Relating Major Depressive Disorder (MDD) to circadian signaling in *Drosophila melanogaster*

Taylor Fox and Mir Zayid Alam

Research Plan

Animal Science

A. Rationale

According to the National Institute of Mental Health (2019), 17.3 million American adults and 3.2 million adolescents aged 12-17 (7.1% and 13.3% of respective U.S. populations) experience Major Depressive Disorder (MDD). 63.8% of adults and 70.8% of adolescents who experienced MDD exhibited severe impairment of quality of life. Also, Greenberg et al. (2015) reported that MDD was responsible for $210.5 billion in productivity losses in 2010, representing a 21.5% increase from the $173.2 billion from 2005. A 2017 National Sleep Foundation poll revealed that 73% of adolescents who experienced MDD did not get enough sleep per night.

Depression in animals has been characterized by lower-than-normal levels of serotonin, or 5-hydroxytryptamine (5-HT). Mushroom body 5-HT1A receptors control 5-HT synthesis/activity, while 5-HT2B receptors in the mushroom body lobes, while the 5-HT1A receptors control behavioral quiescence. Depression-like behaviors, such as the lack of resistance to stimuli such as uncomfortable heat or electric shocks, can be induced via chronic stress, such as heat punishment (Ries et al. 2017).

Serotonin, or 5-HT, is a neurotransmitter ubiquitously present in mammals and insects. It is also implicated in regulating sleep-wake cycles (Yuan et al. 2006). Yuan et al. (2006) implicated 5-HT in sleep promotion (ablating serotonergic cells led to insomnia) as well as wakefulness (the timing of serotonin secretion is correlated with neuronal activity). d5-HT1A, d5-HT1B, d5-HT2, and d5-HT7 serotonin receptors in the brain of fruit flies. Yuan et al. (2006) posit that their stimulation and knockout could be used to tease out the mechanisms of the serotonergic regulation of sleep.

Light is involved in circadian stimulation and entrainment via two main pathways: the photoreception of ocelli, compound eye, and the Hofbauer-Buchner eyelets (HB), as well as the blue-light photo-pigment Cryptochrome. Cryptochrome is known for its role in targeting the Timeless protein in the biochemical circadian oscillator of fruit flies (Shang et al. 2008; Fogle et al. 2011). Light promotes arousal in fruit fly brain arousal circuits, specifically by stimulating the pars intercerebralis (PI) neurons and ellipsoid body (EB) neurons of the central complex (CC).

The large lateral ventral neurons (l-LNvs) and mushroom bodies are specifically implicated in arousal in their action potential firing in response to light (Shang et al. 2008).

The circadian oscillator of flies consists of negative feedback transcription cycle of the *period* and *timeless* genes, which is in part mediated by the presence of the Timeless protein. This system is mediated by 140-150 clock neurons, notably including the pigment-dispersing-factor-expressing (PDF) lateral ventral neurons (LNvs), which specifically include Evening (E cells; include 5th Lateral Neuron (5th LNv), Dorsal Lateral Neurons (lND), some dorsal neurons (DN1s)), and Morning cells (M cells; include small LNvs (s-LNvs)) (Fogle et al. 2011). The small lateral ventral neurons (sLNvs) are critical to the maintenance of circadian rhythms. The action of period-controlling neurons controls arousal, locomotion, aggression, and reproductive tenacity in flies (Dissel et al (2015); Li et al. (2009)).

Ries et al (2017) conducted a study that established the fly as a model for MDD. Flies were exposed to a vibrational stimulus several days. It was found that flies retained motor function, as revealed by a phototaxis assay, but demonstrated a decreased motivation in a climbing assay. Additionally, serotonergic signaling was reduced (an often posited symptom of MDD) in flies exposed to the vibration. Finally, the latency of courtship of flies exposed to vibration was found to be greater than compared to non-exposed flies (Ries et al., 2017).

