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Alcohol Reward Memory in Drosophila: Investigating Changes in Neural Activity in Response To Cues Alcohol Associations and Their Role in Regulating Other Alcohol-Related Behaviors.

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HONORS THESIS

Alcohol Reward Memory in Drosophila: Investigating Changes in Neural Activity in Response To Cues Alcohol Associations and Their Role in Regulating Other Alcohol-Related Behaviors

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ABSTRACT

With more than 200 health conditions associated with Alcohol Use Disorder, excessive drinking can take a serious toll on an individual's health, mental health, and relationships. Despite significant disruptions, there are a few treatments for Alcohol Use Disorder that support long-term abstinence. To develop effective long-term treatments for Alcohol Use Disorder, it is essential to obtain an understanding of the underlying complex changes that alcohol has on the brain, however, the complexity of the mammalian nervous system has precluded researchers from achieving this. *Drosophila melanogaster* is a powerful model organism for investigating how alcohol hijacks neural circuits within the brain due to its complex yet tractable brain. Previous studies in *Drosophila* identified neuronal circuits important for the acquisition and expression of alcohol reward memory. Alcohol Reward Memory expression includes cholinergic and dopaminergic neurons that innervate a learning and memory structure within the brain called the Mushroom Body. Here, we investigate how the neural activity of requisite cholinergic neurons changes while flies learn to associate odor cues with alcohol intoxication and whether these alcohol reward memory neurons modulate other alcohol-related behaviors. Understanding how alcohol changes memory circuits in *Drosophila* and how their behaviors are impacted can inform how alcohol disrupts reward circuitry in other species.

INTRODUCTION

World Health Organization stated that in 2016 alone, over three million people died due to the excessive use of alcohol. That is 5.9 percent of the total number of deaths each year (World Health Organization, 2019). In the United States alone, 140,000 people die each year because of alcohol misuse (Centers for Disease Control and Prevention, 2022). Further, approximately 7 percent of all premature deaths were caused by alcohol use. Additionally, more than 200 health conditions are associated with alcohol misuse, including heart diseases, cancer, and liver diseases (World Health Organization, 2019). In fact, one in five intensive care admissions in psychiatric hospitals is related to alcohol (American Psychiatric Association, 2013). Alcohol misuse can also cause severe impairments in the person's life, which often consist of having a difficult time communicating and interpersonal relationships, and not being able to perform or participate in school, work, or drive. In total these statistics demonstrate that alcohol misuse and Alcohol Use Disorder have significant negative consequences on health and society.

More recent evidence suggests that the COVID-19 pandemic had a significant negative impact on an individual's perceived stress, mental health, and increased susceptibility to developing alcohol use disorder. Research demonstrates that during the pandemic there was an overall increase in alcohol consumption, including populations who regularly drank before the pandemic, thereby exacerbating the harmful effects of alcohol on society (Schmidt et al., 2021). Given these burdens, treatments for Alcohol Use Disorder are essential to reduce these devasting statistics and give people guidance toward alcohol use recovery. Despite this, currently, there are few effective treatments for Alcohol Use Disorder. This is likely due to the complex and long-term changes alcohol has on the brain.

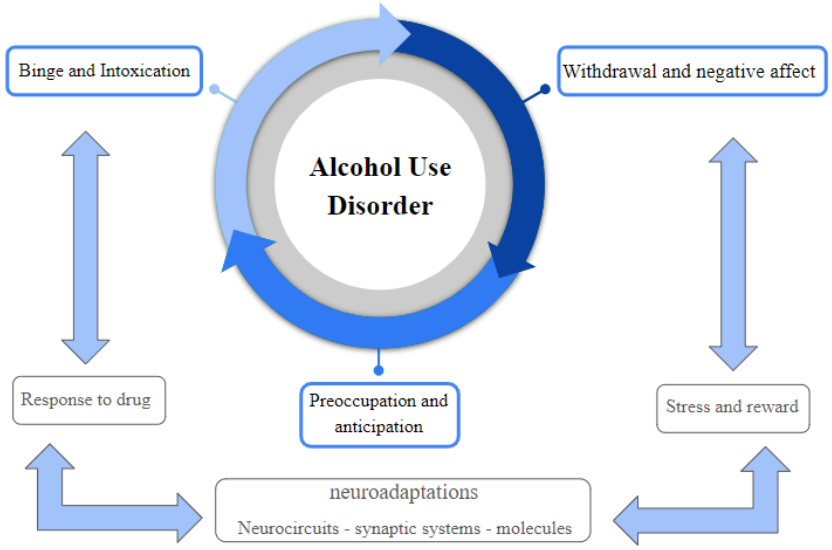
LITERATURE REVIEW

Alcohol Use Disorder is a Cyclical Disorder

Alcohol Use Disorder is characterized by the compulsion to consume alcohol and the inability to control consumption, despite the withdrawal symptoms and negative emotional states that arise (Koob & Volkow, 2010). Alcohol Use Disorder is considered a cyclical disorder because it begins with drinking to intoxication which leads to the buildup of tolerance and therefore more drinking. Following that, abstinence from alcohol results in withdrawal and/or negative affect, which often leads to preoccupation and anticipation or cravings to find the alcohol, thereby repeating the cycle (Koob &Volkow, 2010). Tolerance also contributes to increases in alcohol consumption. As individuals engage in drinking behavior, the body adjusts and creates a new homeostasis, making it harder for an individual to reach their desired intoxication state. According to the *Diagnostic and Statistical Manual of Mental Disorders,* withdrawals and cravings include cognitive, behavioral, and physiological symptoms when an individual cuts back on the consumption of the substance (American Psychiatric Association, 2013). They usually appear after 4-12 hours post intoxication and can consist of shaking or even epilepsy, nausea, and vomiting. Alcohol Use Disorder could also include insomnia, nausea, anxiety, and hallucinations (American Psychiatric Association, 2013). Given the negative consequences associated with reducing the consumption of Alcohol Use Disorder, most individuals suffering either continue to consume alcohol to avoid withdrawal symptoms and cravings or quickly relapse. As a consequence, NIAAA defines Alcohol Use Disorder as a chronically relapsing brain disease that requires further research to appropriately address the management of this disease.

