg/mL

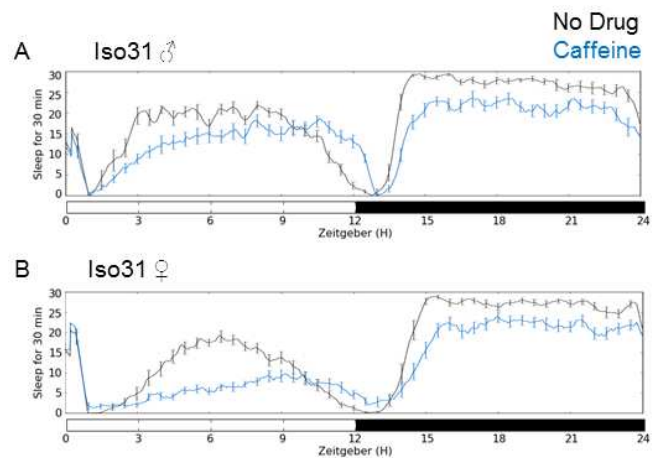


Figure 2.1 - Caffeine Reduces Sleep in *Drosophila*
Sleep profiles for male (a) and female (b) flies assayed on drug-free food (black line) or food containing 0.5 mg/ml caffeine (blue line). Graphs depict minutes of sleep per 30 minute sliding window across a 24-hour period composed of 12 hours of light (white bar) and 12 hours of dark (black bar). Error bars show standard deviation.

caffeine concentration produced the largest, most reproducible loss of sleep with no toxic effects, so this concentration was used for the rest of the experiments reported here.

Evidence from both mammals and *Drosophila* suggests a role for dopamine signaling in the effect of caffeine on arousal (reviewed in Chen et al., 2010). However, those previous studies implicate dopamine receptors, which in mammals are known to interact with adenosine receptors, and do not assay for a requirement of dopamine (reviewed in Xie et al., 2007). We tested the requirement of dopamine in the caffeine response using a transgenic fly line deficient for tyrosine hydroxylase (DTH), the rate-limiting enzyme in dopamine biosynthesis. DTH, encoded by the *pale* (*ple*) gene, is required in peripheral tissue during development, resulting in larval lethality of

the *ple* null mutant (Jurgens et al., 1984; Neckameyer et al., 1993). A nervous system-specific DTH mutant (*DTHg^{FS±}*) was created by transgenically expressing a periphery-specific isoform of

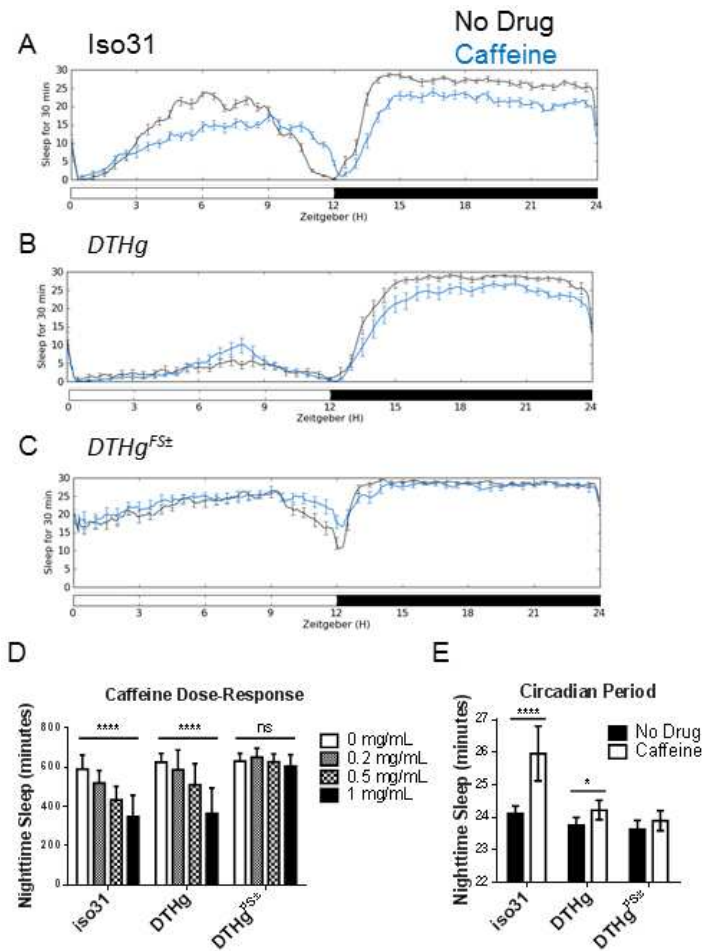


Figure 2.2 - The response to caffeine requires dopamine synthesis in *Drosophila*

Sleep profiles for female (a) iso31, (b) DTHg, and (c) DTHg^{FS±} flies on drug-free food (black line) or food containing 0.5 mg/ml caffeine (blue line). Graphs depict minutes of sleep per 30 minute sliding window across a 24-hour period composed of 12 hours of light (white bar) and 12 hours of dark (black bar). (d) Average number of minutes of sleep per night is plotted for the three genotypes fed either drug-free food or food containing 0.2, 0.5, or 1 mg/ml caffeine. (e) Circadian period of free-running rest-activity rhythms is plotted for the same three genotypes fed drug-free food or food containing 0.5 mg/ml caffeine. Error bars show standard deviation.

DTH in a *ple* null mutant background (Riemensperger et al., 2011). This *DTHg^{FS±}* transgene rescues the viability defect of the *ple* mutants, but they still lack DTH in the nervous system. Control flies contain a wild type copy of the DTH coding sequence in a *ple* mutant background (*DTHg*), rescuing expression in both the nervous system and peripheral tissue.

We measured the effect of chronic caffeine exposure on iso31, *DTHg*, and *DTHg^{FS±}* flies by

concomitantly exposing these flies to caffeine-containing food and monitoring their sleep behavior for five days. The sleep-reducing effect of caffeine was most robust and reproducible during the dark phase, so nighttime sleep was quantified for all experiments. Female iso31 flies

