Effects of micro-RNA 190 on sleep regulation in *Drosophila melanogaster*

Senior Thesis

Presented to
The Faculty of the School of Arts and Sciences
Brandeis University

Undergraduate Program in Neuroscience
Leslie Griffith, Advisor

In Partial Fulfillment of the Requirements for the Degree of Bachelor of Science

By

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April 2016

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1. Abstract

Sleep causes changes in gene expression which result in improved cognition and overall better health. Because micro-RNAs (miRs) are important regulators of gene expression, a screen for miRs that are involved in sleep was performed by the Griffith Lab using the fruit fly *Drosophila melanogaster*. This screen implicated miR-190 as an important regulator of sleep. To verify that miR-190 is required in neurons for its effects on sleep, I used the GAL4/UAS system to express a miR-190 inhibitor in neurons only. Total sleep was significantly decreased, as was mean sleep episode duration. I attempted to further localize the activity of miR-190 by inhibiting the miR in a variety of neuronal subsets and general brain areas. In brain areas such as the fan-shaped bodies, which have been implicated in homeostatic regulation of sleep, lack of miR-190 reduced total sleep and elicited a fragmentation phenotype, suggesting that miR-190 could be partly responsible for regulating sleep consolidation. This phenotype recurred in many brain areas and neuronal populations, suggesting that the need for miR-190 function for consolidated sleep is widespread throughout the brain. Using CRISPR/cas9 genome editing, fly lines with endogenous mutations in the mir-190 gene were created. When their sleep was tested, it was indistinguishable from experiments performed with a miR-190 inhibitor expressed in neurons, increasing the validity of previous results. Overall, miR-190 is important in the *Drosophila melanogaster* brain for sleep regulation. A better understanding of this function may yield insight into human sleep disorders and other chronic illnesses.
1. Introduction

An introduction to sleep

Sleep – that mysterious behavior that faithfully occurs every night, leaving humans immobile and effortlessly cycling through sleep stages. If done “properly,” this strange process results in well-rested Homo sapiens cognitively ready to start their day. However, despite the obvious benefits of this behavior, scientists have not yet discovered the true purpose sleep, if there is one. And, interestingly enough, even in the 21st century, not all scientists are convinced that all other members of the Animal Kingdom partake in the same behavior (Siegel, 2008). The fact that people so readily agree that sleep exists in mammals, but may not in “lower” animals prompts this discussion about theories of the purpose of sleep, leading to a still-precursory definition of sleep and its known components in humans, and corresponding discoveries in animal models. Only after such a relatively-unbiased comparison can conclusions be made about the nature of sleep at all.

Theories on the purpose of sleep

Why do we sleep? Out of this persistent question, a growing body of sleep research has begun to help shed light on this query. Many emerging theories of sleep are just beginning to explain why this behavior is advantageous. One hypothesized role of sleep is to modulate synaptic plasticity in the brain. The “synaptic homeostasis” hypothesis posits that the role of slow-wave sleep is to downregulate the increases in synaptic strength that occurred over the course of waking (Tononi and Cirelli, 2006). One way that synaptic strength can be increased is long term potentiation (LTP), which can be achieved by insertion of glutamate receptors into the neuronal membrane (Cooke and Bliss, 2006). For example, if a certain environmental stimulus is repeatedly attended to during wake, the synapse will strengthen over the course of the day. This synapse experiences an increase in synaptic strength by LTP and has some glutamate receptors inserted into the membrane. Perhaps during a night of sleep, this synapse
experiences a reduction in the number of these AMPA receptors, but only in proportion to every other synapse on that neuron, so that the relative strengths remain the same, presumably as does the memory (Tononi and Cirelli, 2006). This “housekeeping” function would make synapses more maintainable in the extremely compact brain.

However, this “synaptic homeostasis” hypothesis has been challenged in recent years. Tononi et al. specifically emphasized the importance of slow-wave sleep in synaptic downscaling but new research six years later also found an upscaling of synapses during the Non-REM-REM sleep cycle (Chauvette et al., 2012). This might indicate a more general role of sleep in synaptic regulation (which includes both upscaling and downscaling of synapses) during the night (Born and Feld, 2012).

Another interesting hypothesis is that sleep helps restore energy to the brain and helps eliminate toxins and other waste in a “metabolic homeostasis” model (Xie et al., 2013). Finally, sleep has been found to be very important for both learning and memory. This brings some researchers to support the “replay hypothesis”: that sleep helps consolidate memories by essentially replaying them during sleep (Breton and Robertson, 2013). Each of these models poses an interesting possibility for why animals sleep, but overall, there is not enough evidence to strongly support any one hypothesis and exclude the others. Future studies might reveal more than one valid explanation for sleep that combines these current theories.

**An overview of human sleep**

Sleep research in humans has been conducted for centuries, yet there still remains much to learn about this complex behavior. What is abundantly clear is that sleep is a necessary behavior: it cannot be avoided and there are clear cognitive consequences when a certain amount of sleep is not obtained (Alhola and Polo-Kantola, 2007). Currently, a working definition of sleep can be said to include five main criteria: (1) rapid reversibility of the behavior, (2) consolidated circadian periods of immobility,
(3) homeostatic regulation, (4) distinct oscillatory activities in the Central Nervous System, and (5) higher arousal thresholds than those in the wake state (Potdar and Sheeba, 2013).

**The rapid reversibility of human sleep**

One of the most obvious characteristics of sleep – and the one that marks the distinction between sleep and coma – is that it is rapidly reversible. Narcolepsy is a prominent pathophysiological example of just how rapid and occasionally unexpected these wake-to-sleep transitions can be (Akintomide and Rickards, 2011). Astonishingly, by examining patients affected by the influenza epidemic of 1915-1924, Constantin Von Economo formulated the remarkable hypothesis that the reversibility of sleep is mirrored in the underlying neuronal circuitry that controls the transition between sleep and wake states (Kaya et al., 2016). When Saper and his colleagues later elucidated the exact circuitry, they confirmed that there are simple, mutually-inhibitive interactions between two distinct brain regions. They coined this circuit the “flip flop switch” because of this switch-like, rapidly reversible manner in which it controls the transition between sleep and wake (Saper et al., 2010).

Overall, on a very basic level, there are two kinds of neurotransmitters and corresponding pathways: wake-promoting and sleep-promoting. While this binary classification of neurotransmitter systems as “sleep-promoting” or “wake-promoting” is most likely an over-simplification, since one neurotransmitter can be involved in more than one kind of pathway, it is useful to make gross comparisons of neurochemical transmission systems between species. Wake-promoting neurotransmission in humans mainly occurs in two ascending arousal pathways; one that projects from the upper brainstem and innervates the thalamus, and a second which innervates portions of the hypothalamus and the forebrain (Saper et al., 2010). Wake-promoting cell populations include those that are noradrenergic, serotonergic, dopaminergic, histaminergic and cholinergic. The integration of these inputs from ascending arousal pathways occurs in the ventrolateral preoptic nucleus (VLPO) of the
brainstem, which is GABAergic and inhibits the wake-promoting circuitry presumably when indirect circadian inputs from the suprachiasmatic nucleus (SCN) indicate that it is nighttime. When daytime cues arrive again, ascending arousal pathways will inhibit the VLPO, and the cycle begins anew (Saper et al., 2010). This two-component mutual inhibition allows for fast transitioning between sleep and wake, as well as stable maintenance of these states.

(2) Consolidated, circadian clock-controlled periods of immobility in sleep

Although the molecular underpinnings of sleep are just beginning to be understood, the basic molecular components of the circadian clock, which determines when sleep occurs, were elucidated at the turn of the millennium. Overall, the circadian clock is the piece of cellular and molecular machinery that serves as the driving force behind many cyclical behaviors, including the cycling of sleep and wake states on a 24 hour cycle (Zordan and Sandrelli, 2015). These complex molecular forces are at work to synchronize periods of activity and rest with changes in environmental cues such as light, which is why diurnal animals show a pattern of being awake during the daylight hours (or nighttime hours, for nocturnal animals,) and sleep when the world becomes dark (Zordan and Sandrelli, 2015). The cyclical regulation of these pathways comes from self-regulatory transcriptional and translational feedback loops (Zordan and Sandrelli, 2015). Two of the most important transcription factors involved in this process in flies are CLOCK and CYCLE. When they form a heterodimer, they start promoting transcription of the period (per) and timeless (Chauvette et al.) genes (Zordan and Sandrelli, 2015). When the PER and TIM proteins associate, they can then enter the nucleus and inhibit the activity of the CLOCK-CYCLE heterodimer (essentially turning themselves off) (Zordan and Sandrelli, 2015). The mammalian homolog of TIM is called CRY (which is a photoreceptor in Drosophila) and the orthologous CYCLE called BMAL1 (Zordan and Sandrelli, 2015). One of main differences between mammalian circadian clocks and the Drosophila clocks is the way by which light synchronizes the clock (Zordan and Sandrelli, 2015). In
mammals, light enters the eye and activates the Suprachiasmatic Nucleus in the brain, where there is a signal cascade that ends in transcription of Per (Zordan and Sandrelli, 2015). In Drosophila, light synchronization is cell-autonomous (Zordan and Sandrelli, 2015). When light enters the cells, TIM is degraded, which affects how PER is processed in the cell (Zordan and Sandrelli, 2015). Finally, in order to reset the Drosophila clock, the blue-light photoreceptor CRY associates with TIM in presence of light, which then causes TIM to degrade (Zordan and Sandrelli, 2015).