Figure 1:

The cycle of Alcohol Use Disorder. Inspired by (Koob & Volkow, 2010).



Current Treatments for Alcohol Use Disorder

The current treatments consist of non-pharmaceutical therapies, such as counseling and motivational interviewing, and pharmaceutical treatment, which consists of several types of medication (Ramkissoon & Shah, 2022). Non-pharmaceutical therapies assist for instance with building relationships, developing coping strategies, dealing with comorbid disorders, and scheduling follow-ups (Ramkissoon & Shah, 2022). These treatments can be effective, especially with the ability to cope with cravings and to withstand relapses, however, these are typically only effective for a relatively short amount of time (around two years) after the individual terminates the therapies (Berglund et al., 2003).

Furthermore, in pharmaceutical treatment, there are various types of medicines an individual can take like Disulfiram, Naltrexone, and Acamprosate (Stokłosa et al., 2023). However, all these treatments come with varying side effects. Disulfiram is a medical drug that inhibits mitochondrial aldehyde dehydrogenase which results in reduced alcohol consumption. Nevertheless, it has only been proven to work in an open randomized controlled trial (RCT) and not in a blinded RCT (Akbar et al., 2018). Naltrexone binds to the opioid receptor, which weakens the pleasant sensation corresponding to alcohol use and therefore reduces the

cravings (Akbar et al., 2018). Despite these positive effects, Naltrexone has been described as moderately effective in the reduction of alcohol consumption. Acamprosate is a medication that balances the neurotransmitters that help to reduce withdrawals from alcohol use (Akbar et al., 2018). This medication works optimally when combined with non-pharmaceutical treatments and even then, the efficiency is moderate. Additionally, after terminating the drug the individual is susceptible to the substance again (Ramkissoon & Shah, 2022). Therefore, more knowledge about how alcohol affects the brain is needed to provide new treatments to help people abstain from alcohol.

Alcohol Hijacks Reward Circuitry in the Brain

When suffering from Alcohol Use Disorder, an individual often endures cravings for years after abstinence. The persistence of cravings is thought to be directly related to alcohol's actions on reward neural circuitry in the brain. Research shows that the connectivity between neuronal reward circuits changes after exposure to intoxicating doses of alcohol (Koob & Volkow, 2010). Like other substances, alcohol hijacks the reward circuitry in the brain to drive alcohol-associated behaviors, including cravings. Despite decades of research, however, it is still unclear how alcohol disrupts specific reward circuits to drive motivated behaviors and support enduring preference.

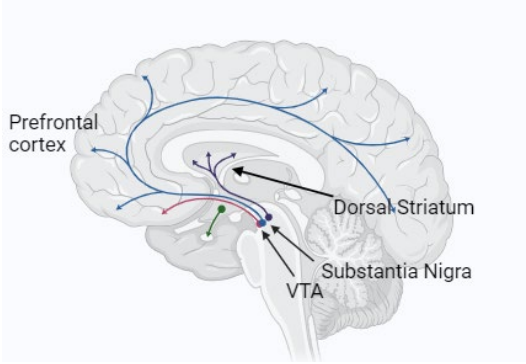
The Reward Circuitry in Mammals is Complex

In mammals the circuits in the brain that process reward are well established and include the medial forebrain and the ventral tegmental area (VTA). For example, VTA dopamine neurons play a crucial role in modulating motivated behaviors and decision-making. They also have the lowest threshold and therefore are most sensitive to reward (Koob & Volkow, 2010). As such they are often disrupted in the context of use disorders, including Alcohol Use Disorder. The synapses of these dopamine neurons that stem from the VTA and Substantia Nigra (SN) are linked with various brain structures as well as diverse types of neurons like GABA, glutamate, and acetylcholine neurons (Morales & Margolis, 2017). Further, cholinergic neurons in the pons innervate dopaminergic neurons in the VTA and SN. Inactivating these neurons decreases motivation for food and cocaine (Mark et al., 2011). This study is one of many that highlights the importance of cholinergic neurons in regulating responses to both natural reward and drugs of abuse. Other studies show that motivated behaviors can be

generated through glutamate and GABA released from VTA-projected neurons, independent of dopamine (Morales & Margolis, 2017). Due to the heterogeneity of connectivity and the sheer number of neurons within the mammalian system, it remains difficult to define how these neuronal circuits work together to drive goal-related behaviors and how they are modified in the context of drug abuse.

Figure 2:

Schematic of Reward circuitry in humans highlighting the dopamine modulation from the VTA and Substantia Nigra (Image made in BioRender).



<u>*Drosophila melanogaster* is a Powerful Organism for Studying How Neuronal Circuits are Modified by Alcohol.</u>

Due to the complexity and heterogeneity that exists within the human brain, researchers have turned to other model organisms that allow better resolution when investigating the effects of alcohol on memory and the brain. *Drosophila melanogaster* is one such model organism. In addition to similar neuronal circuits that support reward processing, learning and memory, as outlined below, *Drosophila* display remarkably similar behavioral responses to alcohol intoxication.

When intoxicated, both species show acute locomotor responses (Kaun, 2012). Initially, intoxication leads to hyperactivity, and longer exposures or higher doses lead to sedation (Rodan & Rothenfluh, 2010). Over time both organisms show an increase in consumption of alcohol and the development of tolerance to the intoxicating effects of alcohol. This consumption is not dependent on caloric intake (Devineni and Heberlein, 2009). The chronic exposure to alcohol can lead to withdrawal-like symptoms, including seizures (Ghezzie et al.,

2014). Furthermore, both organisms will overcome aversive consequences in order to obtain alcohol which are some of the cardinal characteristics of alcohol use disorder (Kaun, 2012; American Psychiatric Association, 2013). Once the flies become intoxicated, they become socially disinhibited (Lee et al., 2008). These similar alcohol-related behaviors and the reduced complexity in the circuits allow us to study these alcohol-related circuits and create a better resolution.