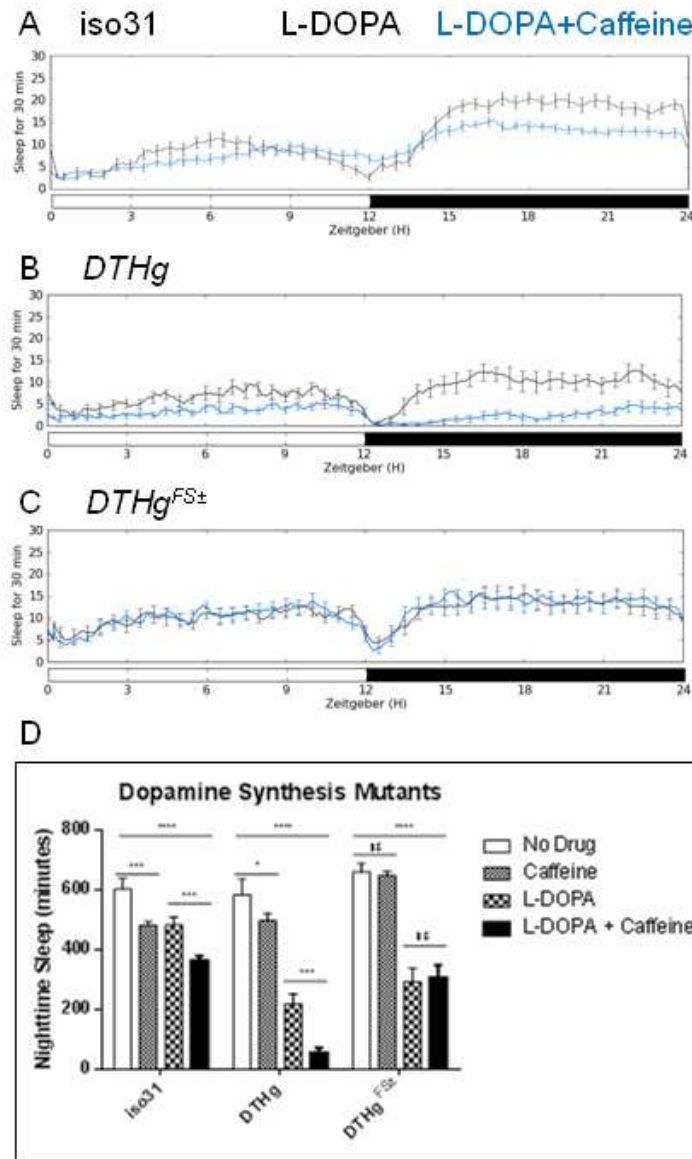
experienced about 160 minutes less sleep during the night when assayed on food containing 0.5 mg/mL caffeine compared to drug-free food ($p < 0.0001$ by 2-way ANOVA with Bonferroni multiple comparisons) (Figure 2.2a, d). *DTHg* control flies also showed a decrease in nighttime sleep when exposed to caffeine, sleeping 86 minutes less than flies fed drug-free food ($p = 0.0008$ by 2-way ANOVA with Bonferroni multiple comparisons) (Figure 2.2b, d). *DTHg*^{FS±} flies, on the other hand, were resistant to the wake-promoting effect of caffeine, sleeping about 650 minutes per night irrespective of drug treatment (Figure 2.2c, d). While these figures depict data for female flies, similar results were observed for males as well (data not shown).

In addition to promoting wake, caffeine lengthens circadian period in both mammals and insects (Wu et al., 2009; Oike et al., 2011). We monitored rest-activity rhythms of flies in constant conditions, and found that 0.5 mg/ml caffeine lengthened the circadian period of these rhythms from 23.8 to 25 hours ($p < 0.0001$; 2-way ANOVA with Bonferroni multiple comparisons). The effect of caffeine on *DTHg* control flies was more modest, lengthening period from 23.5 to 24.1 hours ($p = 0.001$). The effect of caffeine on circadian period seemed to also require dopamine, because the *DTHg*^{FS±} flies did not display lengthened period when monitored on caffeine-containing food ($p = 0.99$).

Caffeine affects dopaminergic signaling upstream of DTH

We next sought to determine if we could rescue the caffeine response by restoring dopamine to *DTHg*^{FS±} mutants. DTH catalyzes the conversion of tyrosine to L-DOPA, which is then converted to dopamine by Dopa decarboxylase (Ddc) (Budnik and White, 1987; Livingstone and Tempel, 1983). Despite lacking neural DTH, *DTHg*^{FS±} flies can produce dopamine if supplied with exogenous L-DOPA. Feeding L-DOPA to iso31, *DTHg*, and *DTHg*^{FS±} flies caused a sleep decrease, consistent with an augmentation of dopamine signaling in all of these genotypes (Figure 2.3d). Both iso31 and *DTHg* flies experienced an even more drastic sleep loss when fed both L-DOPA and caffeine together, as compared to L-DOPA alone.

Iso31 flies slept 483 minutes per night when fed L-DOPA-containing food, and 362 minutes per night when fed food containing both L-DOPA and caffeine ($p=0.0009$ by 2-way ANOVA with Bonferroni multiple comparisons) (Figure 2.3a, b). *DTHg* flies slept 218 minutes per night when fed L-DOPA, and 54 minutes per night when fed L-DOPA and caffeine ($p<0.0001$) (Figure 2.3b, d). Importantly, L-DOPA feeding did not rescue the caffeine responsiveness of *DTHg^{FS±}* flies; they slept 297 minutes per night when fed L-DOPA alone, and 306 minutes per night when fed L-DOPA and caffeine together, a difference which is not significant ($p=0.99$)



(Figure 2.3c, d). Because rescue of dopamine synthesis downstream of DTH did not restore caffeine response, caffeine likely modulates dopaminergic signaling upstream of DTH.

Figure 2.3 - Caffeine affects dopaminergic signaling upstream of DTH

Sleep profiles for female (a) iso31, (b) *DTHg*, and (c) *DTHg^{FS±}* flies on food containing 3mg/ml L-DOPA (black line) or food containing 3 mg/ml L-DOPA and 0.5 mg/ml caffeine (blue line). Graphs depict minutes of sleep per 30 minute sliding window across a 24-hour period composed of 12 hours of light (white bar) and 12 hours of dark (black bar). (d) Average number of minutes of nighttime sleep is plotted for flies fed drug-free food (white bars), food containing 0.5 mg/ml caffeine (small check bars), food containing 3 mg/ml L-DOPA (large check bars), or food containing 3 mg/ml L-DOPA and 0.5 mg/ml caffeine (black bars). Error bars show standard deviation.

The response to caffeine is sensitive to neuronal dopamine levels

Dopaminergic signaling can be disrupted not only by blocking biosynthesis, but also by blocking synaptic release and dopamine inactivation. Synaptic release of dopamine relies on the transport of dopamine into synaptic vesicles by the vesicular monoamine transporter (DVMAT).

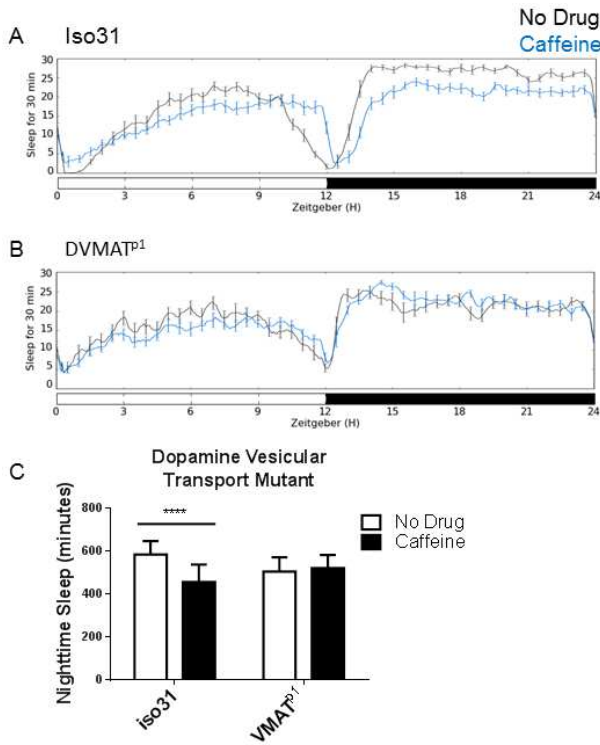


Figure 2.4 - The response to caffeine requires synaptic packaging of dopamine
 Sleep profiles for female (a) *iso31* and (b) *DVMAT^{p1}* flies assayed on drug-free food (black line) or food containing 0.5 mg/ml caffeine (blue line). Graphs depict minutes of sleep per 30 minute sliding window across a 24-hour period composed of 12 hours of light (white bar) and 12 hours of dark (black bar). (c) Average number of minutes of nighttime sleep for flies fed drug-free food (white bars) or food containing 0.5 mg/ml caffeine (black bars). Error bars show standard deviation.