In 2017, Qian et al. studied molecular mechanisms pertaining to the sleep homeostasis of fruit flies, specifically investigating the roles of *Trh*, responsible for the 5-HT synthesizing enzyme, as well as all five 5-HT receptors in the brain of flies. Using microscopy (GAL4-UAS to express fluorescent tagging proteins; immunofluorescence using antibodies), genetic techniques, and a Trikinetics *Drosophila* Activity Monitor, Qian et al. assessed gene expression and downstream ramifications on locomotor behavior/periodicity. Using a 5-HT receptor (*5HT2b*) knockout line, they determined that the 5-HT2B receptor was essential for maintaining sleep homeostasis. Additionally, it was found that genetically ablating *Trh*, *5HT1a*, or *5HT2b* reduced overall sleep duration, and the ablation of *Trh* and *5HT2b* reduced the sleep rebound of the flies after sleep deprivation.

Yuan et al. (2005) studied the role of 5-HT in circadian entrainment of *Drosophila melanogaster* using GAL4-UAS/immunohistochemistry, drugs to alter circadian rhythms, and

assessment of sleep architecture. They found that 5-HT plays a role in preventing dramatic adjustments to the circadian period of arousal, thus slowing entrainment, but contributing to sleep homeostasis. Additionally, immunostaining revealed that aside from lateral neurons (already established), PI neurons, mushroom bodies, and the optic lobes of the fly's brain were implicated in this serotonergic circadian regulatory mechanism (Yuan et al. 2005).

A telling study by Turner et al. (2008) paints a more mysterious picture of MDD than literature suggests, however. This meta-analysis revealed the role of data manipulation impacting the scientific interpretation of MDD and treatment. From 1987 to 2004, 74 studies on supporting MDD remediation centering on serotonin or associated chemicals were performed; these studies were used to validate the use of antidepressants. Out of these studies, 38 yielded positive results, and 37 of these were published. However, 36 studies declared inconclusive results; only three of these studies were published as so. 11 of the inconclusive studies were published with a 'positive outlook' on the future of research in that vein, while the majority of those studies (22 studies) went unpublished entirely (Turner et al. 2008). Clearly, this conflict in literature necessitates further investigation.

Drosophila melanogaster is an appropriate model for a behavioral study because MDD can be reliably modeled, and MDD's pathology in fruit flies has been shown to rely on similar systems to those of humans. This includes the serotonergic signaling implicated in MDD but also includes interlocking circadian mechanisms such as the opposing dopaminergic and gamma-aminobutyric acid-releasing (GABAergic) signaling pathways, which are responsible for arousal and drowsiness respectively in flies (Martin et al. 1998; Yuan et al. 2006). In addition, the fly has great potential for genetic manipulation; for example, the use of fluorescent tagging proteins, and information about each of *Drosophila melanogaster*'s ~14,000 genes is available online (Flybase).

Ries et al. (2017) modeled MDD ('learned helplessness,' or failure to respond to a disturbing stimulus) to flies by exposing them to a 300 Hz vibrating motor periodically for ~10 hours/day for several days. This was quantified using a modified climbing assay in which the number of times a fly attempted to surpass an insurmountable gap via climbing was assessed.

Additionally, reproductive latency can be measured using 'depressed' males and virgin females in a video-recorded chamber (Ries et al. 2017).

Flies overexpressing *Trh* (using bacterial sodium channel UAS-*NaChBac*) can be used to assess how additional serotonin production can modulate assorted behaviors of fruit flies (including in response to vibration). Regulatory system manipulation can be extended to using temperature-dependent signaling (using *Drosophila* dTrpA1, which only transcribes a reporter gene at temperatures above room temperature, such as 32°C) (Ries et al. (2017); Zhang et al. (2013)).

B. Research Question, Hypotheses, Expected Outcomes

Research question:

What is the relationship between circadian rhythm modification and the severity of symptoms of MDD in the model *Drosophila melanogaster*?