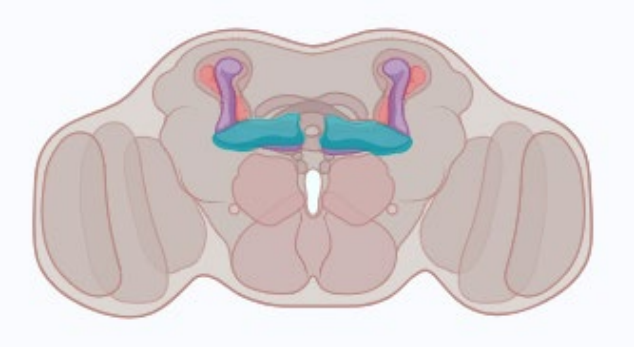
Despite having only 200,000 neurons, the brains of *Drosophila,* or more commonly known as the fruit fly, are built in remarkably similar ways (Scaplen & Kaun, 2016). This reduction in the connections of neurons gives us a powerful opportunity to study neuronal circuits more thoroughly. For instance, similar to mammals, dopamine neurons within the fruit fly communicate with glutamatergic, cholinergic, and GABAergic neurons to support learning and memory (Scaplen, 2016). These neurons come together in a structure called the Mushroom Body (MB), which is thought to play a crucial role in learning and memory (Connely et al., 1996).

The Mushroom Body (MB) Plays an Important Role in Memory and Learning

Similar to mammalian circuitry for memory, the MB comprises many recruit feedback circuits that are essential for stabilizing memories (Aso et al., 2014). The *Drosophila* MB consists of thousands of intrinsic and extrinsic neurons. Intrinsic neurons consist of approximately 2,000 Kenyon cells (KC), which are second-order olfactory cells (Aso et al., 2009, Aso et al., 2014). The KCs and their parallel axons form three lobes in the MB α/β, α′/ β′, and γ lobes (Aso et al., 2014). These MB lobes are functionally compartmentalized by dopamine inputs and MB output neurons (MBONs) (Aso et al., 2014). Cholinergic MBONs innervate the vertical MB lobes (α, α′), whereas glutamatergic and GABAergic MBONs innervate the horizontal lobe (β, β′, γ) (Aso et al., 2014). When dopamine binds to receptors expressed in the MB and/or MBONs, it results in changes in the activation of these compartments. These changes can give important information on reward and punishment behaviors (Schwearzel et al., 2003). This can be compared to the midbrain projections to the basal ganglia in mammals, which plays a role in reward memory and learning too, and highlights how these neuronal circuits are similar between humans and *Drosophila*.

Figure 3:

Schematic of Drosophila Mushroom Body (Image made in Biorender). The α′/ β′ lobe are labeled in pink. The α/β lobes are labeled in purple and the γ lobes are labeled in turquoise.



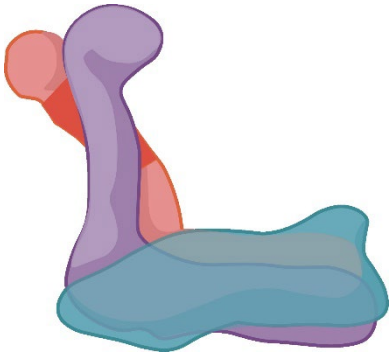
MBONs Modulate Goal-Directed Behaviors

Recent research suggests that MBONs modulate goal-directed behaviors of *Drosophila* and predict goal-directed behaviors. After learning, dopamine modulates approaches and avoidance connections to drive learned responses. The vertical α, α′ lobe is important for learned punishment, whereas the horizontal β, β′ and γ lobes are important for learned reward. The glutamatergic MBONs are responsible for naïve eversion behavior responses, whereas the GABAergic and cholinergic MBONs are responsible for naïve approach. Additionally, the cholinergic and glutamatergic neurons are important for appetitive memory, but not for aversive memory. To get a better understanding of the neuronal population and which exact neurons influence these behaviors, more research is needed.

The current study targets the cholinergic α′2 MBON, which is located in the vertical α′ lobe. This lobe is important for learned punishment. Previous research also identified this MBON to be broadly important for alcohol-associated preference (Aso et al., 2014). More recently, data suggest a specific role for this MBON during the expression of 24-hour alcohol-associated preference (Scaplen et al., 2020). The current study is investigating the importance of the α′2 MBON in other alcohol-related behaviors by using genetic tools that are available in *Drosophila*.

Figure 4:

Schematic of MBON α'2 displayed in the dark red area. (Image made in Biorender).



Various Genetic Tools are Available to Manipulate and Visualize the Genes in *Drosophila*

Drosophila are a powerful model organism because of the ability to use refined intersectional genetic strategies to target individual neurons and manipulate gene expression (del Valle Rodríguez et al., 2012). The most common tool for manipulating gene expression is the *GAL4-UAS* binary transcriptional factor/enhancer system. *GAL-4* is a yeast transcription factor that binds to the upstream activating sequence, called a *UAS* (Brand & Perrimon, 1993). Researchers have capitalized on this system to drive the expression of transgenes in specific subsets of neurons. In this way, the expression of *GAL-4* is contingent on the expression of a specific promoter. The transgene of interest is placed downstream of the *UAS* such that when the *GAL-4* binds, it drives the expression of the transgene in that subset of neurons (Pfeiffer et al., 2010). This expression could then be visualized by expressing, for instance, the red fluorescent protein (RFP), however, multiple *UAS* driver lines can be used to either inactivate, activate, visualize, or record the activity of neurons (Pfeiffer et al., 2010).

The current study capitalizes on three genetic tools within the fly that allow for the temporary inactivation of specific subsets of cells (*UAS*- S*hibire*^{ts}), the ability to monitor and measure neuronal activity post-experience (TRIC), and the ability to disrupt receptor expression (RNAi) in subsets of cells.