Disrupting this transport with a null mutation in the *DVMAT* gene blocks dopamine signaling, and we found that this mutation also blocked the wake-promoting effect of caffeine. *DVMAT* null mutants (*DVMAT^{p1}*) outcrossed into an *iso31* genetic background (Nall and Sehgal, 2012) slept 505 minutes per night when fed drug-free food and 520 minutes per night when fed food containing 0.5 mg/mL caffeine, a difference which is not significant ($p=0.86$) (Figure 2.4).

Dopamine signaling is also regulated by the rate of dopamine inactivation, which occurs when dopamine molecules are conjugated to different functional groups that mark them for degradation. Dopamine is inactivated and degraded in glia and neurons by different pathways. The enzyme Ebony, a β -alanyl

dopamine synthase, inactivates dopamine in glia and the enzyme Dopamine-N-acetyltransferase (Dat) inactivates dopamine in the nervous system and gut

(Brodbeck et al., 1998; Richardt et al., 2002). Ebony mutants (e^1) maintained caffeine response, with caffeine-fed flies sleeping 122 fewer minutes per night than flies fed drug-free food ($p=0.006$, 2-way ANOVA with Bonferroni multiple comparisons) (Figure 2.5b, d). Dat mutants (Dat^{lo}), on the other hand, were resistant to the effect of caffeine, sleeping about 450 minutes per night regardless of food caffeine content ($p=0.13$) (Figure 2.5c, d). Given that these mutants are predicted to have higher levels of presynaptic dopamine, we surmise that maintenance of appropriate dopamine levels in neurons is essential for the arousal response to caffeine. Dat^{lo} mutants may be unable to respond to caffeine due to saturated exocytotic machinery or developmental compensatory mechanisms. The importance of maintaining proper dopamine levels has a precedent in *Drosophila* neurobiology, as flies with either elevated or reduced dopamine levels show impaired memory retention (Zhang et al., 2008).

Mapping dopaminergic neurons required for the response to caffeine

There are many clusters of dopaminergic neurons in the fly brain, characterized by location of cell bodies and the anatomical targets of axonal

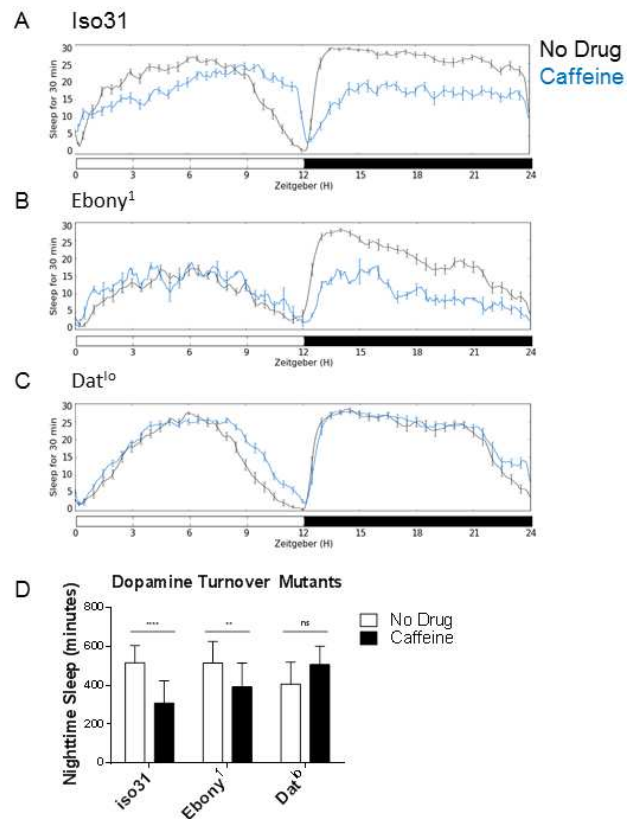


Figure 2.5 - The response to caffeine requires proper neuronal dopamine turnover

Sleep profiles for female (a) iso31 and (b) ebony1 and (c) Datlo flies assayed on drug-free food (black line) or food containing 0.5 mg/ml caffeine (blue line). Graphs depict minutes of sleep per 30 minute sliding window across a 24-hour period composed of 12 hours of light (white bar) and 12 hours of dark (black bar). (d) Average number of minutes of nighttime sleep for flies fed drug-free food (white bars) or food containing 0.5 mg/ml caffeine (black bars). Error bars show standard deviation.

projections (Mao and Davis, 2009). We silenced subsets of dopaminergic neurons using restricted Gal4 drivers to express the temperature-sensitive dynamin mutant *Shibire^{ts}* (*Shi^{ts}*). At the restrictive temperature, 30°C, the targeted neurons have stalled axonal transport and synaptic signaling (Kitamoto et al., 2001). We used six restricted dopaminergic Gal4 lines created by the

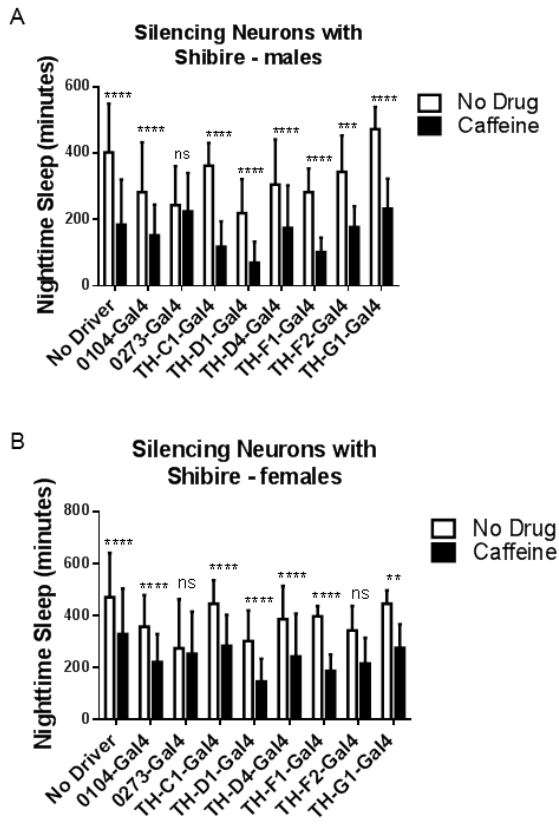


Figure 2.6 - Mapping dopaminergic neurons required for the caffeine response

Average number of minutes of nighttime sleep for (a) male and (b) female flies containing various restricted dopaminergic Gal4 constructs driving expression of temperature-sensitive *Shibire* (*UAS-Shits*). Control flies contain the *UAS-Shits* transgene in the absence of a Gal4 driver. Nighttime sleep is averaged across three nights at the 30°C restrictive temperature on drug-free food (white bars) or food containing 0.5 mg/ml caffeine (black bars). Error bars show standard deviation.

