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Effects of Sleep-Deprivation on Decision-making and Action Selection

A Thesis

Presented to

The Faculty and the Honors Program

Of the University of San Diego

By

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Abstract

This thesis addresses neuroscience research focusing on the brain's mechanisms underlying behavioral choice, or prioritization, and decision-making. The research has been conducted with *Drosophila melanogaster*, the fruit fly – a good model from both the behavioral and neural perspectives. This project specifically observes the co-regulation of sleep with two other behaviors – courtship and oviposition. The overlap between the sleep and courtship circuits in the brain should provide a good model for behavioral prioritization, and the interaction between sleep and ovipositional preference should provide a model for understanding the effects of sleep on decision-making. All three of these adaptive behaviors are well studied at the behavioral level among flies and humans, but not well understood at the neuronal levels. The data presented points toward a neurotransmitter called *octopamine* – the fly's homolog of the human neurotransmitter *norepinephrine* as key in the co-regulation between sleep and decision-making circuitry in the brain. Further research should delve into this pathway for a better understanding of such neural mechanisms.

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Introduction and Literature Review

Basic adaptive behaviors such as sleep, courtship, feeding and even aggression are conserved across species. The simplest and most complex of organisms somehow are capable of prioritizing among these basic behaviors using both external and internal cues to effectively determine which behavior is momentarily more important. Among humans, prioritization to some degree must be inherent; research shows the human brain was not originally evolved to multi-task (D'Esposito et al. 1995). Although behavioral prioritization is highly supported for many organisms, we are only beginning to understand the brain's processes underlying such behavioral choice (McFarland 1977; Mowrey and Portman, 2012; Barron et al., 2015; Esch and Kristan, 2002). Behavioral prioritization or choice can also be referred to as “action selection.”

An added aspect to action selection is that some behaviors, such as feeding, require a degree of decision-making (Itskov and Ribeiro 2013). It seems that even invertebrates such as the *Drosophila* engage in decision-making – rather than simply responding to stimuli with impulsive instincts (Brembs 2013).

When seeking to understand the neural mechanisms underlying observable behaviors, we often turn to model organisms, and this research utilizes *Drosophila melanogaster* – the fruit fly. Behavioral trends representing those common among humans can be identified in the model organism, which can then be further evaluated at the neural level. Several behavioral studies using the *Drosophila melanogaster* have shown trends similar to human behavior, including those requiring cognitive abilities (Kazama 2015). Furthermore, analogous features of the fly and human nervous systems have been identified. Some of the fruit fly's senses, such as olfaction, are involved in

behavioral choices, are simplified, and yet are highly conserved from *Drosophila* to humans (Fiala 2007). Systems including neurotransmitter signaling and the wiring of neural circuitry provide simplified but thorough models for gaining a better understanding of similar features in the more complex human brain (Hendricks & Sehgal 2004).

With all of the similarities described above, *Drosophila* is an excellent model to work with in neuroscience research. Three tangibles that facilitate the use of *Drosophila* are (1) that we can genetically manipulate the fruit flies down to genetically tractable neurons, (2) that stocks can be raised fairly quickly given their short generation time, and (3) that using genetic schemes, we can manipulate neurons in live, active flies (Kazama 2015).

In this research, three well-studied, rich, and intricate basic behaviors of the *Drosophila* are identified: sleep, sex, and oviposition.

Selecting sleep as a focus of this study was based on the growing understanding of sleep behavior and sleep circuitry in the fly brain, as well the relevance of sleep-deprivation and its interactions with various cognitive functions and behaviors of humans. In *Drosophila*, several neuroscience studies have identified key areas in the brain implicated in the sleep circuitry. Findings include dopaminergic neurons in the mushroom body and the octopaminergic neurons that act on insulin-producing cells, both of which act to promote wakefulness (Sitaraman et al. 2015 & Erion et al. 2012).

Courtship is the second behavior observed in this research. A male fly demonstrates various courtship rituals when attempting to mate with a female. These include chasing the female, tapping her with his forelegs, contacting his mouth to her

genitalia, singing a courtship song with his wings, and mounting her and bending his abdomen to attempt copulation (Pavlou & Goodwin 2013). All five have been repeatedly identified and can be easily observed upon close investigation.