TRIC or the Transcriptional Reporter of Intracellular Calcium is a genetic system that utilizes multiple binary transcriptional factor/enhancer systems (*GAL4/UAS* and the split *LexA/LexAop* systems) to monitor the activity of neurons via intracellular calcium dynamics (Gao et al., 2015). At the core of this system the split *LexA/LexAop* system is activated by the intracellular calcium which will bind to the binding protein calmodulin (CaM) (Gao et al., 2015). The calmodulin will then bind to the target peptide MKII. This bond will promote the activation domain (AD), which is attached to the calmodulin, and the *LexA* DNA binding domain, which is attached to the MKII to bind together. This binding of the AD and the *LexA* DBD will then activate the *LexAop* and drive the expression of green fluorescent protein (GFP). Because of the expression of the GFP, the neurons that are activated will be green and therefore will be visualized. Through these fluorescent proteins, the connections and activation between the neurons will be visualized and can give more data on how these neuronal connections change.

Furthermore, the *GAL-4*/*UAS* system can be combined with a temperature-sensitive allele *shibire*^{ts} to inhibit a target neuron. S*hibire*^{ts} encodes the protein dynamin, which is essential for synaptic vesicle recycling (Chen et al., 1991). At temperatures higher than 29 degrees Celcius, the temperature-sensitive allele *shibire*^{ts} is nonfunctioning and therefore will inhibit the synaptic transmission (Koenig et al., 1983). Additionally, the *GAL-4*/*UAS* system can be used with RNAi, which can knock down dopamine receptors using RNA interference. All these genetic tools can be used to manipulate and visualize complex circuitry, in this case, the reward circuitry in the Mushroom Body.

RESEARCH QUESTION

With the use of these three genetic tools and the previous knowledge that the cholinergic α′2 MBON is important for alcohol retrieval, the following questions are asked:

How does the neuronal activity of the cholinergic neurons required for alcohol reward memory in *Drosophila melanogaster* change within 24 hours after acquisition to drive alcohol-associated preference?

Further, is this α′2 cholinergic MBON also important for several alcohol-related behaviors like locomotion and alcohol consumption?

It is expected that the activity of α′2 MBON would increase in response to alcohol-intoxicating experiments and that the α′2 MBON is important for alcohol-related locomotion. Additionally, alcohol consumption will decrease when knocking down dopamine-1 and dopamine-2 receptors in α′2 MBON.

RATIONALE

As shown, Alcohol Use Disorder has significant effects on human health and behavior. Despite these effects, there are not enough long-term effective treatments that restrict the number of side effects, and therefore more research needs to be done about the effects alcohol has on neuronal connections in the brain. Due to the availability of *Drosophila* and the similarities based on the behavior and neuronal circuits of *Drosophila* and mammals, *Drosophila* is a suitable organism to use for this experiment. Additionally, there are genetic tools available to manipulate their genes as well as visualize their neuronal activity. To explain the role of α'2 MBON in alcohol-related behaviors and better understand how its behavior changes during the acquisition of alcohol-related memories, various genetic tools including TRIC Calcium, Thermogenetic, and RNAi are used.

METHOD

Fly Strains

All *Drosophila* lines were raised on standard cornmeal-agar media with tegosept anti-fungal agents and were maintained in humidity-controlled chambers at 18°C or 21°C under 14/10hr light/dark cycles. *Drosophila* stocks were acquired from Bloomington *Drosophila* Resource Center. The current study capitalizes on the *GAL-4*/*UAS* system to drive the expression of the Transcriptional Reporter of Intracellular Calcium (TRIC) in target neurons. To obtain flies that had both the *GAL-4* and *UAS* components, *GAL-4* and *UAS* stocks are flipped in new vials every week to manage reproduction, the number of flies, as well as their age. From these stocks, virgin females are collected for subsequent crosses. Virgin flies are identified as larger than males, with the absence of sex combs, and the presence of meconium in their abdomen.

TRIC (Transcriptional reporter of Intracellular Calcium)

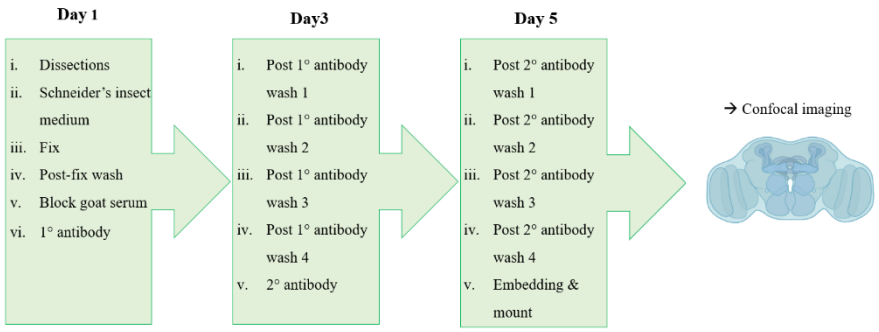
For the visualization of the MB018B neuron, or α'2 MBON, 61679 female virgins (10XUAS-IVS-mCD8::RFP,13XLexAop2-mCD8::GFP; nSyb-MKII::nlsLexADBD/CyO; UAS-p65AD::CaM) were crossed with GMR20G03 male flies. These crosses are flipped into new vials, including yeast, every two days to encourage egg laying. Once the progeny of the crosses emerges from their pupa, the flies are collected for behavioral experiments. When collecting behavioral flies, we selected against chromosomal balancers including CyO (curly wings) and TM6B (tubby and humeral phenotypes). Thus, collected flies that had the darkest eyes indicating multiple transgenes, were of normal size, and had straight wings. Behavioral experiments started 2-3 days post-collection. For behavioral experiments, male and female flies were collected 1-2 days post eclosion, shifted from 21°C to 18°C, and trained 2-3 days post-collection. Odor conditioning is performed similarly to Scaplen et al. (2020). In short, groups of 10 flies were trained in perforated 14ml culture vials filled with 1ml of 1% agar and covered in mesh lids. Training rooms were temperature and humidity-controlled (65%). The training is performed in the dark, preceding 3 times 20 minutes of habituation to the training chambers; 10 minutes in one odor with ethanol and 10 minutes in the other odor without ethanol. After the intoxication, the flies were placed in the 18°C incubator for 24-hour rest before processing the tissue.