Hypotheses:

The alternate hypothesis is that overexpression of *Trh* will reduce the severity of MDD-associated motivational deficits of fruit flies (ex. Climbing, reproductive tendencies), and that post-vibrational-stimulation (to mimic MDD) thermogenetic *Trh* overexpression can play a role in reducing said parameters. Additionally, the extension of the fruit fly's photophase is posited to reduce the severity of MDD-like symptoms.

The null hypothesis is that no genetic or environmental manipulation of fruit flies before or after exposure to the MDD-like vibrational stimulus will reduce the severity of motivational behavioral deficits.

Expected Outcomes:

It is expected that MDD-like symptoms of reduced motivational behavior (including lower climbing tendency per the gap-climbing assay, lower rates of spontaneous walking per Buridan's paradigm, and greater courtship suppression per the courtship latency assay). Additionally, it is expected that inducing *Trh* transcription will remediate these deficits. Finally, it is expected that driving *Pdf* activity in LNVs will lengthen the photoperiod of flies, and is is expected that elongating the photoperiod of animals will reverse expected MDD-like behavioral deficits.

C. Procedures, Risk and Safety, Data Analysis

- **Procedures:**

Culturing Flies

Culturing *Drosophila melanogaster*: Wild type strains will be used and obtained from Carolina Biological. *Elav*-Gal4, *Pdf*-Gal4, UAS-*Trh* and UAS-*TrpA1* will be obtained from the Bloomington Drosophila Stock Center. *fumin* and *w1118* controls will be acquired from Rob Jackson (Tufts University School of Medicine).

Food will be prepared using the following protocol: 1 scoop (using an included measuring cup) of Formula 4-24 Instant *Drosophila* Medium from Carolina Biological will be combined in a ratio of 1:1 with spring water in the vial. Then, baker's yeast will be sprinkled on top. The flies will be transferred to vials containing the fresh media approximately 1 minute after being made to ensure that the medium is not so soggy that the flies will get stuck and die. (Flagg 1988)

Fly stocks will be maintained as follows: Upon receiving the *Drosophila melanogaster*, organisms will be transferred to (a) properly prepared vial(s) of food. Label vial with date of transfer. If the flies are age-synchronized and/or sexed, they will be labeled accordingly. For age-synchronized population generation, flies must be transferred daily or otherwise2+ age-synchronized generations will be lost. (Flagg 1988)

Drosophila melanogaster will be stored in plastic vials with cotton or foam plugs that allow for air circulation as well as firm seals. In order to transfer flies from old vials to new vials, the old vial will be tapped on a flat surface briskly (several times if necessary) to knock flies to the bottom of the vial. Next, the plug will quickly be removed, and new vial will be placed on top. Flies will naturally climb up into the new vial, but the transfer process can be hastened by flipping both vials and then vigorously tapping to knock flies to the bottom of the new vial. Finally, a plug will be inserted into the new vial. (Flagg 1988)

Genetics

Majeed et al. (2016) developed a protocol for 5-HT overexpression: virgin female *elav*-Gal4 driver-bearing flies (Bloomington Stock #8760) will be crossed with male UAS-*Trh* (Bloomington Stock #27638) reporter-bearing flies. This induces expression of tryptophan

hydroxylase, the rate-limiting enzyme in the synthesis of serotonin, in the brain neurons of *Drosophila melanogaster*, thus increasing the concentration of serotonin (Majeed et al. 2016).

Photoperiod extension can be accomplished via manipulating the environment of the flies, but can also be examined using the dTrpA1 cation channel (which drives neuronal activity at temperatures from 26-30+ degrees Celsius). Specifically, female *Pdf*-Gal4 (Bloomington Stock #6899) will be crossed with male UAS-*dTrpA1* (Bloomington Stock #26263) (Berni et al. 2010).