Oviposition is a complex behavior by which female fruit flies decide where to lay their eggs. A variety of factors influences this choice, such as the content of the available substrates and their correspondence with the flies' nutritional needs. The substrates must provide nutrition and sterility to eggs to ensure healthy development. An interesting aspect of oviposition is its discrepancy with positional preference. This means that the female fruit flies sometimes spend more time around one substrate, while laying all of their eggs in another. A recent study showed that female fruit flies highly preferred laying eggs in food containing acetic acid, despite strong positional avoidance for the same food (Joseph et al. 2009). The experiments in this study further suggest that different sense might be taken into account for the two related behaviors – gustatory signaling seemed to be involved in ovipositional preference, whereas olfaction seemed to guide where the females spent most of their time (Joseph et al., 2009). Such observations in regards to oviposition and positional preference further varied if the available substrates changed (Dweck et al, 2013). Altogether, these results display the complexity of ovipositional preference, as well as its interaction with other behaviors. Building upon these findings, this thesis research looks into the overlap between sleep and oviposition, and how sleep-deprivation might mediate the decision-making in oviposition behavior. Because oviposition is clearly a complex decision, it serves as a strong model for decision-making.

This thesis as a whole comprises research on the effects of sleep-deprivation on courtship behavior exerted by male flies, as well as the effects of sleep-deprivation on oviposition behavior of female *Drosophila*. While the first is focused on understanding action selection processes, the latter should have implications for how sleep-deprivation modulates decision-making. To study the underlying mechanisms for the interaction of sleep with courtship and sleep with oviposition, specific groups of neurons in the fruit flies brain must be targeted, for which thermogenetic methods are utilized.

Methods

Thermogenetic targeting of specific neurons

GAL4-UAS gene expression system. The GAL4-UAS binary expression system allows spatial and temporal regulation of gene expression, and can be utilized as a powerful genetic tool in neuroscience research with *Drosophila melanogaster* (Scialo et al. 2016). This system has two factors – a transcription factor called GAL4 that is placed under the control of a tissue-specific promoter, and a gene of interest positioned downstream of a UAS (upstream activator sequence) sequence. The tissue-specific promoter provides spatial control. The promoter selected will express GAL4, the transcription factor will be produced, and if both the transcription factor and UAS are present, the transcription factor will bind UAS and cause expression of the gene of interest. Though UAS will be present in a variety of neurons, GAL4 will be neuron-specific because of the promoter that controls it. UAS present in other neurons will not be activated, and thus in these other locations it will not cause the expression of the gene of interest.

Temporal control can be introduced via several methods, but inserting a temperature-sensitive ion channel called dTrpA1 is the technique used in this project. The dTrpA1 gene is thus the gene of interest, inserted downstream of UAS. Because this is expressed only where the GAL4 transcription factor is present, the dTrpA1 channels can be inserted in specific target groups of neurons. The resulting phenotype allows experimental, temporal manipulation of neuronal activity in neurons-of-choice. When the mature flies containing the full GAL4-UAS-dTrpA1 system are placed in an environment between 27 to 30 degrees Celsius, the channels will be activated, and the opening of these channels will create action potentials directly interfering with the brain's natural signaling in that group of neurons. Neurons identified to be involved in a specific behavior can thus be targeted – in this case, neurons implicated in regulating sleep.

Thermogenetic targeting of dopaminergic clusters. In this experiment, the GAL4-UAS system was used to target clusters of dopamine neurons using the temperature dependent dTrpA1 method. The MB054B neurons are mushroom-body neurons that express dopamine as their primary chemical messenger. This cluster exhibits strong wake-promoting control, and thus is an essential component in the sleep circuit (Sitaraman et al. 2015). When the flies of the desired phenotype – containing dTrpA1 in MB054B neurons – are placed in the appropriate temperature, these dopaminergic neurons are activated, and sleep is disrupted. Any significant results from sleep-deprivation tests done with these flies will indicate that these dopaminergic MB054B neurons are involved in the co-regulation of sleep and the other observed behavior.

Thermogenetic targeting of octopamine clusters. Octopamine is a neurotransmitter that is the invertebrate homolog of the human brain's chemical messenger norepinephrine, and is also found to be involved in sleep behavior by promoting wakefulness (Erion et al. 2012). The neurons with octopamine identified as the *tdc2* cluster are specifically implicated in the sleep pathway, and the UAS-GAL4 system can be utilized to insert temporally and spatially-controlled dTrpA1 channels in this group of neurons. Any significant results from the thermogenetic sleep-deprivation of these flies will indicate these octopamine *tdc2* neurons are involved in the co-regulation of sleep with the other observed behavior.

