SPECIFIC AIMS: Sleep is a known biological behavior in humans. Many studies have aimed to determine the factors that regulate or induce sleeping patterns. *Drosophila melanogaster* has proven to be an extremely beneficial research model, as its sleeping behavior is remarkably similar to that of humans with many comparable reactions to specific drugs (Hendricks et al., 2000; Shaw et al., 2000). However, not much information is known about how neuronal pathways influence sleeping regulation in fruit flies. Our aim is to develop a further understanding of how external biological factors, namely the consumption of common stimulants such as caffeine, affect sleeping patterns in *Drosophila*. Through this, we hope to determine how certain chemicals play a role in sleep regulation in humans. This research may also lead to a further understanding of the pathophysiology of specific sleeping disorders such as insomnia.

Many researchers have suggested that a group of neurons in the dorsal fan shaped body (dFB) of *Drosophila* directly regulate sleeping patterns (Donlea et al., 2014). Essentially, dFB neurons, once activated, release GABA and inhibit wakefulness. Previous studies have also shown that the presence of dopamine (DA) can block the activity of dFB neurons as it inhibits the release of GABA, and thereby, promotes sleep (Crocker et al., 2010). Interestingly, caffeine also affects this sleep/wake activity as it acts presynaptically to increase DA signaling (Andretic et al., 2008). However, the effects of this stimulant are a controversial topic; is it beneficial or detrimental to one's functioning processes?

Overall, the goal of this research is to observe the effect of external stimuli, specifically the caffeine through the dopaminergic pathway, on sleeping behaviors via optogenetic stimulation of dFB neurons in *Drosophila*. We hypothesize that optogenetic activation of dFB neurons will induce sleep in *Drosophila* while the introduction of caffeine will greatly suppress sleep and promote behaviors of wakefulness. We hope to explore these hypotheses and achieve a greater understanding of this through the following aims:

1. **Genetic crossing to express CsChrimson in dFB neuronal area in F1 generation.** To express these exact genes, we will cross Gal-4/dFB male Drosophila with UAS/CsChrimson female Drosophila. Flies will be sexed and a strong Gal-4/dFB male Drosophila will be crossed with a strong UAS/CsChrimson female Drosophila pupae to produce F1 generation and to ensure no contamination of other genetic makeup from other flies is introduced. The male fly and female pupae will be put in a clean vial with food and allowed one week for reproduction to produce F1 generation.
2. **Dissecting of Drosophila brain to visualize neuroanatomical structure of the dFB neurons via immunofluorescence imaging.** To effectively verify a successful cross, we will dissect and mount the Drosophila brain. We will then observe the mounted brain under the fluorescence microscope. Three images will be captured and overlaid to express area we are trying to express.
3. **Examining the role of dFB in the sleep pattern of Drosophila both with and without the treatment of caffeine.** Under the use of optogenetics, sleeping behaviors of Drosophila will be assessed. A control Drosophila fly, with no caffeine consumption, will be optogenetically treated by shining a red light with a wavelength of 625 nm for 60 seconds and behaviors will be recorded. This will set the threshold for sleep onset of Drosophila. We will then treat different Drosophila flies with caffeine and record the same data observed in control. Behaviors of each will be compared to determine if and how caffeine affects sleep onset and sleeping behaviors.

SIGNIFICANCE

Importance of Studying Sleep in *Drosophila*

The notion of sleep remains a scientific mystery. Sleep could be defined as a period of reduced activity or response to one's environment. Moreso, it can be seen that sleep is a necessity for even the smallest of animals, such as *Drosophila*, just as much as it is necessary in humans. However, there are still many unknown underlying factors associated with sleep. The discovery of similarity in sleep patterns in *Drosophila* and humans was a groundbreaking milestone in sleep research (Hendricks et al., 2000; Shaw et al., 2000). In these works, many common features of *Drosophila* were observed and studied. One is in the similarities of sleep cycles between mammalians and fruit flies as they both undergo different stages in a period of sleep, with the similar purpose of maintaining homeostasis and consolidating memory, suggesting an evolutionary advantage for both species (Allada et al., 2017). Another shared feature between the two is their biological response to sleep deprivation as physically diminished and consequent attempt to regain sleep time (Harbison et al., 2009).

Being an extremely critical and evolutionarily-favored process, an altered sleeping pattern caused by internal or external factors can intensely affect behavior of many species, especially humans. For instance, even partial sleep deprivation can have a noticeable impact on cognitive performance. More chronic and extreme loss of sleep can cause patients to become more inclined to other mood disorders such as depression or anxiety (Krause et al., 2017). Despite these negative consequences, not much has been discovered about the innate aspect of sleep and how a biological factor may affect the sleeping patterns. Our research aims to discern the ethological value of sleep in organisms while utilizing *Drosophila* as a model of study to examine the effect of different neurons or external systems on sleep regulation.