Anesthetization

Flies in vials (with vials oriented on their sides to prevent flies from getting stuck in food if there is food in the vial) will be placed in a freezer for 2 minutes. They will remain largely immobilized for 2-3 minutes after removal. Care will be taken not to leave flies in the freezer, for they will perish, and also, it must be taken into account that the older a fly is, the longer it will take to recover from the cold (Flagg 1988).

Sexing

To sex *Drosophila melanogaster*, first 2 fresh vials will be prepared, one for males and one for females. Then, flies will be anesthetized using a freezer (see anesthetization protocol). Anesthetized flies will be placed on ice packs under a microscope. Next, using a fine, soft-bristled paintbrush, males and females will be sorted into separate groups. Males can be distinguished based on their darker, rounder, and smaller abdomens, meanwhile females can be distinguished based on larger, pointier, and lighter abdomens. Males are typically smaller than females, but this will not be the sole criterion used to distinguish males and females. After males and females are sorted, they will be lightly brushed into segregated vials labeled appropriately (Flagg 1988).

Age Synchronization

To age-synchronize flies, flies will be transferred (from stock population or already age-synchronized) into a fresh vial to mate. After 24 hours, these flies will be transferred again to another vial. The vial in which flies mated for 1 day will contain similarly aged *Drosophila melanogaster*, which will eclose 9-10 days later. In order to preserve age-synchronized

generations, flies used for generation age-synchronized generations will be transferred daily; mating periods of more than 24 hours can lead to greater discrepancies in age (Flagg 1988).

MDD Stress Treatment

15-25 males aged 3-5 days old will be placed in acrylic tubes 98 mm long and 4 mm wide. Each side will be plugged with cotton stoppers, and these tubes will be placed on top of vibration motors (Obtained from Tinkersphere, product number TS1680). Controlled using an Arduino, these motors will vibrate for 20 seconds at a time with 10 s in between vibrations in order to prevent habituation. This is to be repeated over 15 minutes and will be followed by 30 minutes of recovery; this cycle will occur approximately 12-14 times per day for 3 days or more. Flies will be transferred from standard medium to these empty tubes for vibration, and at the finish of each day's worth of vibration, they will be returned to standard food. Controls for vibrational stress involve placing flies in an acrylic tube on a stable tabletop or countertop instead of exposed to the vibration motor. (Ries et al. 2017)

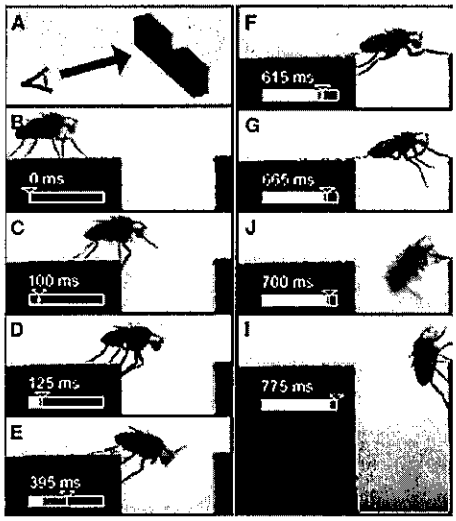
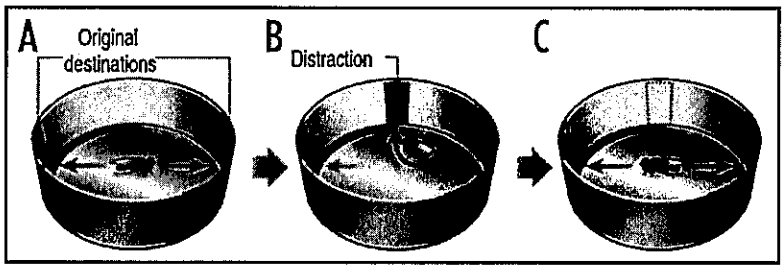


Figure 1: (A) shows what the gap-climbing apparatus will look like, while figures (B)-(I) show an example of the behavior in question (Pick et al. 2005).