Fly Stocks

Raising flies. Flies were kept in vials containing a cornmeal-agar-dextrose medium that is deemed conventional for raising fly stocks (Sitaraman et al. 2015). This medium both provides nutrition to the flies and is appropriate for laying eggs. Flies were kept either in 18-degrees-Celsius or in 22-degrees-Celsius environments. The latter environment speeds up development, but neither cause expression of temperature-sensitive channels in transgenic lines. The flies are low maintenance and typically survive in this lab environment for about a month. From the time flies are mated, their progeny take around 11 days to develop from egg to larva, to mature fly.

Stocks ordered. Four different stocks were ordered and utilized for this research. CS, or wild type, flies were used for much of the experiments. The other stocks were flies bearing UAS-dTrpA1, and split-GAL4 flies that could be crossed together for desired thermogenetic manipulations. Thus, there were three total lines of flies raised for the experiments – (1) CS (wild type), (2) *tdc2*-GAL4-UAS-dTrpA1 (targeted octopamine

neurons with temperature-sensitive channels), and (3) MB054B-GAL4-UAS-dTrpA1 (targeted dopamine neurons with temperature-sensitive channels).

Genetic crosses. Crosses must be set up for the thermogenetic targeting of specific neurons, outlined previously in this methods section. UAS-dTrpA1 eggs can be heat-shocked, such that the only eggs that survive and develop are females. This ensures the females are virgin and in turn, that any crosses set up with them will not be genetically “contaminated.” The UAS-dTrpA1 virgin females are crossed with males of the desired GAL4 stock. The F1 generation will express both UAS and GAL4 on the same chromosome, and as a result, will carry the phenotype that has temperature-sensitive dTrpA1 channels on the targeted neurons.

Sleep Deprivation Methods

Heat Sleep-deprivation. At high temperatures of 30 degrees Celsius or higher, the environment is too hot for fruit flies to sleep, effectively serving as a sleep-deprivation method. Thus, CS flies in this research were sleep deprived at approximately 32 degrees Celsius for 16 hours overnight, encompassing the dark cycle of the incubators, and done consistently around the same time frame for each set of deprivations. Setup required pre-heating the incubator to the correct temperature, then placing the vials of experimental fly groups in the high-heat incubator.

Mechanical Sleep-deprivation. A mechanical-stimulation machine was used to create a mechanically active environment. Vials were placed on the machine, which was set to shake every few seconds such that the flies – attempting to rest on the inner surface of the vials – would react and fly around rather than sleeping. This method of sleep-

deprivation also entailed 16 hours of deprivation overnight – set consistently with the other sleep-deprivation methods.

Thermogenetic Sleep-deprivation. The thermogenetic method entailed utilizing the GAL4-UAS system as described above. Sleep could be directly interfered with at specific parts of the sleep pathway by simply setting temperature parameters between 27 and 30 degrees Celsius, and introducing the transgenic flies into that environment. One notable aspect of this setup is that when raising stocks of flies with this genetic setup, the flies must be kept at temperatures lower than this range. Expression of the dTrpA1 channels during development or outside of the sleep-deprivation time frame among the adults might interfere with the proper experimental findings (Scialo et al. 2016). Thus, a properly controlled environment at all times is especially important when using transgenic lines with temperature-sensitive channels.

Action Selection Courtship Assays

Collection of flies. The observable courtship rituals are identified among specifically the male fruit flies. Thus, male flies must be carefully collected within 6 hours of eclosing to ensure they have not yet mated. Ensuring that all of the males used are virgin is essential for normalizing courtship drive among the males being tested. Female flies are also collected for the males to attempt courting with.

Courtship testing apparatus. The courtship assays are run in custom plates with 8 enclosed chambers. In each chamber a male fly is paired with an immobilized female fly to make tracking the male easier for both manual and software-based tracking of courtship rituals. Flies are allowed ten minutes in for activity after a recovery period of thirty minutes. This recovery period was determined after several trials in which a period

of inactivity was observed following anesthetization with carbon dioxide (used for transferring flies from vials into assay chambers).