Wu lab and two others from the InSite collection (Liu et al., 2012, Gohl et al., 2011). Six of the Gal4 lines still permitted a caffeine-induced loss of nighttime sleep at 30°C when driving *Shi^{ts}*. One fly line, TH-F2-Gal4>*Shi^{ts}*, had a significant caffeine-induced sleep reduction only in males; however, there was a strong trend towards sleep loss in females ($p=0.0639$ by ANOVA with Bonferroni multiple comparisons). One Gal4 line, InSite0273, prevented a caffeine-induced sleep decrease when driving *Shi^{ts}* at 30°C in both males and females (Figure 2.6 a, b). Therefore, this driver defines a group of dopaminergic neurons which, when silenced, block the wake-promoting effect of caffeine.

Caffeine causes increased activity of PAM cluster neurons

The InSite0273 driver line expresses Gal4 primarily in the Paired Anterior Medial (PAM) cluster, which is a group of dopaminergic neurons that projects mostly to the mushroom

bodies. We monitored the effect of caffeine on the PAM neurons using the CaLexA tool, in which neural activity-induced elevation of intracellular calcium results in long-term green fluorescent protein (GFP) reporter expression (Masuyama et al., 2012). Representative images showed a noticeable increase in GFP fluorescence in InSite0273-Gal4 labeled cells following 24 hours of

caffeine feeding (Figure 2.7a, b).

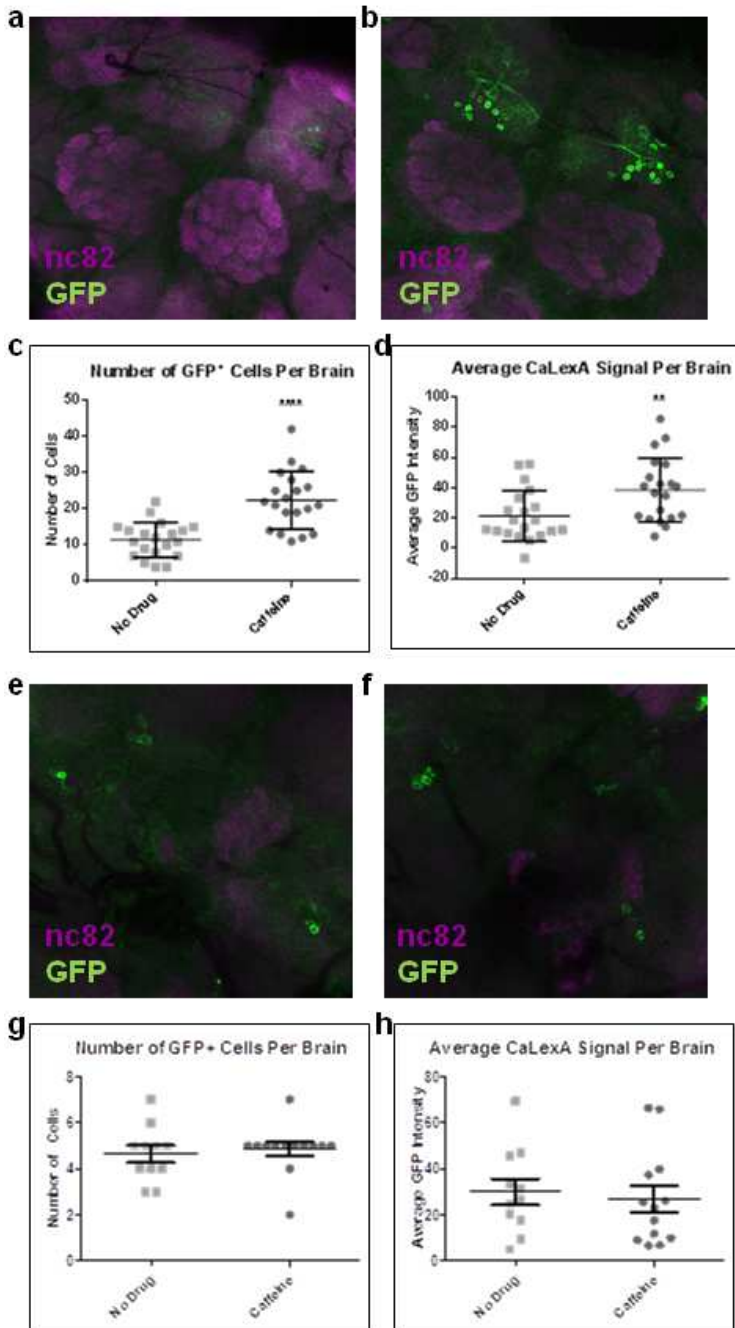
This increase in GFP signal was significant, both in terms of the number of cells with visible GFP expression, as well as the average GFP intensity for all of the visible cells in each brain

(Figure 2.7c, d). This suggests that caffeine ingestion causes

Figure 2.7 - Caffeine causes increased activity of PAM cluster neurons

Immunostaining of the CaLexA signal (GFP, green) and neuropil (nc82, magenta) in brains of flies expressing the CaLexA construct under the control of the 0273-Gal4 (a-d) and TH-Gal4 (e-h) drivers. Flies were fed either (a,e) drug-free food or (b,f) food containing 0.5 mg/ml caffeine for 24 hours prior to dissection and staining. The GFP intensity was quantified on a cell-by-cell basis in each brain, with

n=20 brains per treatment group. (c,g) The average cell intensity for each brain is plotted for drug-free and caffeine-fed groups. (d,h) The number of visible GFP-positive cells in each brain is plotted for drug-free and caffeine-fed groups. Large horizontal line reflects the average, and error bars show standard deviation.



increased neuronal activity in the PAM cluster neurons.

As a control, we quantified the activity-induced calcium signal in a different set of dopaminergic neurons, the PPM3 cluster. These neurons form a wake-promoting circuit projecting to the dFB (Ueno et al., 2012). After 24 hours of caffeine exposure, these flies showed no increase in GFP signal than flies given drug-free food. This indicates that the effect of caffeine on neuronal activity is somewhat specific to the PAM cluster and not all dopaminergic or wake-promoting neurons.