(3) Sleep is under homeostatic control

Homeostasis is a general biological term which refers to dynamic regulation of a process around a set point. Most important biological processes must be kept within narrow ranges in order to function optimally. Since sleep is an essential process, its homeostatic regulation is not surprising. Thus, in terms of sleep regulation, the circadian clock itself constitutes only half of the regulatory machinery. Dubbed the “sleep homeostat,” this piece of molecular machinery has not yet been isolated. However, it is known to interact with the circadian clock much like the components of the circadian clock interact with themselves; in regulatory feedback loops (Daan et al., 1984). The main purpose of the sleep homeostat during normal conditions is to maintain sleep homeostasis; which, on the simplest level can be thought to conserve total sleep time. This accumulation of sleep pressure signals to the body that sleep is needed (Daan et al., 1984). In cases when the goal of sleep is achieved, sleep pressure then decreases as a function of time spent asleep. However, in circumstances when a subject does not have the opportunity to sleep and becomes sleep deprived, it is the sleep homeostat which attempts to bring sleep back to the baseline amount (the set point) (Daan et al., 1984). This compensatory sleep usually occurs at a time when the animal would be asleep (during the day for diurnal animals) in what is termed a “sleep rebound.” Importantly, this rebound is proportional to the amount of sleep that was lost.

(4) Distinct oscillatory activities in the brain associated with sleep and wake states
It may seem logical to think that since the body is asleep, the brain is too. However, EEG studies have revealed that the brain is very active during sleep and has electrical activity that is markedly different than when it’s in the wake state (Hung et al., 2013). This is conserved throughout the animal kingdom although not every animal will have the same kinds of stages (e.g. REM sleep only occurs in birds and mammals) (Campbell and Tobler, 1984).

(5) Heightened arousal thresholds in sleep

Arousal thresholds are determined by inputting sensory stimuli and measuring the level of the intensity of the stimulus that results in sleep disruption and consequent awakening. Different stages of sleep correspond to different “sleep depths”. Thus, arousal thresholds can fluctuate throughout the night, but will generally be higher during sleep than those observed in the wake state, when people are actively attending to their surroundings and its associated stimuli. Arousal thresholds can be measured in flies, and corroborate the similarity between fly and human sleep (Cirelli and Bushey, 2008).

Sleep in other animals

These five characteristics of sleep have been a useful tool to evaluate the presence of sleep in other animal models. Sleep has been found to be a universal behavior in the Animal Kingdom, and is clearly necessary for proper mental and physiological functioning. Although some parameters of sleep differ between animals, most likely due to evolutionary pressures and ecological niches, the basic defining characteristics of sleep are conserved (Campbell and Tobler, 1984).

Drosophila as a model organism for sleep studies

As captured in the title of the Hendricks et al. seminal study “Rest in Drosophila is a sleep-like state,” there was a general hesitance to declare that Drosophila sleep in the year 2000. Despite the lack of precedent in the field at that time, this study and the concurrent Shaw et al. study drew many parallels between fly “rest” and human sleep. Their pioneering arguments for fly sleep were based on
the observations that *Drosophila* exhibit higher arousal thresholds, are in an immobile state, have reduced activity, and are less responsive to external stimuli (Hendricks et al., 2000; Shaw et al., 2000).

Since then, it has been established that *Drosophila* is an extremely useful model organism within which to study sleep. Sleep in *Drosophila* is defined as a period of time longer than five minutes for which the fly is inactive (Hendricks et al., 2000). Males and females exhibit different amounts of sleep (See Figure 1). Newer findings have exhibited that electrophysiological recordings in *Drosophila* detect different power spectra of brain waves during sleep (van Alphen et al., 2013). So, *Drosophila*, like humans, cycle through different stages of sleep, strengthening the parallels between human and insect sleep. Although flies sleep both in the day and the night and have shorter sleep episodes than humans, the fact that flies also have different sleep depths indicates that integral elements of the core behavior are preserved, which validates measuring sleep in *Drosophila melanogaster*.

**Figure 1: Sleep in *Drosophila* is sexually dimorphic**

![Graph A](image1.png)

**A**

![Graph B](image2.png)

**B**

**Figure 1**: As previously reported in (Hendricks et al., 2003), *Drosophila* sleep is sexually dimorphic. **A**) Average baseline sleep for both male (n = 24) and female (n = 17) flies of the genotype w<sup>C</sup> was measured in 12 hr : 12 hr LD over a period of 4 days. Female flies of this and other wild-type strains experience significantly less sleep during the day, and overall than do males. **B**) Quantification of levels of total average sleep for same flies as in A). Total 24 hour sleep is significantly decreased in females.
compared to males (p<.0001, Mann-Whitney U test), as is total daytime sleep (p<.0001, Mann-Whitney U test). Total night-time sleep is not different (p>.05, Mann-Whitney U test).

Drosophila are also genetically tractable, have a low amount of gene duplication, and have a fast rate of reproduction that allows for a large sample size in experiments. Importantly, there are many genetic tools available in Drosophila such as the UAS-GAL4 system that allows researchers to specifically express a gene of interest anywhere in the body or brain of the fly (Brand and Perrimon, 1993). There are also myriad mutant Drosophila lines that are widely available and can be ordered online (http://flystocks.bio.indiana.edu). Finally, Drosophila sleep is comparable to human sleep (Hendricks et al., 2000; Shaw et al., 2000). Thus, Drosophila sleep is a simple but viable model for mechanisms involved in human sleep regulation.

Changes of gene expression during sleep

Sleep has been established as a state with robust electrical activity. These changes in cell membrane potential and release/receipt of cellular signals occur with many changes in gene-expression throughout the course of the night. Examples of such changes include the aforementioned circadian system, which drives a continuous looping of translational repression and activation, turning genes on and off throughout the day (even in the absence of sleep). It has recently been shown in mice that glial morphology can be altered from sleep deprivation due to changes in gene expression (Bellesi et al., 2015).

An introduction to micro-RNAs and their role in gene regulation

Micro-RNAs (miRs) are one such class of genetic regulators which could affect sleep. Micro-RNAs were first discovered in 1993 by Ambros et al., and today there are about 250 known miRs in the Drosophila genome and 1,800 in the human genome (www.mirbase.org) (Lee et al., 1993). These small, non-coding RNAs are encoded in the genome and have a wide variety of regulatory functions (He and
Hannon, 2004). Micro-RNAs can use slightly different mechanisms to regulate gene expression, but most mechanisms end with the inhibition of translation of target messenger RNA (mRNA), halting the production of its target’s specific protein (He and Hannon, 2004). Micro-RNAs are normally around 20-23 nucleotides long, and have a highly-conserved seed region, which is the first 10 nucleotides that are complementary to its target in order to bind (Maziere and Enright, 2007). This high degree of conservation in the seed sequence has indicated that this region in particular serves an important function across species in target mRNA regulation (He and Hannon, 2004). There is also a more variable hairpin loop region, which can sometimes be necessary for the function of the micro-RNA (Chang et al., 2003).

Many miRs sit within introns, while the rest can come from their own genes (Lee et al., 2004). All micro-RNAs undergo extensive processing before they arrive at their mature form (He and Hannon, 2004). The miR is considered “pri-miRNA” after it is transcribed by RNA polymerase II in the nucleus and is several hundred nucleotides long (Lee et al., 2004). Then Drosha and Pasha, an RNA-endonuclease and its partner, respectively, transform the pri-miRNA into a pre-miRNA (He and Hannon, 2004). The pre-miRNA is then transported to the cytosol, where an enzyme called Dicer then cleaves the hairpin region of the miR (He and Hannon, 2004). Binding to the RISC (RNA-induced silencing complex) then occurs, and the miR is considered fully mature and functional. When a miR encounters its target mRNA, it binds to a complementary site on the 3’ UTR, preventing the mRNA from making any protein (He and Hannon, 2004). Since the complementarity doesn’t have to be perfect for a miR to bind to an mRNA, and multiple binding sites can be found in multiple mRNA 3’UTRs, one miR can have many different mRNA targets (He and Hannon, 2004). Taken together, miRs create dynamic changes in gene expression that can respond to environmental conditions.
Many biologists have come to a consensus on the fact that most biological processes involve regulation by micro-RNAs (Luo and Sehgal, 2012). Many micro-RNAs have been implicated in being involved in the circadian clock, which is not surprising, because the circadian clock works by changing levels of gene expression which affects levels of protein: a function for which micro-RNAs are specialized (Luo and Sehgal, 2012). One example of a miR regulating an output pathway of the circadian clock is miR-279 (Luo and Sehgal, 2012). In the same year, a whole cluster of micro-RNAs (miR-959-964) were found to help regulate when Drosophila choose to eat, among other output pathways of the circadian clock (Vodala et al., 2012). Most importantly, miRs have been found to be differentially expressed in sleep and wake (Davis et al., 2007).

*Methods for manipulation of micro-RNAs in Drosophila*

Measuring a micro-RNA’s effect on sleep in Drosophila requires two main components: a genetic construct to inhibit the activity of the micro-RNA *in vivo*, and a behavioral assay to measure any corresponding changes in sleep. The UAS-GAL4 binary expression system can be used to express transgenes that inhibit miR function. The UAS-GAL4 system is a remarkable, easy-to-use tool that was invented over twenty years ago (Brand and Perrimon, 1993) and has been used by scientists ever since. UAS (Upstream Activating Sequence) is an enhancer endogenous to yeast to which the transcription factor Gal4 can bind. The union of UAS and GAL4 results in turning on expression of the inserted downstream gene of interest in a specific tissue.