A video of the courtship behavior is recorded for the ten minutes and can be replayed to measure the time spent in courtship rituals. Such data is calculated for each individual fly, and is converted into courtship index percentages, which can then



Figure 1. Video still of courtship assay.

be used for statistical analysis.

Experimental Setup. All of the flies tested in the courtship, action selection assays are from the CS stock. Thermogenetic lines were raised for this portion of experiments, but discarded due to fluctuations in the lab environment temperature that compromised the integrity of their data. The CS flies were fully rested, sleep-deprived by heat, or sleep-deprived by mechanical stimulation. Thus, both methods of sleep-deprivation could be compared back to the control group.

Decision-making Oviposition Assays

Collection of flies. For the previous courtship experiments the male fruit flies were the focus of the behavioral study. For the oviposition assays, the focus is on the female, egg-laying fruit flies. Collection of virgin females in this case is essential to normalize their receptivity to mating as well as their fertility levels.

Oviposition testing apparatus. Once the desired type and amount of female flies have been collected, they are given two to three days for mating. This mating occurs in the presence of a yeast-based substrate that heightens the sexual drive of the fruit flies. Thus, in the given time frame, the females should lay a substantial amount of

eggs in the vial such that there is no more space available to lay eggs. The females will continue to mate, and thus will be “holding” more eggs. Given enough time most to all of the females should be holding a substantial amount of eggs that can be viewed in the oviposition assay.

As the female fruit flies are mating, the oviposition chambers should be prepared with the appropriate substrates. The plate used for the oviposition assays contains eighteen individual chambers, and it can be disassembled to fill with two substrates that both will be available in each chamber for the female fruit fly to make her oviposition decision. The flies, once introduced into the oviposition assay, are given 16 hours for egg laying. Any sleep-deprived flies are loaded into the oviposition plates immediately after the 16-hour sleep-deprivation period is complete. Sleep-deprivation for these flies is done concurrently with the last night of mating.

At the completion of the oviposition assay, flies are removed and the plates visibly contain their eggs in the substrates. These plates are imaged, and eggs can be counted either manually or using software. After totaling the number of eggs laid on each substrate, a preference index (PI) value can be calculated and utilized for statistical analysis.

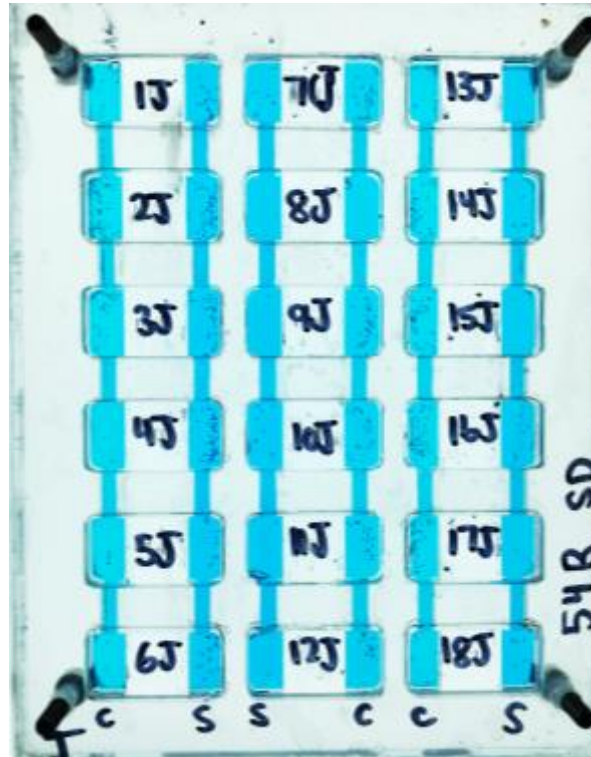


Figure 2. Custom oviposition plate with individual chambers. This image displayed is post-assay, with eggs laid in substrates.

Experimental Setup. 3 different lines of flies were used for the oviposition assays – CS (wild-type), *tdc2-GAL4-UAS-dTrpA1*, and *MB054B-GAL4-UAS-dTrpA1*. The latter two lines required setting up genetic crosses prior to the oviposition assay to obtain females with the desired phenotype. Each of these lines was divided into two groups – the experimental (sleep-deprived) and the control (non-sleep-deprived). CS flies were sleep-deprived using the heat method. Mechanical sleep-deprivation was avoided due to possible interference with egg laying. The two thermogenetic lines of females were sleep deprived by introduction into the temperature-specific environment for targeting those tissue-specific promoters.