DISCUSSION

Many features of human sleep are observed in *Drosophila*, and the fruit fly has been an invaluable tool in identifying sleep regulatory mechanisms. As in humans, caffeine treatment in *Drosophila* increases wakefulness, lowers arousal threshold, and fragments sleep (Andretic et al., 2008; Wu et al., 2009; Roehrs and Roth, 2008). While the arousal-promoting effects of caffeine are beneficial to humans during the day, they can be disruptive to successful sleep at night. Thus, it is important to identify all the mechanisms through which caffeine affects brain function.

Most effects of caffeine, including promoting arousal, have been studied in the context of adenosine receptor antagonism. Caffeine can bind mammalian adenosine receptors, antagonizing A1 and A2a subtypes with equal affinity in vitro and in vivo (Fredholm and Lindström, 1999; Fredholm et al., 2001). Caffeine also shows a psychomotor profile consistent with non-specific adenosine receptor antagonism (Karcz-Kubicha et al., 2003). Studies in mice have implicated adenosine signaling in caffeine-induced arousal, demonstrating that global or nucleus accumbens-restricted knockdown of A2A receptors blocks the response to caffeine (Huang et al., 2005; Lazarus et al., 2011). These studies, however, only measured the acute response to caffeine, measuring wakefulness during a 3-hour window following a single injection of caffeine in a naïve mouse. This paradigm does not mimic a coffee-sipping human consuming caffeine, nor does it account for sleep effects on a longer time scale.

The physiological and behavioral effects of caffeine seem to be dependent on concentration, mode of administration, and chronic versus acute exposure (Reviewed in Ferré, 2008). For example, animals show no locomotor response to chronic caffeine administration, since they rapidly develop tolerance; however, chronic caffeine exposure reduces sleep persistently (Finn and Holtzman, 1986; Roehrs and Roth, 2008). Indeed, chronic caffeine administration has very different effects and pharmacology to acute administration (Jacobson et al., 1996). The divergent effects seen with different administration paradigms support the notion that caffeine likely has a complex mode of action. While adenosine receptor antagonism may be involved in acute behavioral changes following caffeine injection, other mechanisms may be at play in the prolonged effects of caffeine on sleep and arousal. Previously, the wake-promoting effect of chronic caffeine feeding was shown to be independent of the one known adenosine receptor in *Drosophila* (Wu et al., 2009). This finding makes *Drosophila* a unique model for studying adenosine-independent mechanisms of caffeine response.

Here, we demonstrate a requirement of the neurotransmitter dopamine for the effect of caffeine on sleep in *Drosophila*. We used an *ad libitum* feeding of caffeine-containing food while monitoring sleep constantly for five days. We observed a strong reduction of sleep during every night of caffeine exposure and this effect was dose-dependent. Mutants that do not produce dopamine, however, were resistant to the wake-promoting effect of caffeine. In addition to promoting wake, caffeine lengthens the period of circadian rhythms in the bread mold *Neurospora crassa*, flies, and mice (Feldman et al., 1975; Wu et al., 2009; Oike et al., 2011). We demonstrate that the dopamine-deficient *DTHg^{FS±}* flies are also resistant to caffeine-induced period lengthening. While the effect of caffeine on the *DTHg* control flies is much more modest than for iso31 flies, both genotypes do show statistically significant lengthening of circadian period. The difference in magnitude of caffeine response between these two genotypes may have to do with differences in expression level or pattern between the endogenous DTH locus in wild type iso31 flies and the DTH transgene expressed by *DTHg* flies.

Previous studies have suggested that dopaminergic signals modulate some clock-controlled behaviors. One cluster of clock neurons, the large ventral-lateral neurons (ILNvs), are wake-promoting cells that express the dopamine receptor, dDopR, and receive dopaminergic inputs that may promote wake in the absence of light (Shang et al., 2011). The Birman lab found that dopamine-deficient *DTHg^{FS±}* flies are defective in circadian entrainment and phase shifting in response to dim light cues, however the mechanism and relevant cells for this behavior were not identified (Hirsh et al., 2010). No previous report has demonstrated an effect of dopamine on the pace of the circadian clock (Shang et al., 2011). The finding that both the wake-promoting and period-lengthening effects occur through the same dopaminergic mechanism is surprising and novel.

Dopamine is known to be a potent wake-promoting neurotransmitter, so we hypothesized that caffeine promotes wakefulness by enhancing dopaminergic signaling. This was supported by some studies in *Drosophila* although these did not examine a role for dopamine synthesis and release (Andretic et al., 2005; Ganguly-Fitzgerald et al., 2006; Kume et al., 2005). Our data suggest that caffeine acts presynaptically, upstream of L-DOPA. This was indicated by the experiment showing that L-DOPA could not restore caffeine responsiveness to *DTHg^{FS±}* flies even though it did reduce sleep in these mutants, indicating that they contain both the neural circuitry and receptors by which dopamine can promote wakefulness. Lack of a response to caffeine is not due to a floor effect, as the *DTHg* flies show a strong reduction in sleep with L-DOPA and an even more marked decrease with the addition of caffeine.

Caffeine has been linked to dopaminergic signaling in mammals; however, this link has always invoked adenosine receptors, which dimerize with and inhibit dopamine receptors (Salmi et al., 2005). Interestingly, though, two laboratories found that dopamine levels rise in the brains of mice after acute caffeine administration (Solinas et al., 2002, Okada et al., 1996). In addition, increased extracellular dopamine causes caffeine hypersensitivity, as shown in both dopamine transporter (DAT) mutant mice and DAT (*fumin*) mutant flies (Wisor et al., 2001; Andretic et al., 2008). A recent study shows that humans carrying a polymorphism associated with lower

expression of the dopamine transporter are also hypersensitive to caffeine (Holst 2014). In fact, almost all known psychostimulants promote arousal by enhancing dopaminergic signaling via different mechanisms (Nishino et al., 1998). This collection of observations does not suggest a mechanism, but does imply that the dopaminergic mechanisms at play in *Drosophila* may be relevant to humans.

We confirmed that we could block the effect of caffeine on sleep by perturbing other steps in dopamine signaling as well. Mutants for the vesicular monoamine transporter (DVMAT) are also resistant to caffeine. These flies cannot package monoamine neurotransmitters, including dopamine, into presynaptic vesicles, and have depleted tissue dopamine by HPLC (Simon et al., 2009). Proper dopamine balance also depends on dopamine turnover. The turnover process is initiated by a few different enzymes, each of which conjugates dopamine molecules to functional groups that tag them for degradation. In glia, the enzyme ebony conjugates dopamine to a beta-alanyl group (Hodgetts and Konopka, 1973; Richardt et al., 2003). Dopamine is also conjugated to N-acetyl group by Dopamine N-Acetyltransferase (Dat) throughout the nervous system and gut (Brodbeck et al., 1998). Dopamine balance is important for sleep and circadian behavior, supported by the finding that ebony mutants (e^1) have disrupted circadian rhythms of locomotor activity and Dat mutants (Dat^{l0}) have an elevated homeostatic rebound following sleep deprivation (Newby and Jackson, 1991; Suh and Jackson, 2007; Maranda and Hodgetts, 1977). Interestingly, e^1 mutants respond to caffeine, while Dat^{l0} mutants do not. This suggests that maintenance of dopamine levels in neurons, not in glia, is essential for the ability of caffeine to promote arousal.