Drosophila melanogaster as a model of study

Sleep is a known biological behavior in humans. *Drosophila melanogaster* poses as a significant model to study these behaviors as it presents much similarity to humans. In addition, they are readily available which compliments their fast and time efficient reproduction rates. Information about sleep can be easily extracted from *Drosophila* due to their simplistic and openly accessible nervous systems. Performing experiments on humans would be more complicated and highly complex due to the intense circuitry of their central nervous systems. Fortunately, *Drosophila* share neuronal and chemical similarity to those of mammals. In other words, the full genome is sequenced with many similarities to humans (Harbison et al., 2009). For these reasons, *Drosophila* have provided the ability to study a broad spectrum of behaviors including mating, aggression, feeding behaviors, and in our case, sleep. Our studies primarily focus on sleep homeostasis in *Drosophila melanogaster*.

Sleep Homeostasis Neurons in *Drosophila melanogaster*

Sleep has an underlying genetic basis. Previous studies have identified two specific neurons that present astonishing effects on *Drosophila* sleeping behaviors. Dopaminergic neurons suppress sleep when activated while a specific group of neurons in the dorsal fan shaped body (dFB) of *Drosophila* have been found to promote sleep (Ni et al., 2019).
Interestingly, these two neurons work in conjunction with one another. dFB neurons are known to release GABA and inhibit wakefulness. The presence of dopamine (DA) can block this activity of dFB genes, inhibit the release of GABA, which in turn will promote sleep (Ni et al., 2019). An increase of dopamine in the brain correlates with higher levels of motivation and attentiveness.

Caffeine is a widely used stimulant that favors awakeness and alertness. When consumed, caffeine activates dopaminergic neurons by increasing dopamine signaling. Specifically, the paired anterior medial (PAM) cluster of dopaminergic neurons are deemed the most relevant neurons that respond to caffeine in the *Drosophila* brain. When activated, these PAM neurons perform an antagonist effect on dFB neurons which in turn suppresses sleep. (Sitaraman et al., 2015).

The g-coupled adenosine receptor plays a distinct role in the regulation process of sleep. When present in the brain, adenosine binds to these receptors and sleep is induced. Caffeine produces an opposite effect in the brain as it acts as an antagonist for these receptors. When caffeine is present, less adenosine is able to bind to adenosine receptors; therefore, making it less feasible to induce sleep. Interestingly enough, these receptors are found in the brains of both *Drosophila* and humans; however, they present only 30% similarity as *Drosophila* have one known receptor (Nall et al., 2016). With caffeine, adenosine receptors are inhibited and wakefulness can be promoted as a result of this inhibition. This further drives the conclusion that caffeine is a favored stimulant to study its effects on sleep homeostasis in Drosophila as it produces dramatic disruption in sleeping behaviors at a molecular level.

Knowledge Gap

Our studies primarily focus on sleep homeostasis in *Drosophila melanogaster*. While many studies have aimed to determine the factors that may regulate or induce sleeping patterns, extensive research of effects of specific stimulant is much needed in the field. Our main goal is to fully understand dFB neurons, how they influence sleep, and how they are influenced by stimulants such as caffeine via the dopaminergic pathway. Thus, we aim to develop a better understanding of the mechanisms that may alter sleeping behavior in humans, which will hopefully aid in a better understanding of the pathophysiology of several sleeping disorders.

APPROACH (Preliminary Studies)

The GAL4/UAS is a system utilized to regulate gene expression in *Drosophila*. UAS (Upstream Activation Sequence) is an enhancer of a specific gene of interest, which can be activated by the binding of Gal4, a yeast transcriptional activator. Through this system, offspring carrying UAS and the gene of interest will not express that gene's transcription factor unless they also have the activator UAS. Therefore, this system allows for the expression of specific genes. In this study, we will combine the use of Gal-4/UAS system with the mechanism of optogenetics to express our specific gene of interest. Optogenetics is a tool that utilizes the light-sensitivity trait of channelrhodopsins to activate different nerves based on associated light wavelengths. Hence, we will combine these two methods by crossing two sets of flies: ones with Gal-4 protein

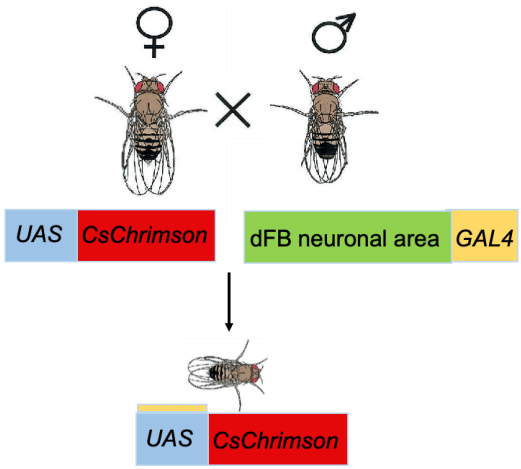

Figure 1. Combinatorial method: After cross, CsChrimson in the dFB neuronal area is expressed only in the F1 generation of GAL4/dFB male and UAS/CsChrimson female.

expression in their dorsal fan-shaped body (dFB) neurons, and ones with their CsChrimson regulated by UAS. With this cross, the CsChrimson channelrhodopsin will now be expressed in the dorsal fan-shaped body subset in F1 flies (Figure 1). By combining these two methods via genetic crossing, we are able to control when the dFB neurons are activated by introducing a red light (625 nm) to the *Drosophila*.