For each of the three lines, two different pairs of substrates were tested. The first pair was caffeine and sucrose, and the second was agarose and acetic acid. For all but the agarose substrate, 5% ethanol was used, determined in previous research to be an ideal concentration (Axanchi, Kaun, & Heberlein, 2013).

In total, with the three lines of flies and two pairs of substrates, there were six different experimental groups, each with a control. For thermogenetic lines, the same line was used for control groups, but not introduced into the temperature range for activation of dTrpA1 channels.

Results

Courtship Assay Results

Calculating the courtship index values is the first step for analyzing the raw courtship data. This value is equivalent to the time spent in courtship behavior, in seconds, divided by the total time of the assay (600 seconds), multiplied by 100. The percentages obtained were useful for the statistical analysis of the data. To compare the data among the three groups – non-sleep-deprived, heat sleep-deprivation and mechanical stimulation – a 3-way ANOVA was run to assess differences in the preference indexes. The results are depicted in Figure 3 below.

analysis. Each “set” of data comprised of the sleep-deprived and non-sleep-deprived (NSD) fruit flies of one line on one of the pairs of substrates. Thus, there were six total sets of data, whose analysis results are depicted in Figures 4 – 9. Each of these sets was analyzed with a T-test comparing the PI values for the sleep-deprived and control groups.

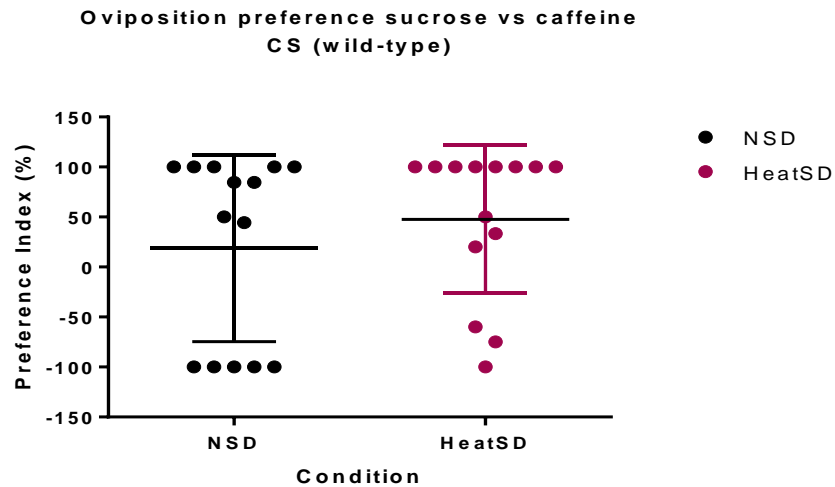


Figure 4. Preference for sucrose versus caffeine among CS *Drosophila* is not distinct. The flies sleep-deprived by heat do not show a significantly different trend from the NSD flies. (n = 14 for each group displayed)

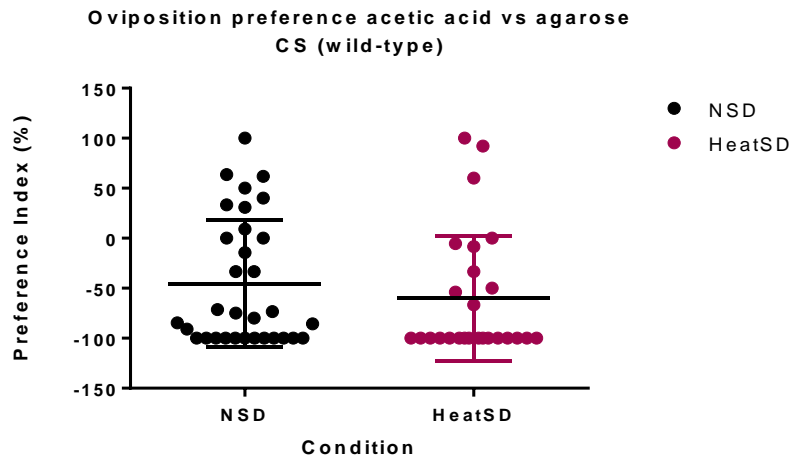


Figure 5. Preference for acetic acid versus agarose among CS *Drosophila* is fairly consistent across NSD and heat-SD groups. No significant difference was found. (n=36 for both groups.)