The *Drosophila* brain contains many clusters of dopaminergic neurons, which are characterized by expression of DTH. These clusters are defined by locations of the cell bodies, as well as the main anatomical targets of their projections (Mao and Davis, 2009). By acutely silencing subsets of neurons, we identified one group whose signaling is required for caffeine-induced sleep loss. This group of neurons is defined by the InSite0273-Gal4 driver line, which expresses primarily in the PAM cluster of neurons (Burke et al., 2012). This cluster of neurons

primarily projects to the medial portion of the mushroom body beta lobe (Claridge-Chang et al., 2009). This is consistent with the finding that the DopR dopamine receptor is required in the mushroom bodies for the caffeine response, suggesting a circuit through which caffeine increases dopamine signaling (Andretic et al., 2008). Interestingly, previously-defined groups of wake-promoting neurons were dispensable in the caffeine response. Th-D4-Gal4, Th-D1-Gal4, and TH-G1-Gal4, which promote wake when driving the heat-sensitive cation channel TrpA1, did not block the wake-promoting effect of caffeine when silenced (Liu et al., 2012). Therefore, the wake-promoting circuit responsible for the caffeine response seems to be distinct from previously-identified circuits.

We confirmed that the PAM cluster neurons are indeed modulated by caffeine by demonstrating that caffeine feeding causes increased neuronal signaling in InSite0273-labeled cells. For these experiments, we used the CaLexA tool, which translates calcium increases from sustained neural activity into GFP reporter expression (Masuyama et al., 2012). 24 hours of caffeine exposure leads to increased calcium-dependent GFP expression in the PAM neurons, both in terms of average fluorescence intensity per cell and the number of visibly labeled cells per brain. The InSite0273-Gal4 line expresses in about 130 neurons; however, only between 4 and 22 showed visible activity-dependent GFP expression at baseline, which increases to between 11 and 42 when the flies were fed caffeine. This indicates that only a subset of PAM neurons is highly active at baseline and is activated by caffeine. Interestingly, silencing cells labeled by the InSite0104-Gal4 line, which expresses in about 40 of the PAM neurons, is not sufficient to block the caffeine response (Burke et al., 2012). Additional studies will be required to characterize the nature of caffeine's effect on PAM neurons; it is possible that all PAM cells increase activity slightly following caffeine feeding, pushing a few additional cells above the detection threshold, or that a distinct group of cells which are not active at baseline respond to caffeine and become GFP-positive.

How caffeine actually increases dopaminergic signaling is still unclear, and will be an interesting topic of further study. The ability of caffeine to promote arousal has been found to

require PKA signaling in the brain, but the relevant neurons have not been identified. It is possible that caffeine activates PKA in dopaminergic neurons by inhibiting phosphodiesterases (PDEs) (Fredholm et al., 1999; Ribeiro and Sebastiao, 2010). Another possible mechanism by which caffeine could increase neuronal activity is by activating ryanodine receptors, which are the major mediators of activity-induced calcium release in the cell (McPherson et al., 1991). Caffeine may also be acting on other cell surface receptors, or perhaps on an as-yet-unidentified adenosine receptor in the fly. Another interesting question for further experiments is why the PAM cluster neurons are specifically sensitive to caffeine. These neurons may express higher levels of the sleep-relevant caffeine target molecules, or may simply be the only sleep-modulating neurons among a broad class of caffeine-sensitive cells. To our knowledge, this is the first set of studies implicating adenosine-receptor-independent modulation of dopaminergic signaling in the arousal-promoting effects of caffeine.

CONCLUSIONS AND FUTURE DIRECTIONS

Many different neurotransmitters, neuropeptides, and neuromodulators have been implicated in the control of sleep and wake behavior. Here, we have studied on class of neuromodulators called monoamines, each of which is required for normal sleep behavior (Cirelli, 2009; Nall and Sehgal, 2014). The sleep-relevant pathways for each monoamine neurotransmitter involve signaling to several different brain regions— the MB, the FB, the PI, and the ventral lateral neurons. Each of these brain regions receives input from several different neuromodulators and in turn sends outputs that contribute to sleep, circadian rhythms, and other behaviors. For example, information about metabolic state, sleep drive, and mating cues must all be evaluated to dictate whether the fly should be eating, sleeping, or courting.

One mechanistic commonality among all of the monoamines is their packaging into synaptic vesicles. All of the monoamines rely on the vesicular monoamine transporter (dVMAT), of which there is only one in *Drosophila* (Greer et al., 2005). Knocking out dVMAT prevents the vesicular packaging of dopamine, octopamine, and serotonin, thereby promoting their breakdown and depleting them from the nervous system. dVMAT-null mutants are defective in exocytotic aminergic signaling from neurons. They display defects in many different behaviors, which is not surprising considering the long list of behaviors previously found to be modulated by monoamines (Simon et al., 2009).

To determine which neurotransmitters are sufficient to drive different behaviors, Chen et al. (2013) went about restoring dVMAT function to subsets of neurons in an otherwise mutant background. Using this approach, the authors found that signaling from octopaminergic neurons alone rescues female fertility, viability, and larval locomotion, whereas either the octopaminergic neurons or the dopaminergic neurons can restore male fertility. dVMAT only has to be restored to a single monoamine system, regardless of which one, to restore wild-type startle-induced hyperactivity. Circadian rhythmicity, which is disrupted in the dVMAT mutant, can be restored by

expressing dVMAT using any two drivers, be they dopaminergic and octopaminergic, dopaminergic and serotonergic, or octopaminergic and serotonergic (Chen et al., 2013). This finding is of particular interest because it illustrates how multiple different neuromodulatory systems can compensate for the lack of others to establish the very robust phenotype of behavioral rhythmicity. Loss of rhythmicity is almost never advantageous; therefore, changes in levels of individual neuromodulators in response to environmental cues should not be able to easily alter circadian control of locomotion.

In a small molecule screen for sleep-altering compounds, an inhibitor of dVMAT was discovered as a strong sleep-inducing drug. The dVMAT mutant was found to also have significantly increased sleep and was resistant to the effects of reserpine. Flies mutant for biosynthetic enzymes for each monoamine were fed reserpine, but each displayed the increased sleep caused by the drug. Therefore, no single monoamine system could be identified as the sleep regulator causing the entire dVMAT mutant sleep phenotype (Nall & Sehgal, 2013). It is likely that the different monoamine systems work in parallel, with each communicating information about physiological and environmental situations into a single or multiple sleep circuits, resulting in finely tuned, contextually appropriate behavior.