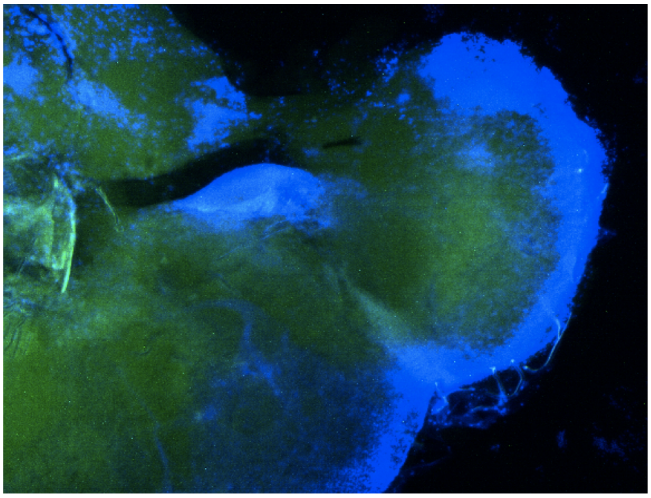

Figure 2. Immunofluorescence labeling of mVenus-tagged CsChrimson in the dFB neuronal area.

The channelrhodopsin CsChrimson we will be using in the experiment is tagged with mVenus, a yellow fluorescent protein visible under the immunofluorescence microscope. Hence, we can utilize that to visualize and confirm expression pattern of the CsChrimson in dFB neurons of the F1 *Drosophila* (Figure 2). In general, we will analyze the morphological position of our gene of interest and the different regions in the *Drosophila* nervous system which will provide a deeper layer of understanding on an anatomical level.

Caffeine is a strong stimulant that has been featured in several *Drosophila* sleep studies. As previous research has suggested, the acute administration (0.25–5.0 mg/ml over 8–12 h) of caffeine leads to rapid reduction in sleep bout duration in fruit flies. In most experiments, the introduction of caffeine to *Drosophila* is done indirectly through mixing with food.

The Boston University lab already has a Gal-4/dFB fly line (81014) and a UAS/CsChrimson flies line (55134). These two lines will be the parent flies for our cross in aim 1 and produce offspring to be further analyzed. The lab also has several microscopes equipped with filter cubes that filters the intense light generated by the mercury burner down to only specific wavelengths. This will make fluorescence microscopy possible for aim 2, allowing for fluorescence micrographs of the *Drosophila* nervous system. Caffeine is also a commonly available stimulant that can be obtained easily from many vendors, providing the material for aim 3. The availability of these tools in the BU lab indicates the feasibility of this proposal should it be conducted.

In summary, by using a combinative genetic approach, the results from this proposal should: create genetic crosses of Gal-4/dFB x UAS/ CsChrimson F1 for further analysis, provide an anatomical representation of the F1 *Drosophila* nervous system, and finally, examine behavior of flies based on activation or inhibition of these neurons.

APPROACH (Experimental Design)

Specific Aim 1: Generation of genetic crosses of CsChrimson expressed on dFB neurons.
Experiment: Crossing Gal-4/dFB male flies with UAS/CsChrimson female flies for F1.

Rationale: In this study, we will use the combinatorial genetic approach between the Gal-4/UAS and the optogenetics technique.
Design: Utilizing the fly lines already present in our lab, we will choose a fly from each line for genetic crosses. For the Gal-4/dFB flies (81014), we will sex the flies to pick out a strong adult male *Drosophila*. For UAS/CsChrimson flies (55134) , we will sex the pupae to find a female pupae about to enter maturation, this will ensure 100% genetic cross with no contamination of other genetic makeups from other flies. Then, we will put the two flies in a vial and allow one week for reproduction.
Possible results, interpretations, and pitfalls: The cross could be unsuccessful if the pupae (F1) are not properly removed from the vial to a fresh vial. It would be highly probable the adult flies (F0) would mate with the newly, maturing F1 generation. This potentially leads to an unsuccessful cross as a result of the F0 adult male not expressing CsChrimson. The possibility of this actually occurring is slim because we will monitor the growth of larvae biweekly.