Buridan's Paradigm

Flies exposed to or not exposed to stress as described by Ries et al. (2017) will have their wings clipped using dissection micro scissors and will be individually introduced into a model of Buridan's arena (8.5 cm diameter circular island surrounded by water, enclosed in a 20 cm

diameter transparent cylinder (ex. Perspex) with dark vertical stripes on opposite ends of the cylinder, which is also illuminated by white ring lights). Flies will spend time walking back and forth between the two dark vertical stripes. The total percent time spent walking per 15 minutes of video observation will be reported as spontaneous walking activity (Strauss et al. 1992). This paradigm will be created using a Makerbot Replicator Z18, a Perspex transparent cylinder, and a



ring light (QIAYA from Amazon; ASIN: B01HXTHPXU).

Figure 2: A model of Buridan's paradigm is depicted above. The wing-clipped fly will walk towards vertical black stripes at certain rates without other stimulation: this walking activity is qualified as spontaneous and motivational (Pitman et. al 2009).

Sleep Analysis

Sleep deprivation and associated effects of locomotion will be measured using a Drosophila Activity Monitor (Trikinetics) in a light-proof box (ex. cardboard). An Arduino microcontroller and an LED light shield (Adafruit) will be used to control the light schedule of the flies. The monitoring system will be housed in a well-ventilated and/or temperature regulated room (~25°C). It is preferable to have a dark room for the monitor; in order to see in the dark room, a lights with red filters can be used (*Drosophila melanogaster* are not sensitive to red light) A PC or Macintosh computer will be dedicated to full-time data collection, preferably with minimal software installed to prevent crashing (loss of data). Data from the DAM must be downloaded onto the device, so significant hard drive space or a USB/similar device is also necessary. Raw binary data from when flies are in the DAM (where periods of inactivity are 0 and periods of activity are 1) will processed using DAM Filescan 102X; circadian parameters are measured in 15 and 30-minute bins, while sleep/rest parameters are measured in 1 to 5-minute bins. 5 minutes of inactivity in a row is generally accepted as sleep/rest in *Drosophila*

melanogaster. Tubes will be occupied by individual flies, with food plugging one end of the tube and a cap covering the other end. Adult male flies from 1-5 days of age will be used to measure locomotor activity since the egg-laying of females can impede locomotion. (Ali et al. 2011)

- **Risk and Safety:**

1. Human Participants: N/A

2. Vertebrate Animal Research: N/A

3. Potentially Hazardous Biological Agents (PHBA)

Organism: *Drosophila melanogaster*

Sources: Bloomington Drosophila Stock Center: *elav*-Gal4, *pdf*-Gal4, UAS-*trh*, UAS-*pdf*

Jackson Lab (Tufts University School of Medicine): *w1118*, *fumin*

BSL: 1

Precautions: All activities will be supervised by instructors.

Disposal: To dispose of all strains of *D. melanogaster*, all vials containing adults, larva, or eggs will be placed in a freezer for 48 hours. Then, they will be sprayed with a 10% bleach solution and disposed of in biohazardous waste containers. If mold develops in medium, bleach will be poured on the mold (not sprayed to minimize transmission of spores) and then the medium will be parafilmed and disposed of (Vanderbilt University School of Medicine, 2018).

4. Hazardous Chemicals, Activities, and Devices

All handling of hazardous chemicals will be supervised by instructors.

-Bleach (Sodium Hypochlorite, 10% in water, 500 mL): Can cause skin corrosion, serious eye damage, and damage to aquatic animals. If ingested, rinse mouth and do not induce vomiting. If on skin/hair, rinse thoroughly. If inhaled, call a Poison Center and remove the afflicted person to fresh air. If in eyes, call a Poison Center, and gently rinse eyes after removing contact lenses, if applicable. Lab aprons and rubber or nitrile gloves will be used while handling. A licensed disposal company will be contacted to dispose of this solution, unless greatly diluted. SDS.