In addition to multiple monoaminergic systems creating a robust and layered regulatory network for behavior, other mechanisms of plasticity can compensate for loss of dVMAT. One study proposed the importance of developmental compensation by observing that reserpine fed acutely in adulthood causes hypoactivity whereas dVMAT mutants that have chronically eliminated exocytotic aminergic signaling display hyperactivity (Simon et al., 2009). The ability of flies to compensate for reduced monoamine signaling is dependent on genetic background. dVMAT mutants with a CantonS background show the same elevated sleep phenotype as flies acutely fed reserpine, but dVMAT mutants in an iso31 genetic background develop relatively normal sleep behavior in adulthood, even without dopamine, serotonin, octopamine, or other monoamines (Nall & Sehgal, 2013). The mechanisms by which this compensation occurs are still unknown, but they are among the important questions in the circuit-wide and systems-wide study

of behavior. Studying compensatory mechanisms will tell us more about how the many tightly integrated signaling pathways interact to confer robustness of behavior.

Thus far, research has largely focused on how individual neuromodulators regulate individual behaviors. However, we are beginning to understand that the signaling molecules are part of a complex network of interconnected circuits that communicate with each other. To determine how these circuits interact, we will need to simultaneously study multiple signaling molecules using combinations of genetic and molecular manipulations. A recent study by Burke et al. (2012) determined epistasis on a cellular level to demonstrate that octopaminergic control of short-term appetitive memory actually signals through dopaminergic neurons, which in turn project to the MB. This approach to elucidating interactions between circuits will prove useful in determining networks underlying other complex behaviors and perhaps shared networks that link multiple behaviors. In the case of the VMAT mutant, mapping neurons where VMAT is necessary and sufficient to drive normal patterns of sleep and wake will be informative. Our work has shown that in this mutant, baseline sleep, homeostatic rebound, and sleep latency seem to be separable aspects of sleep behavior which can each be studied to gain a deeper insight into regulatory circuits which may act separately, convergently, or epistatically.

Various studies have attempted to map the sleep and wake circuits in *Drosophila*, and these studies have implicated multiple different brain structures and neuronal populations. In addition to multiple neurotransmitters contributing to sleep/wake regulation, different brain structures have also been found to be involved. The pars intercerebralis (PI) relays octopaminergic wake-promoting signals and the fan-shaped body (FB) seems to be a sleep-promoting brain region that is inhibited by dopaminergic signals (Crocker et al., 2010; Ueno et al., 2012; Liu et al., 2012). In our studies, we mapped a group of dopaminergic neurons which are required for the wake-promoting effect of caffeine. These neurons, the PAM cluster neurons, are distinct from the PPL1 and PPM3 dopaminergic neurons that have been identified as wake-promoting in the past. While the PPL1 and PPM3 project to the fan-shaped body to promote

wake, the PAM cluster projects primarily to the mushroom body (MB) (Ueno et al., 2012; Liu et al., 2012; Burke et al., 2012).

The mushroom body has previously been identified as a brain region that promotes sleep, as ablation of this structure leads to increased arousal (Pitman et al., 2006; Joiner et al., 2006). However, increasing PKA signaling or neuronal excitability using some mushroom body Gal4 drivers increases sleep while promoting activation of a different, non-overlapping group of neurons in the MB causes a sleep decrease, indicating that the MBs likely contain both sleep-promoting and wake-promoting cell groups (Joiner et al., 2006).

The MB expresses dopamine receptors to allow it to respond to signals from neurons such as the PAM cluster. We hypothesize that the PAM neurons convey wake-promoting signals to the MB, and these signals are amplified by caffeine. The dopamine type 1 receptor, dDopR, which is required in the MB for the caffeine response, is the likely recipient of these wake-promoting signals. The MB is a brain region that was previously implicated in learning and memory. The roles of the MB in sleep and learning may be mechanistically linked. Sleep deprivation impedes learning in fruit flies, similarly to more complex animals, and this decrement is exacerbated by drugs that decrease dopamine signaling and by mutation of dDopR (Seugnet et al., 2008). On the other hand, increasing dDopR signaling, either by feeding agonists or by overexpressing the receptor in the MBs, was able to rescue learning and memory after sleep deprivation as effectively as recovery sleep (Seugnet et al., 2008). Caffeine can also reduce cognitive impairment following sleep deprivation, further underscoring that this dopaminergic innervation of the MB is targeted by caffeine (Alhaider et al., 2010).

In addition to sleep affecting learning and memory, learning also affects sleep. Flies appear to modulate the amount of sleep they get depending on prior waking experience; flies exposed to a socially enriched environment sleep more than those that are individually housed. Context-dependent sleep change is disrupted by either augmenting or impeding dopaminergic signaling (Ganguly-Fitzgerald et al., 2006). Further research is required to understand the true nature of the connection between sleep and learning and the extent to which sleep circuits and

learning circuits overlap. Determining the specific MB neurons that express dopamine receptors and mapping their inputs will be useful in dissecting the various behavioral roles for the MB. New tools are available to assist in this undertaking, including restricted Gal4 lines that express in subsets of MB neurons. These Gal4 lines can be used to monitor and alter the behavior of these cells by driving expression of biosensors or modulators of neuronal activity (Pech et al., 2013).

In addition to the PI, FB, and MB, an increasing body of evidence is also implicating the circadian clock neurons in regulation of sleep and wake behavior. Originally, it was thought that the circadian and homeostatic control of sleep occurred via separate mechanisms and that circadian clock neurons only conveyed time-of-day information to downstream circuits (Borbély, 1982). It is now clear that clock neurons can receive modulatory inputs themselves that may modulate sleep and wake behavior in response to cues besides the normal zeitgebers of light and temperature. One group of central clock neurons, the large ventral lateral neurons (-ILNvs), are light-sensitive wake-promoting cells that express receptors for many different internal neuromodulatory molecules (Shang et al., 2011). Histaminergic control of sleep maps to the clock neurons; expression of HisCl1 in these cells is necessary and sufficient for normal sleep behavior (Oh et al., 2013). An earlier anatomical study identified histaminergic projections from extraocular eyelet photoreceptors to the ventral lateral neurons, which could be the wake-promoting histaminergic circuit (Hamasaka & Nässel, 2006). The serotonin receptor d5-HT1B is expressed in clock cells in the brain, where it likely acts to modulate circadian entrainment (Yuan et al., 2005). However, it is also expressed in the MBs and PI, where it could potentially modulate the sleep circuit on the basis of light and circadian cues. The ILNvs also respond to octopamine by increasing cAMP levels in the dark, and sensitivity of these cells to octopamine is under clock control (Shang et al., 2011).

The responsiveness of the ILNvs to many different inputs makes them an attractive candidate for future studies on the consolidation of internal and external cues into a single behavioral program. Indeed, some previous studies have established a precedent for clock cell involvement in integration of environmental cues and cellular and molecular context to coordinate

sleep and wake behavior at appropriate times and in appropriate situations. For example, the ILNvs promote wake in a dopamine-independent manner in the light phase, but respond to dopamine in the night (Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008). Light suppresses dopamine's wake-promoting effects by upregulating inhibitory dopamine receptors (dD2R) in the ILNvs (Shang et al., 2011). Another study showed that clock cells are involved in prioritizing sleep and foraging behavior, indicating that the clock genes *clock* and *cycle* are required for the suppression of sleep behavior in starvation conditions (Keene et al., 2010). In our studies, we found that caffeine can not only suppress sleep, but can lengthen the circadian period, and that these effects are both dependent on dopamine. It is possible that, while the effect on sleep maps to neurons projecting to the MB, that the period-lengthening effect may act through dopaminergic neurons projecting to the clock cells. This mechanism may be relevant to mammalian systems as well, since activation of dopamine type 1 receptors has been shown to alter clock gene expression, indicating functional interactions of dopamine signaling with clock cells (Imbesi et al., 2009). Future experiments will need to map the dopaminergic neurons responsible for the circadian caffeine effect and query whether dopamine receptors in clock cells are required.