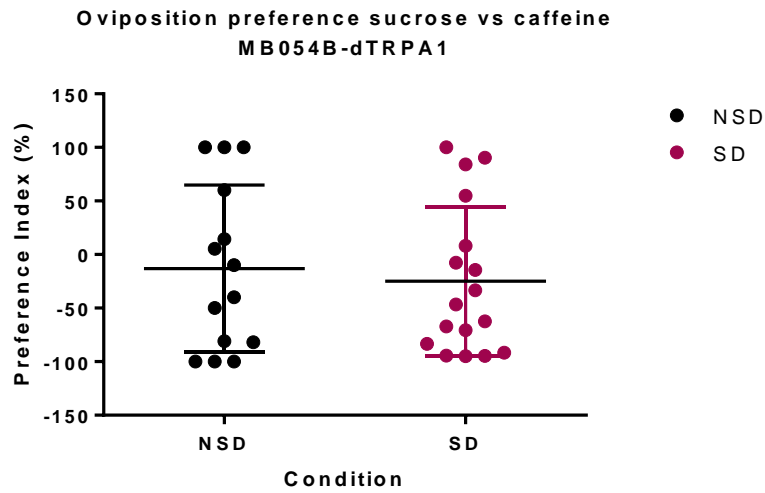


Figure 6. Oviposition preference for sucrose versus caffeine among MB054B-dTrpA1 *Drosophila* is not modulated by sleep deprivation. (n = 14 for NSD, n = 17 for SD)

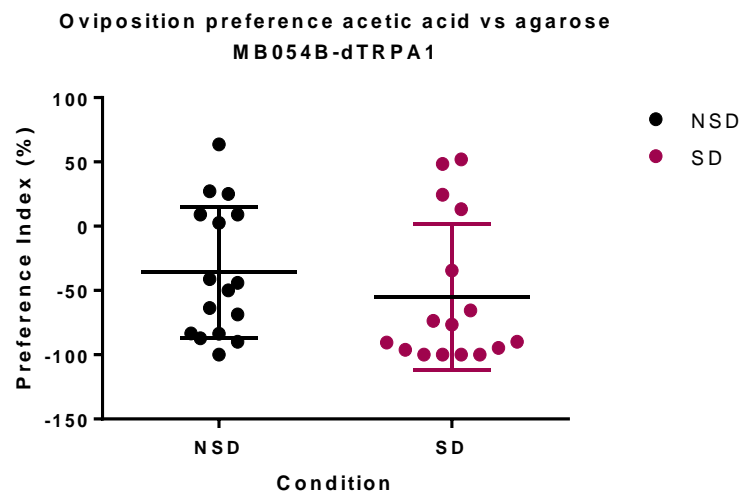


Figure 7. Oviposition preference for acetic acid versus agarose among MB054B-dTrpA1 *Drosophila* is not modulated by sleep deprivation. (n = 16 for both NSD and SD)

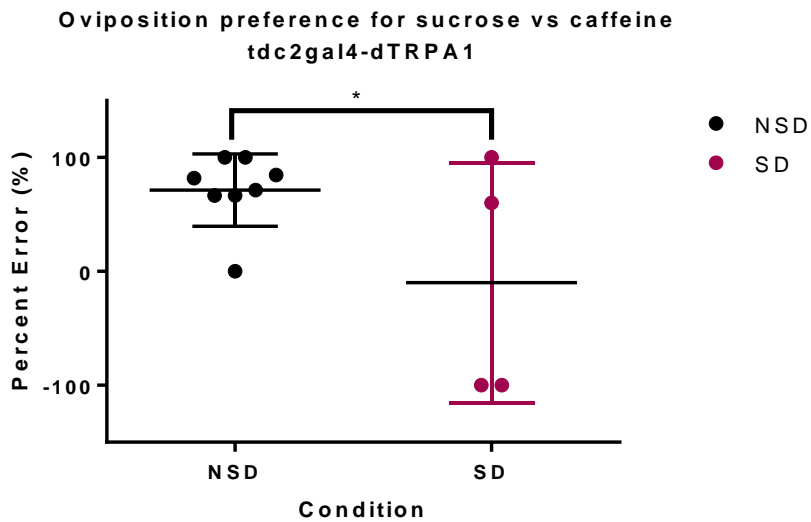


Figure 8. Oviposition preference for sucrose versus caffeine among *tdc2-dTrpA1 Drosophila* changes with sleep-deprivation. (n = 8 for NSD, n = 4 SD. Note this sample size does not include flies that laid zero eggs.)