Immunohistochemistry Procedure

On the day of the dissections, the flies are put on ice to anesthetize them. Once the brains are dissected, they are put into solutions to fix the tissue. Subsequent washes were conducted within the same vial. First, the brains were put into a 2% paraformaldehyde solution (PFA) that fixed the brain tissue for 55 minutes. Next, the tissue was washed in 0.5% phosphate-buffered saline solution (PBT) for four separate 15-minute periods. The tissue was then blocked in goat serum for an hour before being incubated for three nights in primary antibodies at 4°C. These primary antibodies consist of anti-rabbit GFP, anti-chicken RFP, and goat serum. After these 3 nights, the brains are washed with 0.5% PBT for four separate 15-minute periods and put together with the secondary antibodies kept in the fridge for 3 nights. The secondary antibodies are light sensitive and target the animal in which the primary antibodies were made. Post-secondary antibody incubations, the brains are washed once more in 0.5% PBT and mounted in glycerol with DAPI to allow for the visualization of neurons within the brain and allowed to dry overnight.

Figure 5:

Immunohistochemistry Procedure (Schematic created in PowerPoint and BioRender)



Confocal imaging

The day after mounting the brains, they can be visualized by using confocal imaging to show GFP/RFP identification. The Zeiss, LSM800 with ZEN software (Zeiss, version 2.1) with auto Z brightness correction was used for this confocal (Scaplen et al., 2020). We expect to see that these images will show the cholinergic neurons as well as the activated cholinergic neurons in the brain.

FlyGrAM

To investigate the impact of α′2 neurons on alcohol-induced locomotion we used *shibire^{ts}* to inactivate α′2 neurons while flies were exposed to air and intoxicating doses of alcohol. In order to target α′2 neurons, we used the *GAL-4*/*UAS* system. The MB018B-*GAL-4* driver line was used to target α′2 neurons and *UAS-shibire^{ts}* was used to temporarily inactivate neurotransmission. Two genetic controls were used to control for the presence of the *GAL-4* (+/*GAL4*) and the *UAS* (+/*UAS*), thus three crosses were prepared. The first cross is a *GAL-4* control, in which yellow-white, the genetic background for flies, virgin females were crossed with MB018B males. For the second cross, *shibire^{ts}* virgin females are crossed with MB018B males as the experimental cross. The last cross is a *UAS* control, which consists of a cross between yellow-white virgin females, and *shibire^{ts}* males. These crosses are flipped and taken care of in the same manner as the experimental flies for TRIC. After eclosion, the male flies were collected from each cross which consisted of the normal to bigger size flies. 4 vials of 10 male flies of each cross were needed to start the behavioral experiments 2-3 days post-collection.

The laboratory setup and the flyGrAM installations are structured similarly to Scaplen et al. 2019, Nuñez et al. 2023. To start the experiment, the airflow is turned on at 115 and the dark behavioral hood is warmed up to 30°C. Next, the flies were put into three different arenas consisting of four behavioral chambers. The location of the chambers was counterbalanced across genotypes. The three different crosses were placed in various orders in all three arenas to control the amount of airflow in each chamber, as depicted in figure 6. To start, the first arena was put in a 30°C incubator for 20 minutes. Then, the arena was placed in the behavioral hood, the air tubes were connected, and the flies habituated for 10 minutes. During these 10 minutes, the exact number of flies in each chamber was counted, the ROIs on FlyGram were set, and the experiment was started after these 10 minutes. After 5 minutes, the ETOH air tube was opened up and the airflow and the vacuum were closed. In this experiment, we used both an ETOH:Air ratio of 60:55 and 75:45. After 10 minutes, the airflow and the vacuum were opened and the ETOH air tube was closed. The flies were accommodated for five more minutes. Once the five minutes were over, the first flyGrAM run was done. The next arena of flies was then put into the 30°C incubator for 20 minutes and the

following steps were repeated with the same concentration of ETOH. This was done for the third arena as well, to be able to counterbalance all genotypes in the chambers. Furthermore, these three runs were then repeated three times in total for a final n of 9.

Figure 6:
Organization flyGrAM chambers (created in Powerpoint).

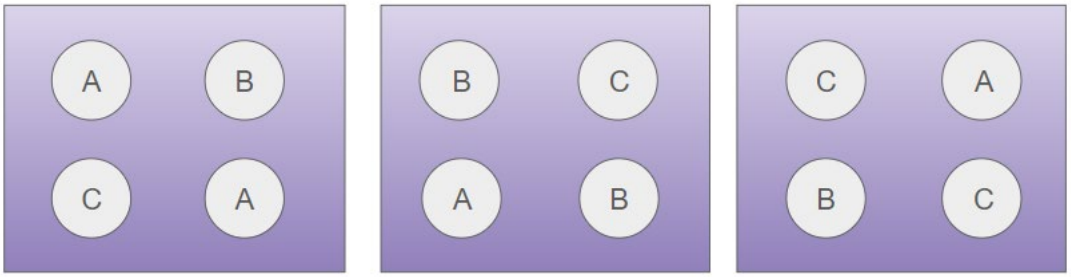
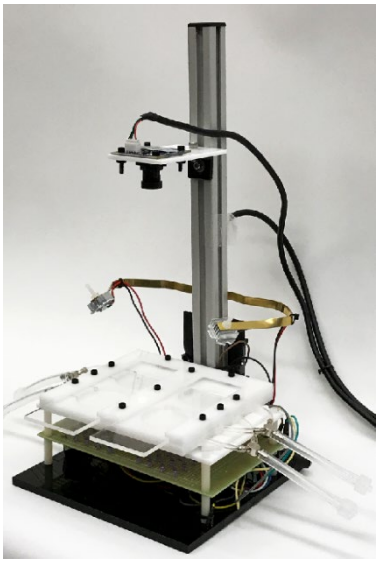


Figure 7:
FlyGrAM installation that shows the arena with four chambers, air tubes, and a camera that records movement (Scaplen et al., 2019).