- **Data Analysis:** All data analysis will be performed using Microsoft Excel or IBM SPSS v.25

-Buridan's Paradigm: the average and standard deviation of the percentage of time spent walking per fly per 15 minute observation period in Buridan's arena will be recorded for 3+ flies for each

treatment. These means will be analyzed using a one-way ANOVA ($p < 0.05$) (Ries et al. 2017). Data will be presented in Microsoft Excel graphs.

-Sleep Analysis: the ShinyR-DAM software package created by Cichewicz and Hirsh (2018) will be used to create actograms and calculate aggregate sleep per night/day, and sleep bouts and bout lengths.

D. Bibliography

Ali, Yousuf O., et al. "Assaying locomotor, learning, and memory deficits in Drosophila models of neurodegeneration." Journal of visualized experiments: JoVE 49 (2011).

Berni, Jimena et al. "Using Neurogenetics and the Warmth-Gated Ion Channel TRPA1 to Study the Neural Basis of Behavior in Drosophila." Journal of undergraduate neuroscience education : JUNE : a publication of FUN, Faculty for Undergraduate Neuroscience vol. 9,1 (2010): A5-A14.

Cichewicz, Karol, and Jay Hirsh. "ShinyR-DAM: a Program Analyzing Drosophila Activity, Sleep and Circadian Rhythms." *Communications Biology*, vol. 1, no. 1, 2018, doi:10.1038/s42003-018-0031-9.

Clarke, Greg, and Allison G Harvey. "The complex role of sleep in adolescent depression." *Child and adolescent psychiatric clinics of North America* vol. 21,2 (2012): 385-400. doi:10.1016/j.chc.2012.01.006

Dissel, Stephane et al. "Sleep restores behavioral plasticity to Drosophila mutants." Current biology : CB vol. 25,10 (2015): 1270-81. doi:10.1016/j.cub.2015.03.027

Druce, Maralyn & Rockall, Andrea & Grossman, Ashley. (2009). Fibrosis and carcinoid syndrome: From causation to future therapy. Nature reviews. Endocrinology. 5. 276-83. 10.1038/nrendo.2009.51.

Fogle, K. J., Parson, K. G., Dahm, N. A., & Holmes, T. C. (2011). CRYPTOCHROME Is a Blue-Light Sensor That Regulates Neuronal Firing Rate. Science, 331(6023), 1409–1413.doi:10.1126/science.1199702

Flagg, Raymond O. *Carolina Drosophila Manual.* Carolina Biological Supply Company, 1988.

Greenberg, Paul E., et al. "The Economic Burden of Adults With Major Depressive Disorder in the United States (2005 and 2010)." The Journal of Clinical Psychiatry, vol. 76, no. 02, 2015, pp. 155–162., doi:10.4088/jcp.14m09298.

Joiner, William J., et al. "Sleep in Drosophila Is Regulated by Adult Mushroom Bodies." *Nature*, vol. 441, no. 7094, 2006, pp. 757–760., doi:10.1038/nature04811.

Kasture, Ameya Sanjay et al. "Big Lessons from Tiny Flies: Drosophila melanogaster as a Model to Explore Dysfunction of Dopaminergic and Serotonergic Neurotransmitter Systems." International journal of molecular sciences vol. 19,6 1788. 16 Jun. 2018, doi:10.3390/ijms19061788

Li, Xinjian, Feng Yu, and Aike Guo. "Sleep deprivation specifically impairs short-term olfactory memory in Drosophila." Sleep 32.11 (2009): 1417-1424.

Ly, Sarah et al. "The neurobiological basis of sleep: Insights from Drosophila." Neuroscience & Biobehavioral Reviews 87 (2018): 67-86.

Majeed, Zana R., et al. "Modulatory Action by the Serotonergic System: Behavior and Neurophysiology InDrosophila Melanogaster." Neural Plasticity, vol. 2016, 2016, pp. 1–23., doi:10.1155/2016/7291438.