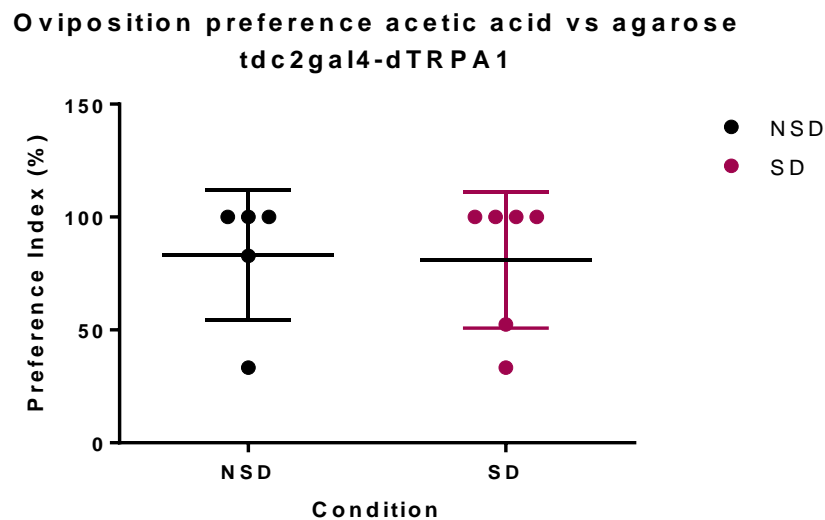


Figure 9. Oviposition preference for acetic acid versus agarose among *tdc2-dTrpA1 Drosophila* is not modulated by sleep-deprivation. (n=5 NSD, n=6 SD. Note this sample size does not include flies that laid zero eggs.)

Figure 4 depicts the data for CS flies tested for sucrose versus caffeine preference, with positive values indicating a preference for sucrose and negative values indicating preference for caffeine. Both the control and experimental groups had up to eighteen flies, including any that did not lay eggs, that escaped, or that were deceased due to human error in experimental setup. These three scenarios are possible for all six data sets presented. Thus, the number of data points represented in each group is less than the total number of flies. Flies that did not lay eggs were excluded from the data because a preference index value could not be calculated, and represent most excluded data points. In Figure 4 we see that there is no significant difference in ovipositional preference indexes for sucrose versus caffeine between CS heat sleep-deprived and non-sleep-deprived fruit flies.

Figure 5 represents the data for CS flies tested for preference of acetic acid versus agarose. Preference in this case is in terms of acetic acid, such that negative values indicate preference for agarose. Two assay plates were run for each group in this set – amounting to 36 data points for each group. No significant difference was found between the heat sleep-deprived and non-sleep-deprived CS flies for ovipositional preference of acetic acid versus agarose. Both groups demonstrate some level of preference for the agarose substrate.

Figure 6 contains the data for the MB054B-dTrpA1 line of flies with preference in regards to sucrose versus caffeine. Positive values indicate a preference for sucrose, and negative a preference for caffeine. In this data, there is no significant difference between the thermogenetically sleep-deprived and the non-sleep-deprived groups. Figure 7 has the data for the same line of flies tested for preference between acetic acid and

agarose. This data also showed no significant difference between the thermogenetically sleep-deprived and non-sleep-deprived flies.

Figure 8 visually represents the statistical data for the *tdc2-dTrpA1* flies on sucrose versus caffeine, and contains the only statistical significant result out of the oviposition data. A significant difference is found between the thermogenetically sleep-deprived and non-sleep-deprived flies in this set. It is notable that the sample size for this set is considerably small – with only 8 data points, or flies, represented in the non-sleep-deprived group and only 4 represented in the sleep-deprived. Both groups had 9 female flies in the behavioral assay, meaning that 1 of the 9 non-sleep-deprived flies did not lay any eggs, and 5 of the 9 sleep-deprived flies did not lay any eggs. The numbers for the latter are more numerically and percentage-wise than the amount of flies not laying eggs in other trials. Altogether, the sample size of 9 is still smaller than ideal.