Specific Aim 2: Imaging of the neuroanatomical structure of the dFB neurons in *Drosophila* brain.
Experiment: Dissecting F1 dFB/ CsChrimson fly brains and fluorescence imaging of the nervous structure.
Rationale: This study will use an anatomical level approach to visualize and confirm a successful cross in the F1 generation. In addition, data collected from this study will also provide visual references of CsChrimson expression within F1's dFB neurons. For that purpose, we will first dissect the brain and then employ immunofluorescence for color imaging of the brain.
Design: This experiment involves two methods. The first method is dissection in which we will put flies under the microscope and physically handle its body to separate a full-mount brain. The brain will then be observed under confocal microscope for better clarification. Since the dFB neuronal area is subjected to contain the mVenus-tagged CsChrimson, the confocal microscope can detect its presence under the fluorescent light. Then, the second method will be immunofluorescence microscopy imaging. We will put the mounted brain under the microscope with mercury burner and adjust it to different magnification levels. At each level, we will capture images from both the green (for neurons) and blue (for DAPI counterstain) filter at the same location (dFB neurons). Then, we will overlay the images using ImageJ to further examine the location of each region in the brain and how their neuroanatomy indicates about their functions.
Possible results, interpretations and pitfalls: Due to the miniscule nature of their brains, it can be difficult to perform the dissections. We have to assume total accuracy and be certain to solely dissect the brain of the *Drosophila* rather than brain fragments or tissue.

Specific Aim 3: Examining the role of dFB in sleep pattern of flies, and how caffeine's influence in its countering dopaminergic neuron may affect it.
Experiment: Activation of CsChrimson in the dFB neurons in flies that have and have not been exposed to caffeine.
Rationale: This experiment will mostly utilize the method of optogenetics to observe the effects of activation of dFB neurons on sleep. This study will also look at the effect of caffeine in inhibiting this neuron. For these purposes, we will mostly do observations of the flies behavior, as well as comparing analysis of both the control and experimental group.
Design: This experiment begins with the employment of optogenetics. In order to enhance the effect of optogenetics, we will feed the flies with all-trans retinal food for three days consecutively. This is because once the all-trans retinal food is consumed, it will undergo many transformations until it becomes 11 cis-conformation which is needed for phototransduction,

specifically the mechanism of pigment regenration. Since all-trans retinal food is extremely sensitive to light, we will incubate the flies in complete darkness after consumption of the food. This filtration from light can be done through wrapping of aluminum foil around the transparent vial. Then, we will conduct two separate experiments with the control group and experimental group with caffeine. For the control, we will put one fly each in two separate petri dishes and set up recording devices for each dish. After set-up, we will shine a red light for one minute to activate dFB neurons and induce sleep behavior in the flies. Sleep behavior in flies is mostly defined by inactivity of at least 5 minutes. Based on the recording, we can infer the sleep time period of flies. For the experimental group, we will use an extraction of caffeine solution and feed it to the flies in their food. Caffeine is a strong stimulant that can be fatal to *Drosophila* in high doses. The acceptable doses for most flies that can elicit a reaction is of 0.03-0.8% solution. Therefore, our solution will be a ratio of 1mg of caffeine/21ml of water or 0.047% solution. Caffeine is a very strong stimulant which can take effect after a short period of time. Hence, its effect becomes noticeable within 10 minutes. Therefore, we will wait 15 minutes for the caffeine to take effect and carry on optogenetics observations as done before with the control group. Finally, we will compare the sleep time period between the control and experimental group.

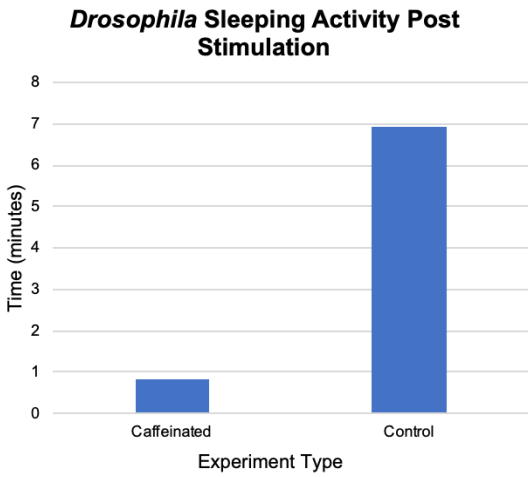

Figure 3. The fruit flies were optogenetically stimulated for one minute and the time it took for them to move or engage in regular activity was recorded.

Possible results, interpretations and pitfalls: There are a few possibilities to consider when conducting the experiment. One includes the fact that caffeine can be fatal to *Drosophila* so we have to have the proper dilutions and amounts within their food. They also cannot consume a lot of it before it leads to their death. Also, the flies might not eat the food or be attracted to it once placed into the petri dish. It would be better to starve the flies in order to ready them for their food.

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