Multiple methods have been developed to alter the functioning of micro-RNAs. An example mainly used in fish is the anti-sense morpholino. This method knocks down gene expression by penetrating into cells and translocating into the nucleus, where it binds to mRNA, sterically blocking other molecules from binding to it and thus preventing transcription of its genes (Summerton, 1999).
However, since morpholinos are not cell-type specific, they can cause unintended “off-target effects,” and penetrating tissues with them is a challenge. (Summerton, 1999).

A newer method called the “micro-RNA sponge” is a genetic construct that is easier to express in specific cell types. These so-called sponges have a name informative of their mechanism: they “soak up” miRs like a sponge. Sponges have inverted tandem repeats of the miR’s target mRNA (Ebert et al., 2007). By expressing the miR sponge at high levels using the UAS-GAL4 system, it becomes more likely that the miR will bind to the sponge rather than to its target mRNA. Overall, this inhibits the ability of the miR to inhibit its target, thus rendering the miR inactive and its target mRNA active. The lab of David van Vactor has created a library of *Drosophila* micro-RNA sponge lines that target 150 different miRs in the genome and can be used to test the function of specific miRs in vivo (Loya et al., 2009).

*Measurements of Sleep in Drosophila*

Once the micro-RNA of interest is inhibited in a certain cell-type of the fly, sleep can be assessed using the Drosophila Activity Monitor system (TriKinetics Inc., Waltham, MA). This consists of plastic “boards” filled with holes to fit 5 mm x 40 mm glass tubes. These DAM boards are kept in incubators that have a set light schedule and temperature to control for potentially confounding environmental factors. One fly is housed per tube, which has food at one end (usually either a cornmeal or agar-sucrose medium) and is capped at the other so the fly can't escape. An infrared beam traverses the middle of the sleep tubes in the board (Figure 1). If a fly is active and awake, it tends to “patrol” up and down the length of the tube, breaking the infrared beam. The DAM board will record a beam-break as an activity count. More than five minutes without a beam-break is classified as sleep (Hendricks et al., 2000). While this is a useful and logistically-easy model with which to measure sleep, evaluating activity as a parameter of sleep duration can be difficult, as there is always the possibility that a fly could be experiencing locomotor difficulty due to transgenic insertions or injury. If this were the case, it would be
impossible to tell when these flies were sleeping. As a proxy to control for this, activity levels (number of beam breaks) can be compared across genotypes. Any genotype which has activity levels that are significantly lower than controls can be considered to be hypoactive and a recently described video-recording method can be implemented to measure their sleep (Donelson et al., 2012).

Sleep can be quantitatively broken up into many interrelated parameters: total amount of sleep, mean sleep episode duration, frequency of sleep episodes and sleep latency, among others. The sleep data are continuously recorded by a computer, and can be analyzed with programs such as MATLAB (Mathworks, Natick, MA). Sleep analysis in *Drosophila* can thus look at any one of those aspects individually, or in combination. For example, when episode number and duration are considered together, they can act as a proxy to measure sleep consolidation. Let’s say, for instance, a normal, wild-type, well-rested fly has 20 sleep episodes throughout the day and sleeps an average of 20 minutes per episode. If that is consolidated sleep, a fly that had 40 sleep episodes and sleeps an average of 10 minutes per episode experiences the opposite of consolidated sleep, or, sleep fragmentation. Research is currently being conducted to investigate sleep fragmentation itself as a way to disrupt the beneficial aspects of sleep in *Drosophila* (Chang 2015, unpublished data) (Lin et al., 2012).

The DAM system can also evaluate sleep homeostasis. Flies can be deprived of sleep by placing DAM boards in a machine that exerts a mechanical shaking according to a programmed schedule. A typical shaking regimen might shake the flies for two seconds every ten seconds for twelve hours (the whole of their subjective night). This is believed to sufficiently deprive flies of sleep so that their compensatory “rebound” sleep can be measured. However, the stress that the flies endure from the shaking is potentially problematic: physical stress has its own effects on sleep (Brown et al., 2012), so it is unknown if the loss of sleep is purely due to the deprivation itself, or if the stressful shaking method has side effects. Hence, other, less stressful methods of sleep deprivation are currently being developed.
Sleep homeostasis is most easily observed through the lens of sleep deprivation: when one gets two hours of sleep one night, the following day arrives with an increase in “sleep pressure” (meaning one feels more tired than if they had been well rested). This sleep pressure, brought about by the sleep homeostat, is an attempt to restore homeostasis and lessen stress on the body. Mechanical sleep deprivation is a method which naturally causes an increase in sleep need in *Drosophila*, enabling researchers to examine and monitor the resulting homeostatic component of the sleeping behavior. Seidner et al. acknowledged that homeostatic “rebound sleep” is highly variable following mechanical shaking, and thus used specific criteria to distinguish between rebound sleep and regular sleep or waking states. These criteria include the observations that recovery sleep surpasses the amount of sleep an animal would normally have following a night of sleep. Secondly, this type of rebound sleep is typically contained within a temporal window of 6-12 hours after sleep deprivation. This homeostatic

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**Figure 2: Drosophila Activity Monitor System**

A) Each tube pictured on the left contains an individual fly and sucrose-agar food with a black plastic cap on top (right side of apparatus). The other end (left end of apparatus) is sealed with Parafilm so the fly cannot escape. The infrared beam is transmitted down the middle of the plastic board and records each beam break as evidence that the fly is awake and moving.

B) A board placed into the sleep-deprivation-inducing shaking machine apparatus inside of an incubator. The board is bolted in place to ensure that it is not shaken around during the course of the sleep experiment. A maximum of four boards can fit into this machine. There are two dials on the machine that can change the amount of force that’s applied to the shaking.
rebound sleep also has a higher depth than does normal sleep, which is marked by a higher, measurable arousal threshold. Another factor distinguishing rebound sleep from regular sleep is a decrease in sleep latency, (a decrease in the time it takes to transition from a waking to a sleeping state) (Seidner et al., 2015).

**Micro-RNA 190**

MiR-190 is a 21 nucleotide-long micro-RNA in humans (www.mirbase.org) that is postulated to have huge and widespread effects on the physiology of an animal. Of all the animals that have been studied for miR-190, 77 known homologs of the miR190 family on miRbase, (a database which collects experimental information about micro-RNAs) four of which have high confidence levels and are conserved in mice, humans, fruit flies, and silkworms (www.mirbase.org). One of the few experimentally-verified targets of miR-190 is the NeuroD transcription factor, which aids in neurogenic differentiation (Zheng et al., 2010). NeuroD has been shown to vary in levels along with miR-190 when a mu opioid receptor agonist was delivered, suggesting that miR-190 regulates levels of NeuroD (Zheng et al., 2010). Interestingly, miR-190 is predicted to target the vesicular acetylcholine transporter (VChat) (www.targetscan.org). Even though there is a list of potential targets of miR-190, there remains much to be learned about how the micro-RNA operates.

The miR-190 total knockout has been found to be embryonically lethal in *Drosophila melanogaster* (Chen et al., 2014). This indicates that miR-190 is essential for development. Since miR-190 levels are enriched in the mammalian brain relative to the rest of the body, (He and Hannon, 2004) it is possible that miR-190 helps develop important circuitry in the brain, perhaps even circuitry promoting sleep.

Future studies might further explore the role of miR-190 in sleep. Its potential connection with sleep homeostasis is particularly interesting, especially in terms of connections to sleep disorders. More
generally, studying circadian clock dysfunction could also yield major insight into psychiatric diseases such as major depressive disorder and Seasonal Affective Disorder (SAD) (Zordan and Sandrelli, 2015). Since many people experience interrupted sleep episodes during the night but still have to wake up early for work, they experience a net sleep loss for the night. If this sequence of events is repeated for a long enough time, the effects of chronic sleep deprivation can accumulate and affect cognitive functions (Alhola and Polo-Kantola, 2007). These effects can be modeled in flies using behavioral paradigms that can measure learning and memory before and after continued sleep deprivation (Zordan and Sandrelli, 2015). When a behavioral paradigm such as this is combined with endogenous mutants for a micro-RNA or malfunctioning portion of the circadian clock, it becomes a powerful tool to simulate human disease in a model organism (Zordan and Sandrelli, 2015). Many such paradigms already exist, for example, manipulating a fly’s environment so it must learn to avoid and travel away from noxious stimuli such as heat and chemicals (Zordan and Sandrelli, 2015). Video-recording technologies have made it possible to typify the social interactions of the flies while they move about in this dangerous environment (Zordan and Sandrelli, 2015). This has far-reaching implications in examining how sleep can affect social behaviors in humans.

Inhibition of miR-190 decreases sleep in Drosophila

The Griffith Lab had previously performed a reverse genetic screen in Drosophila melanogaster to determine which micro-RNAs could potentially regulate sleep (Goodwin 2013, unpublished data). Micro-RNA sponges were used as a tool to evaluate whether inhibition of a particular micro-RNA affected total amount of sleep. When micro-RNA 190 was inhibited in flies, it was found to significantly reduce sleep overall. Additionally, miR-190 was unique in that it also decreased the typical homeostatic “sleep rebound” response to sleep deprivation. This evidence, along with the high expression of miR-190
in heads and its predicted neuronal targets led the Griffith Lab to specifically investigate the effect of inhibition of miR-190 in neurons.