Receptors that receive monoaminergic inputs are usually G-protein coupled receptors (GPCRs; Marraziti et al., 2009). These traverse the membrane and couple ligand binding to intracellular signaling by activating different G-proteins. Most of these receptors either activate or inhibit adenylate cyclase, which produces cyclic adenosine monophosphate (cAMP; Uzzan and Dudai, 1982; Nall and Sehgal, 2014). Among other signaling cascades, cAMP activates protein kinase A (PKA), which in turn activates the transcription factor cAMP response element-binding protein (CREB; Lonze and Ginty, 2002). This signaling pathway is essential for regulating sleep and wake behavior, with CREB mutant mice showing an increase in time spent in non-rapid eye movement (NREM) sleep and a decrease in time spent awake (Graves et al., 2003). Consistent with a wake-promoting role for CREB, acute inhibition of CREB signaling in *Drosophila* causes increased homeostatic rebound following sleep deprivation (Hendricks et al., 2001). Conversely,

expressing a constitutively-active form of PKA in all neurons results in a marked reduction of nighttime sleep (Joiner et al., 2006). More focused studies have shown that PKA signaling in the MB underlies the control of sleep and wake by that brain structure (Joiner et al., 2006). PKA signaling in the PI is responsible for the control of sleep by octopamine (Crocker and Sehgal, 2008). In addition, blocking PKA signaling acutely in all neurons using a pan-neuronal inducible Gal4 (elav-Geneswitch) increases the number of minutes of sleep per day (Wu et al., 2009). Interestingly, blocking PKA acutely using a mushroom-body-specific inducible Gal4 (MB-Geneswitch) did not cause a change in sleep behavior (Wu et al., 2009).

PKA signaling is required for the ability of caffeine to promote wakefulness in *Drosophila*. This PKA requirement does not map to the mushroom bodies, however, since blocking PKA signaling using MB-Geneswitch does not prevent the wake-promoting effect of caffeine (Wu et al., 2009). Interestingly, the dopamine receptor dDopR is required in the mushroom body for the response to caffeine, and this receptor is coupled to adenylate cyclase (Sugamori et al., 1995). It is possible that the regions of the mushroom body receiving the dDopR1-mediated wake-promoting caffeine signal are not targeted by MB-Geneswitch or that dDopR1 couples to a different second messenger cascade in the MB as has been observed in the other *Drosophila* type 1 dopamine receptor dDopR2 and in mammals (Reale et al., 1997; Beaulieu et al., 2011). The requirement of PKA signaling in non-MB neurons suggests that perhaps this signaling molecule is required in a different step of the caffeine-sensitive circuit. We find that calcium release is increased in the MB-projecting PAM neurons following caffeine feeding, but further experiments will be required to demonstrate whether cAMP/PKA signaling is also upregulated in these cells. In addition, it will be interesting to observe whether inhibiting PKA signaling in this set of cells can block the caffeine response.

Caffeine increases PKA activation and CREB phosphorylation in the brains of mice, suggesting shared mechanisms with *Drosophila* (Zeitlin et al., 2011). Increases in PKA activation may be attributable to inhibition of cAMP-degrading phosphodiesterases or perhaps to direct interactions with adenylate cyclase-interacting receptors. Another similarity between work done

in *Drosophila* and mammalian systems is the observation of impacts on dopaminergic signaling, such as increases in extracellular dopamine, following caffeine administration (Solinas et al., 2002, Okada et al., 1996). Even caffeine-induced dopamine release has been attributed in mammals to antagonism of adenosine receptors – in this case, presynaptic adenosine receptors which inhibit dopamine autoreceptors. However, it is also possible that the presynaptic action of caffeine in mammals is adenosine-receptor-independent and shares the same mechanisms we observe in *Drosophila*. Indeed, adenosine-receptor-independent effects of caffeine have been described; identical aversive and appetitive responses to caffeine are observed in both wild type mice and mice lacking both A1 and A2A adenosine receptors (Sturgess et al., 2010).

Activation of ryanodine receptors may be another physiologically-relevant caffeine action, as ryanodine receptor type 3 mutants have attenuated dopamine release following caffeine administration (Wan et al., 1999). Ryanodine receptor activation may underlie the increases in calcium signaling that we observed in the PAM cluster neurons in *Drosophila*. Mobilization of calcium stores by ryanodine receptors triggers dopamine release in mammals, and ryanodine receptor activation can lead to circadian clock phase shifts (Ding et al., 1998; Patel et al., 2009). It is possible that these effects occur in *Drosophila* as well, and may explain the link between neuronal activity, dopaminergic signaling, sleep, and circadian period. Further studies will be required to determine the sleep-relevant caffeine targets in both mammals and *Drosophila*, whether it be PDE inhibition, ryanodine receptor activation, direct dopamine receptor interactions, or some other mechanism. Discovering the pathways by which caffeine can promote wake will also inform us about wake-promoting signaling pathways and neuronal circuits that may yet be unidentified.

In the combined experiments of this document, we have demonstrated an importance of multiple interacting neurotransmitter systems in establishing various aspects of baseline sleep behavior and of presynaptic modulation of these systems by pharmacological agents that change sleep/wake patterns. Acute blockade of synaptic transport of monoamines causes increased sleep; however, chronic lack of dVMAT can be bypassed by other mechanisms, demonstrating

how plastic the neuronal controls of this essential behavior can be. We also demonstrated that caffeine can promote wake by upregulating dopaminergic signaling presynaptically, which has not yet been demonstrated. In addition, we showed that the relevant caffeine-sensitive neurons belong to a cluster that had previously not been shown to be involved in the regulation of sleep and wake. Both of these bodies of work highlight the power of acute pharmacological approaches to discovering the regulation of behavior in adulthood. They also both underscore the myriad parallel and interacting pathways that regulate sleep. It is still not clear how the various sleep pathways interact, and what is the relative contribution of the various monoamine neurotransmitters – dopamine, serotonin, octopamine, and histamine – as well as the multiple sleep-regulatory brain structures – the PI, FB, MB, and ILNvs. Whether these circuits converge spatially and how they can turn a complex body of contextual information into a unified behavioral program are still open questions. Studying how these different circuits are prioritized in different environmental contexts or in response to different stimuli will be an essential next step in determining the purpose of this apparent redundancy. This work will contribute enormously to our understanding of how we maintain the behavioral plasticity necessary for survival in an ever-changing, unpredictable world.

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