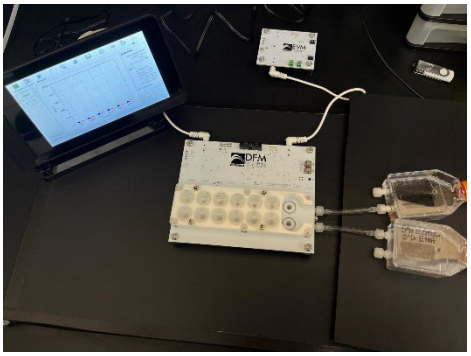
FLIC (Fly Liquid-Food Interaction Counter)

To investigate alcohol consumption behavior in *Drosophila*, dopamine receptors in MB018B were knocked down using RNAi. There are two specific dopamine-1-like receptors (D1R1 and D1R2), which are important for olfactory learning and memory, and one dopamine-2-like receptor (D2R), which is important for addiction (Cognigni et al., 2018). In this experiment, the D1R1 and D1R2 were knocked down at the same time to investigate the importance of the dopamine-1-like receptor in MB018B. The dopamine-1-like receptor virgin females were crossed with MB018B males as well as with an empty *GAL-4*, which lacks the enhancer to drive the gene expression and was used as a control (Pfeiffer, 2010). Furthermore, the dopamine-2-like receptor virgin females were crossed with MB018B as well as with the empty *GAL-4*. This creates the possibility to analyze both the importance of the dopamine-1-like receptors and the dopamine-2-like receptors on alcohol consumption separately from each other. These crosses were flipped every other day. After eclosion, the male flies were collected and starved in agar for 24 hours at 18°C.

After 24 hours of starvation, the flies were put in the FLIC assay, where single flies were placed in feeding areas similar to Ro et al., 2014. Furthermore, two different solutions were added to the FLIC assay. These consist of magnesium chloride water and 5% sucrose, as well as magnesium chloride water, 5% sucrose, and 15% ethanol. The FLIC assay monitored when *Drosophila* consumed, what they consumed, and how much they consumed (Ro et al., 2014).

Figure 8:

FLIC set-up; two solutions connected to the DFM, which is connected to the monitor that records the consumption.



ETHICAL CONSIDERATIONS

In contrast to mammals, there are no major ethical issues when using *Drosophila melanogaster*. They do not have to be evaluated by the IRB or IACUC. These flies have a short life cycle and reproduce quickly, which is convenient for the experiment. Furthermore, power analyses were conducted to determine the number of flies required for each experiment.

DATA SOURCES

To be able to analyze the differences between the cholinergic neuronal connections in the MB before and after alcohol intoxication, the brains were visualized. These visualizations and images were used to collect data for this experiment by looking at the connections between these cholinergic neurons. Furthermore, we used R to custom code graphs and analyze data from FlyGram and FLIC to investigate the locomotion and the alcohol consumption of *Drosophila*.

RESULTS

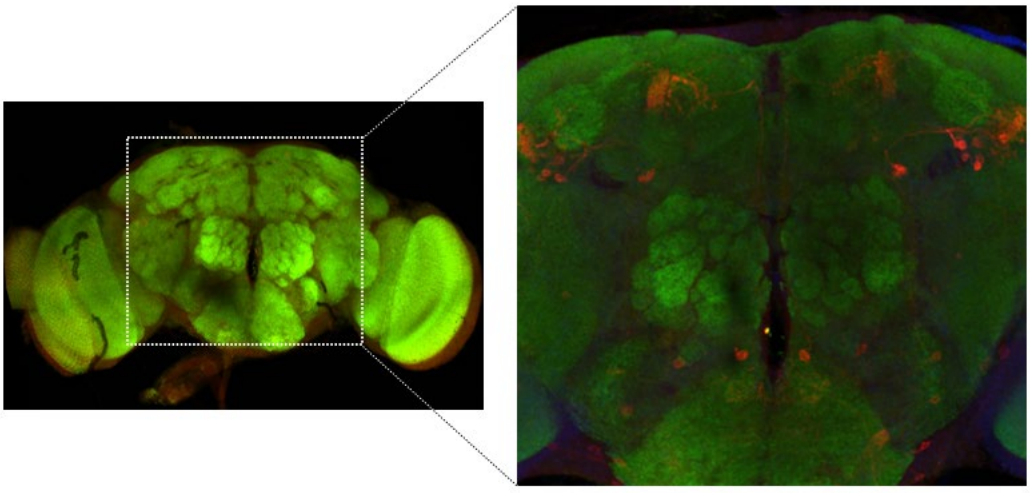
TRIC

This research hypothesized that the activity of α′2 MBON would increase in response to alcohol-intoxicating experiments. We measured activity using the TRIC system where increases in GFP signal are indicative of increases in calcium levels and thus neuronal activity. Despite dissecting and imaging approximately 25 brains, only some showed RFP signaling, indicating the activation of the *GAL-4*, whereas others had no expression at all suggesting the lack of transgene. Therefore, this experiment has inconclusive results, and more research is needed to demonstrate the changes in activation in the α′2 MBON during alcohol acquisition.

Figure 9:

RFP signaling of α'2 MBON:

The red signaling shows the RFP displaying all the cholinergic neurons. No GFP signaling is found, therefore no activity was captured.

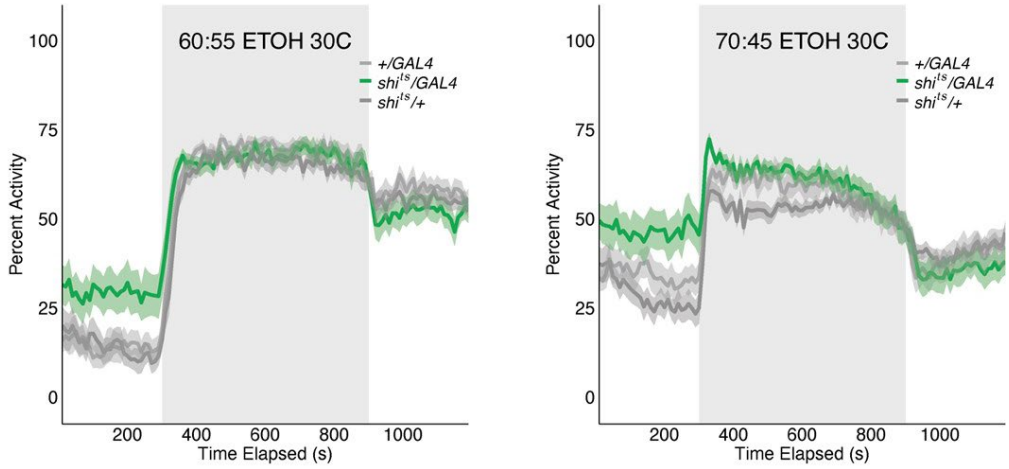


FlyGrAM

This research hypothesized that the α′2 MBON is important for alcohol-related locomotion. After running the experiment three times and silencing this neuron using *shibire^{ts}*, the results did not support this hypothesis at 60:55 and 70:45 ETOH:Air concentration. The results show no effect of the α′2 MBON being important for alcohol-related locomotion. Nonetheless, silencing the α′2 MBON did show an increase in locomotion during baseline suggesting that this neuron might be important for overall locomotion.