To do this, Dr. Patricia Goodwin used a pan-neuronal driver (Nsyb-Gal4) to express the miR-190 sponge in the *Drosophila* brain. The total amount of sleep in these experimental flies was drastically reduced, and there was no difference between the total amounts of daytime vs nighttime sleep between sexes (Figure 3A). Since flies usually sleep less during the day than they do during the night, this raised an interesting question about potential circadian involvement: perhaps this lack of difference could be attributed to an inability to distinguish between night and day because of an influence by miR-190 on the circadian clock. Along with this, Goodwin also found that male flies expressing the miR-190 sponge in neurons had a significantly decreased mean sleep episode duration during both the day and night periods compared to scramble control, and females exhibited a significantly decreased mean sleep episode duration relative to scramble control at night (Figure 3B). As previously discussed, shortened episode duration can be a hallmark of an altered homeostatic system, and the sleep rebound data after mechanical sleep deprivation further suggested this was the case. Specifically, the rebound of miR-190 sponge-expressing female flies suggested that sleep homeostasis was abnormal, as rebound sleep usually occurs directly following sleep deprivation (since the flies were deprived of their sleep at night, their rebound would typically occur the following morning). However, it is clear that these female flies recover only in the nighttime, and this recovery does not taper off when baseline levels of sleep are reached, but rather extends to extreme hyper-recovery past their baseline (Figure 3C). In contrast, males expressing the miR-190 sponge in neurons do not recover from sleep deprivation, which further implicated miR-190 in regulating a homeostatic component of sleep (Figure 3D). This study inspired our following experiments investigating the role of miR-190 on sleep regulation in *Drosophila*.

**Figure 3:** Inhibition of miR-190 in neurons abolishes day/night and sex differences in sleep
Figure 3: Unpublished data from Dr. Patricia Goodwin, 2013. A) Normal sexually-dimorphic sleeping patterns are abolished in flies of both sexes expressing the miR-190 sponge in neurons relative to scramble controls. B) Mean sleep episode duration is significantly decreased during both the day and nighttime periods for male flies expressing the miR-190 sponge in neurons, and during the night for females expressing the miR-190 sponge in neurons relative to scramble controls. C) Female flies expressing the miR-190 sponge in neurons exhibited a hyper-recovery sleep phenotype past their level of baseline sleep after sleep deprivation. D) Male flies expressing the miR-190 sponge in neurons do not recover from sleep deprivation.

Overall, the above study led by Patricia Goodwin sparked many questions about miR-190 that were investigated in the following study. Four main investigations probed a potential role of miR-190 in the circadian clock, attempted to localize miR-190’s effects on sleep homeostasis in the Drosophila brain, and searched for areas miR-190 could be required in for regulation of consolidation of sleep.
Previous results that had used the micro-RNA-190 sponge were then verified using an endogenous mutant of mir-190 that was generated by CRISPR.
2. **Materials and Methods**

**Animals**

Flies were grown in plastic bottles or vials with standard agar/cornmeal and yeast food in an incubator at 25 °C. The incubator operated on a 12 hour light-dark schedule, unless otherwise mentioned. Flies were flipped into a new bottle every 3-5 days and discarded after an age of one month.

**Crosses**

Genetic crosses were performed by collecting ten virgin females of desired genotype at time of eclosion and placing them in a vial of agar-yeast food with five males of the desired genotype. The parent flies from this vial were flipped into a new vial every two days until there was a total of at least four vials per genotype. The parent flies were discarded exactly two weeks after the first vial of the cross was made, so as not to confuse flies of the G0 generation with their progeny (F1). Desired flies for experiments were then collected. In order to minimize variance due to differences between genetic backgrounds, between mir-190 mutants and wild-type controls, outcrossing to Cantonized w' genetic backgrounds was performed three to five times.

**Table 1: List of genotypes used in all experiments with shorthand abbreviations and description**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Shorthand</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>w; miR190 spg/+; miR190 spg/+</td>
<td>miR-190 sponge</td>
<td>Micro-RNA sponge construct which inhibits expression of miR-190</td>
</tr>
<tr>
<td>White-eyed, canton-type special</td>
<td>w^cs</td>
<td>A lab-derived strain which lacks red eye pigment</td>
</tr>
<tr>
<td>Canton-type, special</td>
<td>Cs</td>
<td>A lab-derived strain</td>
</tr>
<tr>
<td>w-; nsyb-GAL4/TM6b or TM3</td>
<td>Nsyb-GAL4</td>
<td>Neuronal-synaptobrevin Gal4; expresses GAL4 in all neurons</td>
</tr>
<tr>
<td>w-; tubulin-GAL4-DCR1014/TM3</td>
<td>Tub-GAL4</td>
<td>Expresses GAL4 in cells which contain tubulin - ubiquitous</td>
</tr>
<tr>
<td>Tubulin-GAL80 temperature-sensitive</td>
<td>Tub-GAL80^{ts}</td>
<td>Expresses a GAL4 inhibitor that is inactivated at high temperature (29 °C)</td>
</tr>
<tr>
<td>White^{1118}</td>
<td>w^{1118}</td>
<td>A lab-derived strain lacking red eye pigment</td>
</tr>
<tr>
<td>w; Cry^{39}/&quot; or Cyo; +/- or Sb</td>
<td>Cry-GAL4</td>
<td>Expresses GAL4 in Cry-expressing cells</td>
</tr>
<tr>
<td>w ; Tim°GAL4/Cyo</td>
<td>Tim-GAL4</td>
<td>Expresses GAL4 in Tim-expressing cells</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>w ; Tim°GAL4, PdfGAL80/Cyo</td>
<td>Tim-GAL4, Pdf-GAL80</td>
<td>Expresses GAL4 in Tim-expressing cells, but not in LNvS</td>
</tr>
<tr>
<td>w-; scrambled RNA/Cyo; scrambled RNA/TM6b</td>
<td>scramble</td>
<td>Expresses scrambled RNA and results in an inactive miR sponge</td>
</tr>
<tr>
<td>Tyrosine-hydroxylase – GAL4</td>
<td>TH-GAL4</td>
<td>Expresses UAS in dopaminergic neurons</td>
</tr>
<tr>
<td>w¹¹¹; 104y-GAL4</td>
<td>104y-GAL4</td>
<td>Expresses UAS in the fan-shaped bodies</td>
</tr>
<tr>
<td>w¹¹¹; 23E10-GAL4</td>
<td>23E10-GAL4</td>
<td>Expresses UAS in the fan-shaped bodies</td>
</tr>
<tr>
<td>Gene-switch ELAV</td>
<td>Elav&gt;gsg</td>
<td>Expresses the gene-switch in the brain</td>
</tr>
<tr>
<td>mir-190 heterozygote</td>
<td>mir-190 het</td>
<td>One copy of mir-190</td>
</tr>
<tr>
<td>mir-190²⁴/mir-190²⁴</td>
<td>mir 190 mutant 24</td>
<td>CRISPR mutant; missing the first nucleotide in the mature 5p miR sequence</td>
</tr>
<tr>
<td>mir-190²³⁻²⁵/mir-190²³⁻²⁵</td>
<td>mir190 mutant 21</td>
<td>CRISPR mutant; missing the first three nucleotides in the mature 5p miR sequence</td>
</tr>
<tr>
<td>Wcs;; [Ti]mir190 knockout/+</td>
<td>mir190 knockout</td>
<td>From Cohen Lab. Knockout of mir-190 on the third chromosome</td>
</tr>
<tr>
<td>Wcs;; TM3/TM6b</td>
<td>TM3/Tm6b</td>
<td>Yields an “ebony”-colored fly. Useful for outcrossing</td>
</tr>
<tr>
<td>VT004849-GAL4</td>
<td>n/a</td>
<td>Expresses the GAL4 in the dorsal fan-shaped bodies</td>
</tr>
<tr>
<td>White minus</td>
<td>w⁻</td>
<td>A lab-derived strain lacking red eye pigment</td>
</tr>
</tbody>
</table>

**Behavioral Assays**

a. **Sleep experiments** – Depending on the experiment, male or female flies were collected. Flies were 3-5 days of age unless otherwise mentioned. Each fly was loaded into a glass tube which fit into Drosophila Activity Monitor boards (Trikinetics Inc., Waltham, MA). Each tube contained sucrose-agar food at one end (unless otherwise mentioned), and Parafilm on the other to prevent the flies from escaping. An infrared beam bisected each tube, and interruptions in the beam signal were recorded by a computer as activity counts. Any period of inactivity greater than five minutes was classified as sleep. Flies were kept in the DAM system for 7-14 days depending on the experiment.

b. **Sleep Deprivation** – Flies were sleep deprived in the DAM apparatus mentioned above. Groups of flies to be sleep deprived were previously designated and loaded together into separate DAM boards, which were in turn fastened to a custom modified vortexor. This programmable shaking
machine was set to shake the flies for two seconds every ten seconds for a total of twelve hours on the night of sleep deprivation.

c. *Data Analysis* – Results were analyzed in MATLAB (Mathworks, Natick, MA) using the custom-written “SCAMP” program as previously described (Donelson et al., 2012). This program analyzes the many quantitative parameters of sleep such as total amount of sleep, mean episode duration, episode number, and activity counts, among others. The first day in the incubator was discarded from data analysis as an entrainment day. Then followed three days of baseline sleep measurements (for experiments with sleep deprivation), and if deprivation was done, it occurred on the fifth night. Rebound sleep was calculated directly following sleep deprivation for 24-48 hours. Statistics tests were performed using SPSS (IBM, Armonk, NY).