Figure 9 is the data for *tdc2-dTrpA1* flies tested for preference between acetic acid and agarose. In this data only 9 flies were present in each group, also with a few not laying eggs. T-test analysis of this data implicated no significant difference between ovipositional preference of the thermogenetically sleep-deprived and the non-sleep-deprived flies.

Discussion

It was initially hypothesized that, if action selection between basic adaptive behaviors does occur, an interaction between sleep and courtship would be observed, reflecting that a sleep-deprived fly engages in less courtship rituals and is thus prioritizing sleep. This courtship data from this research is useful because replicates such expected action selection. That sleep-deprived fruit flies are engaging in significantly

less courtship – or none at all – means there might be some sort of co-regulation between sleep and courtship consistent with the idea of action selection. At some level, their brain seems to prioritize sleep-recovery over sex when sleep-deprived.

The next step with the courtship data is to utilize thermogenetic lines of flies to target specific neurons in the sleep circuit. Such lines were created and raised; however, were discarded due to fluctuations in lab temperatures. Despite the fluctuations, trials were initially run, with results reflecting some sort of interference by sporadic activation of temperature-sensitive ion channels. Obtaining a better, meaningful set of thermogenetic data will be key in identifying the location in the sleep circuit where co-regulation with courtship behavior might be occurring. This would provide a direct understanding of the underlying mechanisms for action selection.

A second hypothesis of this research was that, given oviposition is a good model for decision-making, if sleep-deprivation affects decision-making then the ovipositional preference observed among sleep-deprived flies would not reflect the preference observed among the non-sleep-deprived fruit flies.

The oviposition data overall did not show trends in favor of this hypothesis. In some cases, the data was extremely consistent for both the sleep-deprived and control groups, such as in Figures 4 and 5. Furthermore, most of the data sets showed a wide distribution of preferences, suggesting that perhaps a pair of substrates more distinct in value should be identified for this research. The bottom line of the oviposition data suggests that flies sleep-deprived by either heat or by targeting the dopaminergic MB054B neurons do not show impaired decision-making, as modeled by ovipositional preference. The one anomaly, and thus potential lead, from the oviposition data is the

data for the *tdc2* octopamine-targeted line tested with sucrose and caffeine. If the significance of this data is valid, it is likely that the octopamine neurons may be involved in the co-regulation of sleep and decision-making. The fault with this data is the small sample size, which is the result of a smaller amount of flies crossed and an inconsistent fly-collection schedule. It is necessary to reproduce this data to further assess the significance. Furthermore, the finding that 5 of the 9 sleep-deprived flies in this set did not lay eggs is highly suggestive of action selection – that sleep-deprivation is not modulating decision-making, but rather decreasing oviposition behavior overall due to prioritization of sleep – in other words this might demonstrate action selection. However, the same trend is not confirmed with the *tdc2* data with acetic acid and agarose, in which more of the non-sleep-deprived control flies refrain from laying eggs than the sleep-deprived fruit flies. Because both data sets have a small sample size, both would need to be reproduced to confirm any of the suggested trends.

Future Implications

The human brain has an estimated 86 billion neurons. Compared to this number, the 250,000 neurons that comprise the *Drosophila* brain seem much less intimidating. Given analogous features already identified between the two, studying neuroscience with *Drosophila* is a practical tool for gain a better understanding of the brain's mechanisms. This thesis seeks to provide a better understanding of the neural mechanisms underlying action selection and decision-making processes, particularly with regards to sleep-deprivation, and utilizes *Drosophila melanogaster* to do so. The overall conclusions of this research support (1) that sleep and courtship behavioral interactions in fruit flies effectively model action selection, and (2) that octopaminergic *tdc2* neurons in

Drosophila might be involved in mediating negative effects of sleep-deprivation on ovipositional decisions. This research will need to be continually built upon as outlined in the discussion for better support, and for further understanding of the mechanisms being studied.

Understanding neural mechanisms in the fruit fly's brain can be generalized to our understanding of similar processes in the human brain. Findings can often translate to tangibles, such as the possibility of targeting identified points of co-regulation with chemical agonists or antagonists that may alleviate symptoms of chronic sleep-deprivation and even sleep disorders. Furthermore, such findings may even present us with a better understanding of related systems beyond the sleep circuit. The possibilities in this line of neuroscience research are vast, and must be further pursued to expand our scientific understanding of the brain and to utilize for ethical applications in the future.

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