Figure 10:

Results flyGrAM: The green line is the experimental group in which the α'2 MBON is silenced. Furthermore, the gray block represents the time at which the ETOH was turned on.



FLIC

When knocking down the dopamine receptors in the α′2 MBON, it is expected that alcohol consumption will decrease because these receptors are important for aversive taste memory and addiction. After knocking down the dopamine-1 receptor, however, it only showed a significant result in the number of licks taken by the flies ($F = 4.447$, $p = 0.00817$). The time between feeding events, and the duration of consumption did not show any effect on their alcohol consumption. Additionally, the dopamine-2 receptor did not show to have a significant effect on alcohol consumption as well. All analyses have similar results for the experimental RNAi group as well as the control, empty-*GAL-4* group. Also, the flies were still preferring sucrose over ethanol in all groups.

Figure 11:
Results FLIC D1R1;D1R2 – RNAi. The green boxes show the experimental group in which the dopamine-1 receptors are silenced through RNAi. The amount of licks taken in 24 hours is significant.

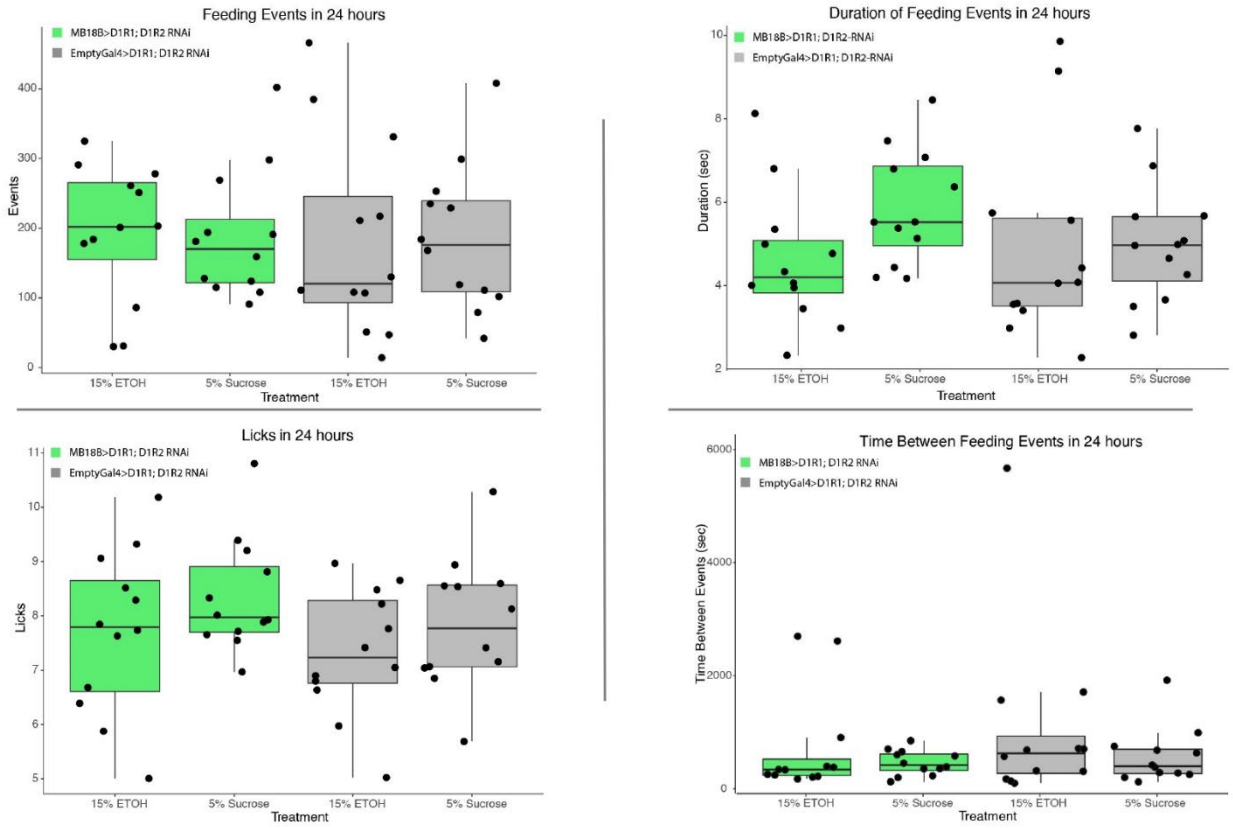
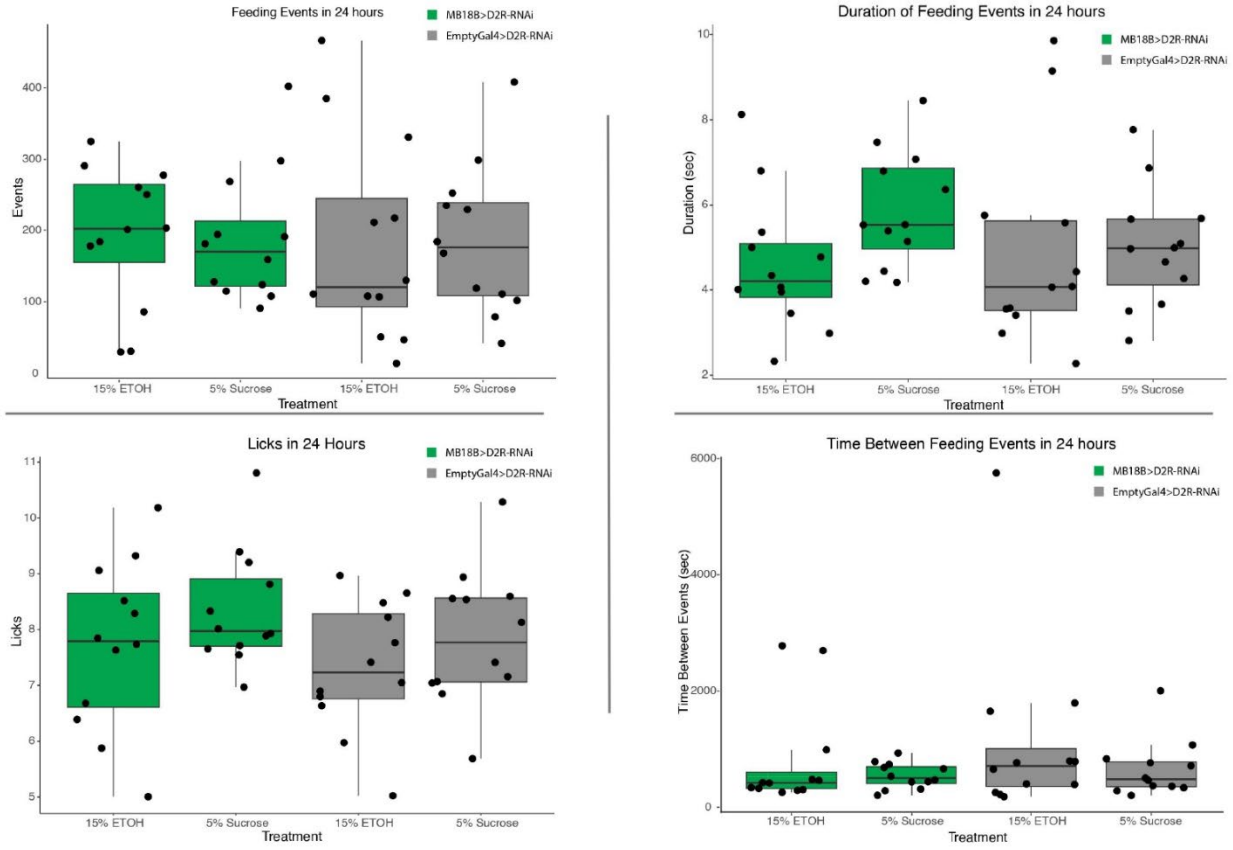