**DNA sequencing of mir-190 mutants**

Polymerase Chain Reaction was used to amplify the mir-190 genetic locus in CS flies, *mir-190<sup>KO</sup>* mutant heterozygous flies from the Cohen lab and *mir-190<sup>Δ24</sup> CRISPR* mutants. The following forward and reverse primers were used:

<table>
<thead>
<tr>
<th>mir-190 sequencing primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FWD:</strong> CCGGATCTGCTGAGTTGATT</td>
</tr>
<tr>
<td><strong>REV:</strong> CCGGCCCTCAGAGTTGGATT</td>
</tr>
</tbody>
</table>

The amplified PCR products were then sequenced to confirm deletions in the *mir-190* gene.
3. Results:

The role of miR-190 in circadian sleep regulation in Drosophila

Previous work in the Griffith Lab found minor changes in circadian rhythms in *nsyb>*miR-190 sponge-expressing flies (Goodwin, 2014 unpublished data). *Nsyb>*miR-190 sponge females had a small increase in circadian period (scramble control: 23.9 ± 0.09 hours; miR-190 sponge 24.5 ± 0.2 hours; t-test p<0.05), and a small but significant decrease in percent of rhythmic flies (data not shown). Thus, I first investigated if miR-190 affects circadian clock cells and their ability to produce rhythmic sleeping and waking behaviors in *Drosophila*. CRY+ neurons were of particular interest because Cry helps synchronize light cues to the circadian clock. Since these flies do not show a difference between day and night time sleep, we proposed that they might have trouble integrating such light cues. In order to test this, a *Cry-GAL4* driver was used to express the miR-190 sponge in Cry+ cells. The male flies were first incubated for four days in normal 12 hour light, 12 hour dark conditions to entrain their circadian clocks to external light cues (zeitgebers). Under these conditions, we saw no change in the amount of sleep in *Cry>*miR-190 sponge-expressing male flies relative to scramble controls (data not shown, see Appendix B). This result did not support the hypothesis that miR-190 is required in CRY+ neurons to regulate the amount of day vs. night sleep.

After the four days in light-dark conditions, the flies were then subjected to constant dark conditions to essentially allow their circadian clock to “free-run” without any environmental light-entraining cues. In flies with normal circadian clocks, the constant-dark condition and the light-dark condition should yield similar circadian periods overall, with the caveat that under constant darkness even these “normal” flies have circadian rhythms that are shifted a minute amount each day since the natural period of flies (or almost any animal), is very rarely an exact 24 hours.
All groups remained rhythmic with a period of ~24.4 +/- .1 hours (data not shown). This evidence, coupled with the fact that there was no observed change in the flies’ rhythmicity indices does not support the hypothesis that miR-190 is involved with or regulates the circadian clock-controlled rhythmicity of sleep.

However, because Cry-GAL4 does not express in all neurons that make up the circadian clock, we did a similar experiment investigating the potential role of miR-190 in the circadian regulation of sleep using a Tim-GAL4 driver to express the miR-190 sponge. Recall that Tim protein is encoded by the timeless gene in Drosophila, and is a key regulator in circadian rhythms. In this experiment, there was also an experimental group with male flies that possessed both a TimGAL4 and a PdfGAL80 construct. This method which uses the GAL4 inhibitor called GAL80, is common in fly genetics because it allows more specific expression in a desired subset of neurons that contain “A” but not “B”. In our case, this meant specific expression of the miR-190 sponge in cells that contained Timeless protein, but not Pigment-Dispersing Factor, which is an extremely important neuropeptide necessary for coordinating circadian rhythmicity. Previous experiments done in the Griffith Lab had found that PdfGal4-driven miR-190 sponge expression had no effect on sleep (Goodwin 2014, unpublished data). Nonetheless, we were interested in exploring the potential role of miR-190 in TIM+PDF- neurons because we could validate any effect seen by expressing the miR-190 sponge in TIM neurons was just due to Timeless and not other circadian gene products also present.

For the sleep experiment, male flies were loaded into the DAM system in an incubator running on a 12 hours of light: 12 hours of dark (LD) schedule. Mechanical sleep deprivation was performed on the fifth night. Overall, animals expressing the miR-190 sponge in Tim+ cells had a reduction in total daytime sleep amount. These flies also experienced a decreased sleep episode duration. When fragmentation is comorbid with decreased total sleep amount, it points to potential
problems with the sleep homeostat (since the normal increase in sleep episode duration seen in rebound after deprivation did not occur.

**Figure 4: Inhibition of miR-190 in Tim+ neurons induces sleep effects:**

![Graph showing sleep patterns and episode durations](image)

**Figure 4: Expression of the miR-190 sponge in Tim+ cells using TimGal4 and TimGal4, Pdf-Gal80**

A) Baseline sleep for all groups. B) Mean episode duration is significantly decreased between TimGal4 control and TimGal4>miR-190 sponge male flies (***p<.0001, **p<.001, *p<.05 , ns; p>.05 Kruskal Wallis Independent sample Test). TimGal4,Pdf-Gal80 scramble; N = 13, TimGal4, Pdf-Gal80>miR-190 sponge; n =21, w1118 x miR190 sponge; n=24, TimGal4 scramble; n=24, TimGal4>miR-190 sponge, n=21, TimGal4,Pdf-Gal80>miR-190 sponge, n=22.

**miR-190 is implicated in the homeostatic control of Drosophila sleep**

While the molecular correlations and underlying mechanisms of the sleep homeostat machinery remain unknown, the concept of sleep homeostasis has been well characterized. Since sleep homeostasis involves both the detection and the response to sleep need, we utilized the technique of sleep deprivation to decrease the amount of total nighttime sleep for one night. The natural homeostatic sleep response could then be measured.
To test if miR-190 is involved in homeostatic sleep regulation, we first repeated the experiment previously done with *nsyb-GAL4* to express the miR-190 sponge in neurons, this time in male flies. In terms of the flies’ baseline sleep (prior to sleep deprivation), we reliably replicated the results, which led us to the conclusion that miR-190 is necessary in neurons to produce a typical level of total sleep. Of note, male flies expressing the miR-190 sponge in neurons showed a sleep fragmentation phenotype; increased episode number in conjunction with decreased mean sleep episode duration. However, I was not able to replicate the previous sleep deprivation recovery results that had been seen in female flies (data not shown).

**Figure 5: Inhibiting miR-190 in neurons decreases baseline sleep and episode duration**

A) Average Male Baseline Sleep

B) Male Mean Sleep Episode Duration

C) Male Recovery Sleep

![Graphs showing sleep patterns and episode durations for male flies expressing the miR-190 sponge in neurons.](attachment:figure5.png)
Figure 5: Inhibiting miR-190 in neurons of male flies. A) Average baseline sleep measured in male flies (nsyb-GAL4>miR-190, n = 14; nsyb-GAL4 control, n = 17) is severely decreased in flies expressing the miR-190 sponge in neurons. B) Mean sleep episode duration is decreased in flies expressing the miR-190 sponge in neurons (***p<.0001, Kruskal-Wallis Test). C) Flies expressing the miR-190 sponge in neurons exhibit hyper-recovery over initial baseline sleep compared to the GAL4 control.

miR-190 is required in the Fan-Shaped Bodies for consolidated sleep

After finding that miR-190 is required generally in neurons for regulation of sleep, the next objective was to localize its activity in the brain. The fan-shaped bodies constitute an intriguing portion of Drosophila neuroanatomy; located in the central complex region of the brain, this area has been previously implicated in sleep homeostasis. This made the fan-shaped body an excellent region in which to inhibit miR-190 activity and examine corresponding sleep effects. To do this, we used two different GAL4 drivers of different strengths and specificities to express the miR-190 sponge; 104y-GAL4 and 23E10-GAL4 (Figure 6A). Experimental male flies of both drivers exhibited a fragmentation phenotype along with less total sleep duration overall. (Results were more pronounced in the stronger 23E10-GAL4 driver. Data for 104y-GAL4 had same results, data not shown.) While these results (fragmentation, reduced total sleep) are qualitatively equivalent to the effect seen by expressing the miR-190 sponge ubiquitously in all neurons, the effect size of total sleep abolished was not nearly as large, indicating that miR-190 must be performing similar functions in other brain areas as well.
Figure 6: Expression of the miR-190 sponge in the Fan-Shaped Bodies prevents consolidated sleep

A) Expression pattern of 23E10-GAL4 in the Drosophila Brain (Jan et al. 2012)
B) 23E10-GAL4 driving expression of the miR-190 sponge in the fan-shaped bodies in male flies results in increased number of sleep episodes and C) Decreased mean episode duration
D) Average baseline sleep is significantly decreased in flies expressing the miR-190 sponge in the fan-shaped bodies
E) Recovery sleep after 12 hr sleep deprivation. Male flies expressing the miR-190 sponge in the fan-shaped bodies have improved recovery after twelve hours compared to w^cs and scramble controls. This was calculated by Σ[(sleep after deprivation) − (night baseline sleep)]. *p<.05, **p<.005, ***p<.0005 Kruskal-Wallis Test.
miR-190 is not required in the dorsal fan-shaped bodies for its effects on sleep

Since the fan-shaped body elicited such a clear sleep-loss phenotype, we wanted to attempt to localize the subset of neurons in which miR-190 was creating this effect. VT-004849-GAL4 was used to express the miR-190 sponge in the dorsal fan-shaped body. Male flies were loaded into the DAM system and their sleep was measured for 8 days. Sleep deprivation via mechanical shaking occurred on the fifth night. No significant differences in any parameter of sleep were found between experimental flies expressing the miR-190 sponge in the dorsal fan-shaped body and controls (data not shown, see Appendix B). These data may suggest that miR-190 does not play an important role in sleep regulation specifically in the dorsal fan-shaped bodies. However, the experiment should be repeated before more definite conclusions are drawn. Further attempts to localize the specific neuronal subset within the fan-shaped bodies would be interesting, as would an experiment with a specific Ellipsoid Body driver, since this area is a downstream synaptic partner of the fan-shaped body and receives feedback from the area.