Martin, Jean-René, Roman Ernst, and Martin Heisenberg. "Mushroom Bodies Suppress Locomotor Activity in Drosophila Melanogaster." Learning & Memory 5.1 (1998): 179–191. Print.

Pick, Simon, and Roland Strauss. "Goal-Driven Behavioral Adaptations in Gap-Climbing Drosophila." *Current Biology*, vol. 15, no. 16, 2005, pp. 1473–1478., doi:10.1016/j.cub.2005.07.022.

Pitman, Jena L., et al. "There Are Many Ways to Train a Fly." *Fly*, vol. 3, no. 1, 2009, pp. 3–9., doi:10.4161/fly.3.1.7726.

Qian, Yongjun, et al. "Sleep Homeostasis Regulated by 5HT2b Receptor in a Small Subset of Neurons in the Dorsal Fan-Shaped Body of Drosophila." ELife, vol. 6, 2017, doi:10.7554/elife.26519.

Ries, Ariane-Saskia et al. "Serotonin modulates a depression-like state in Drosophila responsive to lithium treatment." Nature communications vol. 8 15738. 6 Jun. 2017, doi:10.1038/ncomms15738

Shang, Y., Griffith, L. C., & Rosbash, M. (2008). Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proceedings of the National Academy of Sciences, 105(50), 19587–19594.doi:10.1073/pnas.0809577105

Strauss, R., Hanesch, U., Kinkelin, M., Wolf, R. & Heisenberg, M. No-bridge of Drosophila melanogaster: portrait of a structural brain mutant of the central complex. J. Neurogenet. 8, 125–155 (1992).

Turner, Erick H., et al. "Selective Publication of Antidepressant Trials and Its Influence on Apparent Efficacy." New England Journal of Medicine, vol. 358, no. 3, 2008, pp. 252–260., doi:10.1056/nejmsa065779.

Vanderbilt University School of Medicine. "Disposal Procedures for Insects Used in Research." Vanderbilt Environmental Health and Safety. Vanderbilt University Medical Center, 2018. Web. 14 May 2018. <https://www.vumc.org/safety/waste/biological-waste-insects>.

Wnuk, Alexis. "Rethinking Serotonin's Role in Depression." *Brainfacts.org*, Society for Neuroscience, 8 Mar. 2019, www.brainfacts.org/diseases-and-disorders/mental-health/2019/rethinking-serotonins-role-in-depression-030819. Accessed 15 May 2019.

Yuan, Q., Joiner, W. J., & Sehgal, A. (2006). A Sleep-Promoting Role for the Drosophila Serotonin Receptor 1A. Current Biology, 16(11), 1051–1062.doi:10.1016/j.cub.2006.04.032

Yuan, Q., Lin, F., Zheng, X., Sehgal, A., (2005). Serotonin modulates circadian entrainment in Drosophila. Neuron 47, 115e127

Zhang, T., et al. "Octopamine-Mediated Circuit Mechanism Underlying Controlled Appetite for Palatable Food in Drosophila." Proceedings of the National Academy of Sciences, vol. 110, no. 38, 2013, pp. 15431–15436., doi:10.1073/pnas.1308816110.

"Depression & Sleep." National Sleep Foundation, 2017, www.sleepfoundation.org/articles/depression-and-sleep. Accessed 30 Apr. 2019.

"How To Drive A Vibration Motor With Arduino And Genuino." *Tech Blog*, Precision Microdrives, 2019, www.precisionmicrodrives.com/content/how-to-drive-a-vibration-motor-with-arduino-and-genuino/. Accessed 17 May 2019.

"Is DMAP1 an Essential Gene in Drosophila?" *SmallScienceWorks*, 29 May 2015, smallscienceworks.com/tag/uas-gal4/. Accessed 17 May 2019.

"Major Depression." National Institute of Mental Health, U.S. Department of Health and Human Services, Feb. 2019, www.nimh.nih.gov/health/statistics/major-depression.shtml#part_155033.

Project Summary:

NO ADDENDUMS EXIST