Figure 12:
Results D2R – RNAi. The green boxes show the experimental group in which the dopamine-2 receptor is silenced through RNAi.



DISCUSSION & CONCLUSION

TRIC

We initially hypothesized that α′2 MBON would increase activity following multiple alcohol intoxication experiences. Unfortunately, most of the brains collected lacked RFP and GFP signals, suggesting that the flies collected did not have all of the required transgenes. Of the remaining brains, RFP signal was present, GFP signal, which indicates activity was absent. It is possible that the α′2 MBON is not very active during alcohol acquisition or that the GFP did not have enough time to show gene expression. To be able to show the GFP expression, the 24 hours between alcohol acquisition and retrieval might need to be extended so that the GFP transgene can develop. Future research could use two-photon imaging that can capture deeper tissue and higher resolution during live activity (Yuste & Denk 1995, Svoboda et al. 1997, Helmchen et al. 1999, Wang et al. 2000, Delaney et al. 2001, Wang et al. 2001), instead of using confocal imaging to evaluate the activity of the α′2 MBON post experience in fixed tissue.

FlyGrAM

For this experiment, we hypothesized that α′2 MBON would be important for alcohol-associated locomotion and expected either an increase or decrease in activity upon inactivation. However, results from this experiment suggest that the α′2 MBON is not important for alcohol-associated locomotion. The tracking through FlyGrAM was useful for this experiment because it can detect all activity in real-time which can be used for quick analysis (Scaplen et al., 2019). One of the reasons why this specific MBON might not be important for alcohol-associated locomotion might be because previous research showed that the coactivation of MBONs can be additive meaning that the MBONs with the same type of action can strengthen the response (Aso et al., 2014). This means that the activation of more MBONs might be required for alcohol-associated locomotion and therefore future research can silence several MBONs at the same time to understand the effect of these MBONs together on alcohol-associated locomotion. Additionally, research showed that the dopamine-2 receptors are important for locomotion (Draper et al., 2007) and that the cholinergic α′2

MBON is important for memory retrieval (Scaplen, 2020). Silencing the dopamine-2 receptor in the α′2 MBON might therefore be influential in alcohol-associated locomotion.

FLIC

We hypothesized that alcohol consumption will decrease when knocking down dopamine-1 and dopamine-2 receptors in α′2 MBON. The results, however, showed there was no effect on alcohol consumption. This can be due to the potential co-activation of both dopamine-1 and dopamine-2 receptors (Masek et al., 2015) and therefore more research is needed to investigate the effect of this co-activation on alcohol consumption. Additionally, previous research showed that naïve *Drosophila* build-up preference between three and five days (Devineni & Heberlein, 2009, Ojelade, 2014). This means that in future experiments the 24 hours of tracking alcohol consumption needs to be extended to at least three days to develop an alcohol preference. Despite having hardly any significance in this experiment the FLIC system was beneficial because it analyzes four different consumption behaviors in a more precise way than the CAFE assays and without any disturbance (Ro et al., 2014).

This research can conclude that the α′2 MBON is not important for alcohol-associated locomotion. Additionally, the dopamine receptors in α′2 MBON did not show to be important for alcohol consumption. Further research is still needed to confirm the neuronal activation and changes in the cholinergic MBON α′2 during alcohol acquisition. These results give us more insight into which specific MBONs are responsible for which alcohol-associated behavior. Additional research should focus on identifying the importance of other MBONs related to alcohol use, which can give us a comprehensive and complete view of the structure and function of the MBONs in the MB. These neuronal connections and functions can be related back to the reward circuitry in humans, which can help us better understand how Alcohol Use Disorder develops and persists.

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