miR-190 is not required in dopaminergic neurons for its effects on sleep

Dopaminergic neurons have long been implicated in sleeping and waking (Holst et al., 2014), and thus are also a potential site of miR-190 regulation. As previous studies have shown that two dopaminergic neurons synapse on to the dorsal fan-shaped body (mentioned above) to help produce wakefulness (Liu et al., 2012), we wondered what would happen if we inhibited miR-190 in all dopamine neurons. To find out if miR-190 is necessary in dopaminergic neurons to produce an effect on sleep, tyrosine-hydroxylase-GAL4 (TH-GAL4) was used to express the miR-190 sponge in this subset of neurons, in both the male and female fly brain. Flies were loaded into the DAM system for a total of eight days, and were subjected to sleep deprivation on the fifth night. The only difference detected in sleep across male or female groups was a slight decrease in the female activity peak (data not shown, see Appendix B). This led us to conclude that miR-190 is not required in dopaminergic neurons for its effects on sleep.
The effect of food on sleep in miR-190 flies

Sleep and metabolism are intimately linked, thus different foods have the potential to produce different sleep patterns in flies. Traditionally, in *Drosophila* sleep studies, sucrose-agar food is placed into the DAM tubes because of its congealing properties. However, using this type of food in the sleep tubes means that the flies are abruptly switched from the yeast and cornmeal food on which they were raised. While this protocol is repeated across all aforementioned experiments and is typical in our lab and the field, we decided to investigate the effect of keeping flies on cornmeal food for both their upbringing and the sleep experiment. A previous study from the Griffith Lab studied the effect of food on the sleep of populations of flies, and found that female flies that were assayed on standard complete food vs the traditional sucrose and agar food had more consolidated sleep at night. They also found that both sexes on this standard complete food slept more during the day, suggesting that the nutritional quality of food can impact sleeping patterns in populations, perhaps in a sex-specific manner (Liu et al., 2015). For our experiment, we focused on individual male flies and expressed the miR-190 sponge in neurons using *nsyb*-GAL4. UAS and GAL4 controls were used, and their sleep was measured for 8 days with sleep deprivation occurring on the fifth night. Since the food type did not affect the amount of baseline sleep, rebound sleep was measured (Figure 7) to gauge their homeostatic response to sleep deprivation. Interestingly, only flies expressing the miR-190 sponge that were raised and tested on cornmeal food did not recover from sleep deprivation, even though they also lost the least amount of sleep compared to all other groups. This could indicate that food type is a factor in miR-190 regulation of sleep in neurons, perhaps due to its nutritional content.

Figure 7: Food types yield different effects on sleep recovery in miR-190 sponge-expressing flies
Male flies were raised on either standard yeast and cornmeal or sucrose-agar food. Sleep deprivation occurred on the 5th night and recovery sleep was measured by calculating minutes of:

\[\text{minutes of recovery} = \frac{\left( \text{sleep}_{\text{recovery day}} - \text{sleep}_{\text{baseline}} \right)}{\text{sleep}_{\text{baseline}}}\]

Nsyb>miR-190 flies raised on cornmeal were the only group that did not recover to or past baseline.

*miR-190 may regulate different neuron subsets during development for its effect on sleep*

Micro-RNAs have been shown to affect the differentiation and development of neurons (VanVactor, 2012). Therefore, miR-190 could be producing its effects on sleep by altering the development of neuronal circuits that control sleep. A temperature-sensitive inhibitor of GAL4, tubulin-Gal80 (TubGal80ts) along with a tubulin-GAL4 specifically expressed the miR-190 sponge during development up until time of eclosion as adults. This particular GAL80 inhibits GAL4 at low temperatures (<18°C), and is GAL4-permissive at high temperatures (>29°C). By raising the flies at high temperature, we inhibited miR-190 during development until we moved them to the cooler incubator for the sleep experiment when the flies were adults at 3-5 days old. Any resulting differences seen in sleep phenotypes could be due to the role of miR-190 during development.
The efficacy of the construct was verified by examining the color of the flies; the UAS construct contains cherry mRNA which turns flies purple only when Gal4 is active. We confirmed that high temperature effectively disinhibited Gal4; the expected fraction of larvae was purple at the Gal4-permissive temperature (29 ºC). However, we were not able to use cherry expression to confirm that Gal80 effectively inhibited Gal4 after a temperature shift to 18 ºC because of the strong stability of the cherry protein.

Male flies that expressed the miR-190 sponge throughout larval and pupal development, but not in adulthood experienced less total daytime sleep than their scramble controls (Figure 8). This was similar to the decrease in sleep seen in the nsyb>miR-190 sponge flies (Figure 5A), and could indicate that miR-190 has a role in the developing fly which affects the total amount of daytime sleep in the adult fly. However, there were multiple aspects of sleep that were changed in nsyb>miR-190 flies that we did not observe in this experiment, including decreased nighttime sleep and decreased mean sleep episode duration. We were unable to examine the effect of larval expression of the miR-190 sponge on larval sleep, because no method to measure sleep in fly larvae has yet been described. Evidently, the exact role of miR-190 on sleep in the developing fly remains an active area of investigation.

Figure 8: Inhibition of miR-190 in fly larvae decreases daytime sleep in adult flies
**Figure 8: A)** Average baseline sleep for tub-Gal80\textsuperscript{ts}>miR-190 sponge males (n=20), tub-Gal80; tub-Gal4>miR-190 sponge males (n=23) and tub-Gal80\textsuperscript{ts}; tub-Gal4>scramble males (n=20). **B)** The Gal4-negative control only experiences significantly increased sleep compared to the experimental males during the Day (*p<.05, Mann-Whitney U test). ***p<.0001 Mann-Whitney U Test.

*Sleep in miR-190\textsuperscript{knockout} heterozygote mutants*

A miR-190 sponge construct with a copy of the sponge present on two chromosomes was used for all aforementioned experiments. While transgenic techniques are extremely useful, it is good practice to confirm results with other techniques as well. To employ a different type of miR-190 inhibition, we obtained a line of mir-190\textsuperscript{KO}/+ heterozygous flies and assayed male and female sleep (**Figure 9A**). This mutant had to be assayed as a heterozygote, because the homozygote mutant is not viable; in fact, it is embryonic lethal (Chen et al., 2014). We sequenced this knockout to ensure that the mutation was correct and unlikely to affect other nearby genes. The heterozygous knockout female flies exhibited an unexpected phenotype; they had more total sleep (**Figure 9C**) as well as more consolidated sleep compared to controls (**Figure 9B**). Despite these unexpected results, when the flies were sleep deprived and their homeostatic rebound measured, the w\textsuperscript{cs} controls recovered more sleep (i.e. attained a level closer to their baseline sleep before the deprivation) than did the heterozygote knockout flies of both sexes (**Figure 9D**). Overall, we would expect to see similar sleep phenotypes from the miR-190 sponge-expressing flies and the heterozygous knockout flies. The fact that the miR-190 knockout and the miR-190 sponge exhibited different sleep phenotypes may be due to the differing nature of the methods of inhibition of miR-190.
Figure 9: Sleep in mir-190 heterozygote knockout males and females

**A**

*Average Baseline Sleep*

- **Female Mean Sleep Episode Duration (mins)**
  - **24 hr total**
  - **Day**
  - **Night**

**C**

*Female Average Total Sleep (min)*

- **Total 24 hr**
- **Day**
- **Night**

**D**

*Recovery Sleep*

- **SD starts**
- **SD hr 12**
- **Rec hr 12**

Ns: p>.05; *=p<0.05; **=p<0.001; ***=p<0.0001, Mann-Whitney U Test

Figure 9: A) Average baseline sleep for male mir-190\(^{KO}\)/+(n=24), male wcs controls (n=24), female mir-190ko/+ (n=21) and female wcs controls (n=17). B) Female mir-190\(^{KO}\)/+ flies experience significantly increased mean episode number for Total 24 hr compared to w\(^{cs}\) controls. C) Female mir-190\(^{KO}\)/+ flies experience significantly increased total sleep for Total 24 hr and Day compared to w\(^{cs}\) controls. D) Recovery sleep measured for 24 hours after 12 hours of sleep deprivation. 12 hr: 12 hr LD conditions were used.

*mir-190 endogenous mutants have reduced sleep and shorter sleep episodes*
In recent years, the CRISPR/cas9 (Clustered Regularly-Interspaced Short Palindromic Repeats, and CRISPR-associated proteins, respectively) system has emerged as a prime genome-editing tool. It can be used to produce insertions/deletion mutations in the genetic locus of interest by inducing double-strand breaks in the DNA breaks, which can result in DNA-repair errors (Bassett and Liu, 2014). To investigate the role of miR-190 in sleep regulation in Drosophila, Patricia Goodwin utilized CRISPR to create flies with deletion mutations in the mir-190 gene. This induced many different insertion/deletion mutations in the seed sequence of the micro-RNA. One of the recovered mutant fly lines was viable as a homozygous adult (Figure 10 C), and I tested this fly line for differences in sleep. Importantly, I found that these flies showed the same sleep phenotypes as did flies from the previous experiment expressing the miR-190 sponge in neurons; there was a large decrease in total baseline sleep with daytime and night time amounts of total sleep being roughly in both sexes of flies (Figure 10 D). Both sexes of these mir-190Δ24 mutants also had severe sleep fragmentation (Figure 10 E-F). Average recovery sleep post sleep-deprivation retained the lack of sexual dimorphism of sleep (Figure 10 G). Finally, the rebound sleep of the mir-190Δ24 mutant females showed a hyper-recovery past their original baseline before even 12 hours of recovery sleep was attained (Figure 10 H). A similar recovery phenotype was also seen in Patricia Goodwin’s experiment with female flies expressing the miR-190 sponge in neurons (Figure 3 C), although the nsyb>miR-190 sponge flies exhibited recovery mainly at night, while this group seemed to recover rapidly during both the day and night. Despite these small nuances inherent in comparative data, this endogenous mutant experiment further confirmed our belief that miR-190 is important in homeostatic sleep regulation, and will undoubtedly be repeated in the future.

Figure 10: mir-190 structure and sequence of endogenous CRISPR mutants, and their sleep
C

miR-190 CRISPR mutant

WT  GATGGTCCAGTGAGATGTTGATATTGCTTTCTGTTGTTCTCAGAAAAGTTCAACCAGAACTCAA

190C24 GATGGTCCAGTG-GATATGTTGATATTGCTTTCTGTTGTTCTCAGAAAAGTTCAACCAGAACTCA

D

Average Baseline Sleep

E

Mean Sleep Episode Duration (min)

F

Mean number of Sleep Episodes (min)

G

Recovery Day Sleep

---
Figure 10: A) The sequence of miR-190 in Drosophila melanogaster. Pink letters denote the mature 5p sequence (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0005808). B) The sequence of miR-190 in Homo sapiens for comparative purposes. Pink letters denote the mature 5p sequence (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0000486). C) Top: the wild-type sequence of miR-190. Bottom: the mir-190Δ24 CRISPR-generated endogenous mutant is missing the first nucleotide in the seed sequence. Note that this nucleotide is not conserved in humans. D) Average baseline sleep for mir-190Δ24 endogenous mutants. Day and night as well as sexual dimorphisms are abolished. E) Sleep episode duration is significantly decreased in female mir-190Δ24 mutants compared to wcs controls for Total 24 hours and Night and significantly decreased in males compared to wcs controls for Total 24 hr Day and Night. F) Mean number of sleep episodes is significantly increased in female mir-190Δ24 mutants (n = 10), for Total 24 hr Day and Night compared to wcs controls (n = 14), and significantly increased in male mir-190Δ24 mutants (n = 7), for Total 24 hr, Day and Night, compared to wcs controls (n = 7). G) Male (n = 8) and female (n = 5) mir-190Δ24 mutants have a comparably lower amount of recovery compared to male (n = 15) and female (n = 10) wcs controls. H) Recovery sleep represented as the cumulative percent of sleep lost from nighttime baseline sleep. ***p<.0001, **p<.001, *p<.05, ns: p>.05, Mann Whitney U Test for M. 1 way ANOVA with Tukey post-hoc for F: tot 24 hr and night, Mann – Whitney for Day. *starred-significance remains same.

D-F) Female mir-190Δ24 mutants (n = 10), Females wcs (n = 14), Male mir-190Δ24 mutants (n = 7), Male wcs (n = 7). G-H) Female mir-190Δ24 mutants (n = 5), Females wcs (n = 10), Male mir-190Δ24 mutants (n = 8), Male wcs (n = 15).
4. Discussion

Sleep is an essential behavior and is conserved throughout the Animal Kingdom. Remarkably, despite its universality, the function and purpose of sleep remain mysterious. It is clear that sleep is influenced by a wide range of regulatory molecules, some of which still elude identification. Recently, micro-RNAs have emerged as prominent regulators of gene expression, which implicates them as potential candidates that could change gene expression pertaining to sleep. A reverse-genetic screen done by the Griffith Lab identified many miRs that cause changes in total amount of sleep in Drosophila melanogaster. Micro-RNA 190 in particular showed potential as a regulator of the homeostatic component of sleep, as sleep recovery after deprivation was hindered following inhibition of the miR. This study aimed to further investigate the role of miR-190 in sleep regulation in Drosophila.

Currently, very little is known about miR-190. Nevertheless, since it is known to be widely conserved from humans to fruit flies, we were able to perform basic behavioral experiments on a model organism that could shed light on the function of miR-190 in mammals. Drosophila melanogaster was our choice for many reasons: the organism is genetically tractable, easily behaviorally manipulated, and has near endless tissue-specific GAL4 transgenic fly lines and endogenous mutants readily available. Of crucial importance was the miR sponge library created by the David van Vactor Lab, as their miR-190 sponge allowed us to easily examine the effects of its inhibition on sleep. We expressed the sponge in different regions of the fly brain using the GAL4/UAS system, and measured the quantitative changes in different parameters of sleep.

As previous results had shown that the inhibition of miR-190 resulted in severely decreased total sleep, we first used a pan-neuronal GAL4 driver to express the miR-190 sponge. The resulting sleep was decreased significantly, and total amount of daytime and night time sleep were equivalent, in both sexes. This is incredibly interesting because Drosophila exhibit sexually dimorphic sleep patterns; males
sleep more during the day than do females. Since our circadian-clock studies could not account for the fact that the sleep of both sexes looks identical after miR-190 inhibition, it could mean that miR-190 plays a role in regulating sex-specific sleep needs. However, this is an area which warrants further research before conclusions are made.

In order to make sure that our results were not due to some peculiarity of the sponge construct, we decided to test the effect of endogenous mir-190 mutations on sleep. Initial studies of a miR-190 knockout heterozygote fly gave a somewhat surprising result; experimental flies slept more than controls, and even had more consolidated sleep. While this may seem non-intuitive, this effect may be due to the different method of inhibition of miR-190. miR sponge-mediated inhibition is partial because the sponge is only formulated to target the 5p miR product (Loya et al., 2009). miR sponges are also not necessarily completely specific to one miR, and could thus be affecting the activity of other micro-RNAs. On the other hand, the heterozygous knockout-mediated inhibition affects both the 5p and the 3p miR product, essentially “knocking-out” the miR on the whole chromosome. This could lead to a dosage compensation effect where the fly expresses a relatively high amount of miR-190 from the normal, wild-type chromosome compared to the mutated chromosome in an attempt to make up for the loss of function of this important, well-conserved miR. Overall, both experiments should be repeated with higher n so that more precise and reliable results can be obtained. Perhaps superior transgenic techniques will emerge that will allow more complete analysis of the relation between sleep phenotypes and miR expression without the inherent underlying complications of dynamic genetic compensation mechanisms.

Even though this greater consolidation in the heterozygous knockout flies was essentially the opposite of the phenotype we observed in nsyb>miR-190 sponge flies, the rebound sleep data of the experimental flies showed that they did not recover as well as w1118 controls to sleep deprivation. This
corroborated previous results that had expressed miR-190 sponge throughout the entire body. Because complete mir-190 knockout is homozygous lethal, we used CRISPR to generate smaller deletions in the mir-190 gene, and recovered viable homozygous mutants. When their sleep was tested, they showed almost the exact phenotypes as we had seen when we expressed the miR-190 sponge in neurons using nsyb-GAL4: loss of sexually dimorphic sleep patterns, and severe fragmentation. This effect was striking, and suggested that the previous result was valid. Perhaps the effect of the deletion of that one nucleotide in the seed sequence has an overall effect on the activity of miR-190 that is similar to the method of inhibition that the miR-190 sponge uses.

In addition to the mir-190Δ24 mutant described here, multiple adult-lethal mutants were also generated using CRISPR. For example, the mir-190Δ23-25 mutant is missing three nucleotides in the seed sequence, so it would be interesting to test how it affects miR-190 activity. Trans-heterozygotes of mir-190Δ24 with these lethal lines, (if viable), could further validate our findings. Since these miR-190-deficient homozygote mutants are adult lethal, it would be useful to specify the stage of development when death occurs. This knowledge could help us to pinpoint time periods in development when miR-190 is most essential for sleep regulation.

*The role of miR-190 in the circadian clock*

A second aspect to consider regarding the equal levels of day and night-time sleep seen in nsyb>miR-190 sponge flies and mir-190Δ24 mutants, is that wild-type flies of both sexes exhibit less sleep during the day (which is why it is nicknamed their “siesta”) and more sleep at night. Additionally, the “siesta” is made up of shorter sleep episodes compared to nighttime sleep. The lack of difference in day and nighttime sleep in flies expressing the miR-190 sponge in neurons could be due to flies of both sexes being stuck in a female daytime sleep pattern. However, the similarities between day and night sleep in nsyb>miR190 sponge flies could indicate that the circadian clock is affected by miR-190. The molecular
machinery that comprises the circadian clock receives sensory inputs from the external environment (called zeitgebers) and these inputs synchronize regular, daily biological rhythms to that zeitgeber. If some of the circadian light sensors are not working properly, it could result in the fly not being able to distinguish between certain levels of light that would normally initiate a cascade of molecular responses resulting in sleep or wake. To investigate this possibility, we inhibited miR-190 in Cry-positive neurons and measured circadian rhythmicity in constant darkness. If miR-190 was disrupting the circadian clock, the flies would be expected to have a significantly different circadian period or reduced rhythmicity compared to controls. However, this was not the case. The only difference was that females exhibited a slight fragmentation during the LD period (this phenotype was not present in experimental males).

Even though there was not a positive result in terms of potential circadian regulation by miR-190 in CRY+ circadian cells, we wanted to verify this effect. Therefore, we expressed the miR-190 sponge more broadly in the circadian circuitry using Tim-GAL4 and in a different subset of cells using the combination of Tim-GAL4 and Pdf-GAL80 and saw a slight decrease in daytime sleep.

**The role of miR-190 in the sleep homeostat**

miR-190 was found to be required for homeostatic (recovery) sleep by the Griffith Lab in a screen that used flies that had miR-190 function inhibited throughout the entire body. As the fan-shaped bodies have been implicated in playing a role in sleep homeostasis (Donlea et al., 2014), we next expressed the miR-190 sponge in this area of the brain, using 23E10-GAL4 and 104y-GAL4. While inhibition of miR-190 in the Fan-Shaped Body did not alter the amount of rebound sleep after sleep deprivation, it did cause a decrease in total amount of sleep and the flies demonstrated a fragmentation phenotype. Next, we looked at a direct synaptic partner with the fan-shaped bodies; the dorsal fan-shaped bodies. However, no significant changes in sleep were observed in the experimental group of flies, thus it is assumed that either the GAL4 driver strength was not as strong as the 23E10GAL4 driver
used for the fan shaped bodies, or that miR-190 is not involved in sleep regulation in this more specific area. Further attempts to localize the activity of miR-190 examined the dopaminergic subset of neurons in the fly brain. Again, no significant differences in sleep parameters were observed.

*The role of miR-190 in sleep consolidation*

There are upper limits to the number of sleep episodes, or total amount of sleep one can have in one day or night. If total amount of sleep remains constant, a highly increased episode number can indicate that the sleep homeostat is working hard to maintain a constant amount of total sleep by increasing the number of sleep episodes in response to shortened episode duration. We commonly observed extreme sleep fragmentation phenotypes in our experiments, which may be due to a homeostatic regulatory component that future studies may reveal.

*The role of miR-190 in development*

After these multiple attempts to localize miR-190 to a certain brain area in adult flies, we decided to examine a different angle: perhaps miR-190 was acting early on in development rather than in adulthood for its effects on sleep. In order to investigate this, we first tried using a transgenic “gene-switch” Gal4, which is a variant of Gal4 whose activity can be controlled by ingestion of a drug. Ideally, this method could be used to express the miR-190 sponge at a desired point in development. We confirmed that our pan-neuronal “gene-switch” GAL4 (elav>gsg) was drug-responsive by driving expression of a temperature-sensitive cation channel (dTRPA1) with the GAL4 and then testing flies at a high temperature (>29°C) for paralysis. This test revealed that there was Gal4 activity in absence of the drug. Additionally, elav>gsg/+ control flies showed abnormal patterns of nighttime sleep. Thus, this tool was not useful and another transgenic method, temperature-sensitive Tub-GAL80TS, was used along with a tubulin-GAL4 to specifically express the miR-190 sponge during development up until time of eclosion as adults. The experimental female flies experienced a significant reduction in daytime sleep, but not
night-time sleep. This result might suggest that miR-190 plays a role in development to regulate total daytime sleep. Since this does not account for the observed reduced nighttime sleep seen in tub>miR-190 sponge and nsyb>miR-190 sponge flies, it leaves the possibility that miR-190 is required in adulthood for this effect. However, more experiments should be done in order to draw any conclusions about the role(s) miR-190 plays in early larval development. An optimized gene-switch tool would also be ideal to help try to pinpoint the timeframe in which miR-190 is most important in sleep regulation.

miR-190: essential in Nature, mysterious in nature

Overall, there remains much to be learned about miR-190. Future experiments may include testing newer, more specific GAL4 drivers to attempt to localize the activity of miR-190 in the brain. Notably, we did not test miR-190 sponge expression on glial populations, which could be involved in sleep regulation and would thus constitute a viable avenue for future research. However, since we tested for the necessity of miR-190 in multiple areas in the fly brain and did not see an exclusive area where the miR is responsible for sleep regulation, it might be that either miR-190 is widespread in the fly brain, or that the neuronal circuitry is set up in such a way that can compensate for loss of miR activity in one area, which makes it difficult to localize in this way. Different approaches to localizing the miR include overexpression of miR-190 and rescue of the endogenous mutant sleep phenotypes. Areas of the brain that have been specifically implicated as being important for sleep regulation by the miR-190 sponge would be of particular interest. It would also be interesting to know what is happening on the level of gene expression of miR-190, as its targets remain mere hypotheses. Some of the more intriguing targets for miR-190 out of the 185 total conserved targets listed on TargetScan include Gad1, which encodes a glutamic acid decarboxylase, and Cha, which encodes a cholinergic acetyltransferase (www.targetscan.org). Cholinergic neurons have recently been implicated in playing a major role in sleep homeostasis (Seidner et al., 2015), and should thus be investigated in the context of miR-190.
Perhaps with a better understanding of the workings of micro-RNA 190, we can better understand both sleep physiology and pathophysiology.

**Possible connections between miR-190, human aging and sleep disorders**

Sleep disorders are unfortunately very common and can be highly disruptive to a person’s life. Sleep is so deeply entangled in psychiatric disease that the two are often diagnosed together (Zordan and Sandrelli, 2015). These pathophysiological facts present a convincing case that we as a society are obligated to attempt to further understand the underlying circuitry of sleep, as well as more general purposes of this behavior. miR-190 presents an opportunity to further explore the avenue of sleep disorders that specifically result from extreme sleep episode fragmentation. When we inhibited miR-190 in all neurons, as well as in specific neuronal populations, we observed a severe sleep fragmentation phenotype in many genotypes that decreased total sleep and which was typically composed of increased episode number along with decreased mean sleep episode duration. This encompasses the main problem associated with sleep-maintenance insomnia, as people with this disorder have a difficult time staying asleep, (as opposed to sleep onset insomnia where people have trouble falling asleep, which is associated with heightened sleep latency) (http://www.sleepdex.org/insomnia.htm).

The unavoidable process of human aging also brings about disruptive changes in sleep. As mentioned before, an increase in sleep fragmentation in the aging population has been observed, and is associated with a decrease in slow-wave activity. Recent studies have gone as far to link this increased fragmentation with declines in different areas of cognition (Lim et al., 2012). Both the fragmentation sleep phenotype and its correlated electrophysiological properties suggest that the strength of the overall homeostatic sleep system decreases with age. Seeing as changes in age come with changes in gene expression, micro-RNAs could be playing a role in regulating the amount of sleep fragmentation.
The strong suggested role of miR-190 in regulating homeostatic sleep in *Drosophila*, along with the fact that miR-190 is highly conserved, might indicate the miR as being important in this process in humans.

Perhaps studies of miR-190 and other such genetic regulators in model organisms will further reveal the molecular underpinnings of psychiatric and age-related sleep disorders. Hopefully, any findings of this nature will contribute to our growing knowledge about human neurophysiology pertaining to sleep and overall health.
5. Acknowledgements

I am so incredibly grateful to so many people. First and foremost I would like to thank Dr. Leslie Griffith for allowing me the opportunity to work in her lab four years ago. A special thank-you goes to my mentor Dr. Patricia Goodwin for her endless patience and for all of her help and guidance on this project. This work would not have been possible without you. I would also like to thank everyone in the Griffith Lab for welcoming me into the lab and for all of the help they have given me over the years. This has been a formative experience for me, to say the least. Thank you to all of my friends and family who supported me throughout this process. I also thank the David van Vactor lab for providing the miR sponge lines and the Cohen lab for providing the miR-190 transgenic heterozygous knockout. Finally, thank you again to Dr. Leslie Griffith, and to Dr. Paul Garrity and Dr. Nelson Lau for taking the time to be on my committee and for your careful consideration. Thank you.
6. References


**Appendix A: Table Summary of main results**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Where Expressed</th>
<th>Total Sleep Time</th>
<th>Sleep Episode Duration</th>
<th>Sleep Episode Number</th>
<th>Recovery Sleep After Deprivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>*NsybGal4&gt;*miR-190 sponge</td>
<td>Neurons</td>
<td>Decreased</td>
<td>Decreased</td>
<td>_____</td>
<td>Increased</td>
</tr>
<tr>
<td>*23E10Gal4&gt;*miR-190 sponge</td>
<td>Fan-shaped body</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>*TubulinGal80ts&gt;*miR-190 sponge</td>
<td>Ubiquitously in development</td>
<td>Decreased during day</td>
<td>_____</td>
<td>Increased</td>
<td>n/a</td>
</tr>
<tr>
<td>*miR-190 KO/+</td>
<td>Ubiquitous</td>
<td>Increased</td>
<td>Increased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>*miR-190Δ24</td>
<td>Ubiquitous</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>*TimGal4&gt;*miR-190 sponge</td>
<td>Tim+ Neurons</td>
<td>Decreased during day</td>
<td>Decreased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>*THGal4&gt;*miR-190 sponge</td>
<td>Dopaminergic Neurons</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
<td>n/a</td>
</tr>
<tr>
<td>*CryGal4&gt;*miR-190 sponge</td>
<td>Cry+ Neurons</td>
<td>_____</td>
<td>Decreased at night</td>
<td>Increased at night</td>
<td>n/a</td>
</tr>
<tr>
<td>*VT004849Gal4&gt;*miR-190 sponge</td>
<td>Dorsal fan-shaped body</td>
<td>_____</td>
<td>Decreased at night</td>
<td>Increased 24 hr, night</td>
<td>_____</td>
</tr>
</tbody>
</table>

*Not shown in figures:*

*increases or decreases in parameters are relative to all control groups, and apply to 24 hr, day and night time bins unless otherwise indicated*
Appendix B: Raw Data from MATLAB and SCAMP program:

**THGal4>miR-190 sponge:**

Females Baseline Average

Males Baseline Average:
CryGal4>miR-190 sponge:
Dorsal Fan-shaped body:
Temperature-sensitive Tubulin-Gal80>miR-190 sponge:

TimGal4>miR-190 sponge: