# Mechanisms of alcohol-induced sleep dysregulation in *Drosophila melanogaster*

**By**

**Maria Ramirez Roman, B.S., MPH**

**Dissertation**

Presented to the Faculty of the Department of Biology of
The University of Puerto Rico, Rio Piedras Campus
in partial fulfillment of the requirements
for the Degree of

**Doctor of Philosophy**

**University of Puerto Rico, Rio Piedras Campus**

**June 6, 2022**

**University of Puerto Rico**
**Rio Piedras Campus**
**College of Natural Sciences**
**Department of Biology**

## CERTIFICATE OF APPROVAL

### PH.D THESIS

This is to certify that the Ph.D. thesis of **Maria E. Ramirez Roman**
has been approved by the faculty of the Graduate Program for the thesis requirement for the Doctor of Philosophy degree in Biology.

**Thesis Committee:** _____
**Alfredo Ghezzi, Thesis Supervisor**

_____
**Jose L. Agosto Rivera**

_____
**Carmen S. Maldonado Vlaar**

_____
**Andrew M. Seeds**

_____
**Rudolf A. Bohm**

# Dedication

To my parents, Mayra Roman and Rafael Ramirez and my sister Ingrid Ramirez

To my loving husband, Luis M. Santiago Tirado

Special dedication to my brother, Rafael Ramirez Roman (R.I.P)

# Acknowledgments

I want to thank my mentor, Dr. Alfredo Ghezzi Grau for enabling me to do this project and providing me with his mentorship. I also like to thank my committee members, Dr. Jose Agosto, Dr. Carmen Maldonado, Dr. Rudolf Bohm and Dr. Andrew Seeds for their guidance throughout the making of this thesis and their constructive feedback. I would also like to thank the graduate and undergraduate students that helped me with my research: Genesis Ayala-Santiago, Nicolas Fuenzalida Uribe, and Amanda Anqueira. Also, I would like to thank the support of the RISE Program and Dr. Orestes Quesada for partially funding this research.

# Abstract

Alcohol consumption is known to disturb a variety of biological processes that affect normal physiological function. In the nervous system, alcohol is generally known to affect several molecular targets leading to an overall suppression of neuronal activity. As alcohol influences the nervous system, the organism produces a series of neuroadaptive changes that help restore neuronal homeostasis and that may lead to alcohol tolerance, dependence and ultimately addiction. Alcohol abuse can also disturb a number of biological processes, including a disruption of normal sleep patterns. As both alcohol addiction and sleep regulation are under homeostatic control, we hypothesize that these processes share a common mechanism. In this thesis, I explored the effect a set of circadian cells known as pdf neurons have in alcohol sensitivity and tolerance, using *Drosophila melanogaster* as a biological model. I found that these cells are important for the development of alcohol tolerance. Afterwards, I studied the effect alcohol has on sleep in general and found that alcohol affects not only sleep parameters but also the architecture of sleep and morning anticipation. Finally, we focused on understanding the role PDF neurons have in alcohol-induced sleep behaviors. As a result, we found that PDF neurons are important molecular mediators of alcohol-sleep interactions to occur.

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# Chapter 1: Introduction

## 1.1. Biological, emotional, and economical effects of alcohol use

Alcohol addiction is a serious mental illness and public health issue that affects society on a biological, emotional and economical level. According to the National Survey on Drug Abuse and Health performed in 2017, it was estimated that there were 19.5 million Americans that struggled with Substance Use Disorder, of which around 74% specifically suffered from alcohol use disorder. In addition, data from the CDC suggest that around 140,000 deaths occur by excessive alcohol use each year, which sums up to around 380 deaths a day (CDC., 2012). Finally, a report of the National Council of Alcoholism and Drug Dependence in Maryland (NCADD) points out that around 40% of hospital beds around the US are occupied with patients that struggle from a health condition that steamed from alcohol consumption.

Alcohol use can lead to a myriad of physical and psychological conditions that can range from anxiety and depression to liver damage, high blood pressure and stroke (Anderson & Baumberg., 2006). In fact, alcohol is known as a dirty drug, which means that it can have multiple effects depending on the dose and time of exposure, possess various molecular targets and affects many organs in the body (Nestler et al., 2000; Anni et al., 2002; Pfefferbaum et al., 2004; Harper et al., 2009). It can even be a stimulant or a depressant depending on how it is used (Hendler et a., 2013). This complex nature of alcohol makes it somewhat difficult to study as alcohol can be affecting various processes at a time.

Thus, it is of no surprise to find that studies that focus on the effect alcohol has on the psychological profile of the individual are bidirectional and dynamic. For example, some studies suggest that alcohol has anti-anxiety-like properties and serves as an anxiolytic, motivating individuals to ingest more alcohol in order to alleviate stress (Conger.,1956; Levenson et al., 1980; Mezue et al., 2021). This is the basis of the stress reduction hypothesis established in 1956 by Conger. On the other hand, other studies report that alcohol consumption significantly leads to stress, specifically during hangovers (Isbell et al., 1955; Trevisan et al., 1988; Badrik et al., 2008). This is termed “hangxiety” and occurs due to the physiological stress the body endures after drinking (Karadayian et al., 2013; Marsh et al., 2019). Symptoms linked with hangxiety can even manifest themselves in a physical increase in the release of the stress-hormone cortisol (Badrik et al., 2008). In alcoholics, hangxiety occurs through signs of withdrawal symptoms such as delirium tremens, hallucinations and seizures (Isbell et al., 1955; Trevisan et al., 1988). The relationship between alcohol and depression is bidirectional and also complex (Madden 1993; Conner et al., 2004; Wu et al., 2006; Amiri & Behnezhad., 2020). Studies report that people that suffer from alcoholism have a higher chance to self-harm or even commit suicide (Madden 1993; Amiri & Behnezhad., 2020). For instance, it has been estimated that alcoholics are at a 10-15% risk of committing suicide (Conner et al., 2004). In addition, both depression and alcoholism were found in 85% of 100 cases that completed suicide attempts (Barraclough et al., 1984; Beautrais et al., 1996; Conwell et al., 1996). Interestingly enough, depression itself also leads to alcohol use. For instance, research suggests that children who suffer from moderate

and major depression are almost twice as likely to use alcohol in adulthood (Wu et al., 2006).

Alcohol can cause serious physical damage as well (Rosito et al., 1999; Seppa & Sillanaukee., 1999; Rem et al., 2013; Lancker et al., 2019; Roerecke et al., 2019; Mount Sinai., 2022). A condition that may develop over the years in alcoholic patients is called Alcoholic Liver Disease (Rehm et al., 2013; Roerecke et al., 2019). This condition is typically characterized by swelling and inflammation of the liver. However, serious cases can lead to cirrhosis and scarring of the liver, causing liver damage and ultimately failure (Lancker et al., 2019; Mount Sinai., 2022). In addition, alcohol use can lead to an increase in blood pressure and stroke (Rosito et al., 1999; Sacco., 1999; Seppa & Sillanaukee et al., 1999; Christensen et al., 2018). Specifically, it has been found that regular consumption of alcohol increases blood pressure in a dose dependent fashion (Rosito et al., 1999) and that binge drinking increases systolic blood pressure from 4 to 7 mmHg and diastolic blood pressure from 4 to 6 mmHg as compared to normal cardiac pressure values (Seppa & Sillanaukee., 1999). Although, low and moderate consumption of alcohol has been linked with reduced risk of stroke (Sacco., 1999; Christensen et al., 2018), heavy drinking does increase the risk of having a stroke significantly (Sacco., 1999).

From a sociocultural view, alcohol can severely affect the daily habits of alcoholic patients and wreak havoc on their workplace, family and friend circles. In terms of the workplace, data obtained by the European Alcohol and Late forum suggests that alcohol

use affects work attendance and overall work productivity. In addition, this study points out that alcohol consumption influences the time period in which a person stays working because it influences their physical health and/or can potentially increase their chances of an early death. Family dynamics are altered when an alcoholic member is present as drinking can significantly affect the family budget, cause alcoholics to neglect their responsibilities, even if they have children (Keller et al.,1994) and increase divorce rates (Caces et al., 1999).

Finally, it has been estimated that the cost of alcohol use results in some serious economic consequences. In fact, it was estimated that the annual cost during 2010 of excessive alcohol use in the United States was around $249 billion (Sacks et al., 2010). Most of these costs were associated with a decrease in productivity in the work environment, healthcare expenses relating to excessive drinking, expenses relating to law enforcement and the criminal justice system and losses of motor vehicles relating to car crashes because of alcohol usen (Sacks et al., 2010; CDC., 2022).

## 1.2. The addictive nature of alcohol

Addiction is described as a long term and recurring disorder that is characterized by a strong need to continue taking the drug despite the detrimental consequences it has on the individual that consumes it (NIDA., 2018). Alcohol is one of the most used substances that cause addiction among Americans; it is legal to drink since 21 years of age, easily accessible and inexpensive (National Survey on Drug Use., 2017). According to the National Survey on Drug Use, it's the third most addictive substance in

the US with statistics pointing out that 1 out of 12 Americans suffer from a type of alcohol abuse or dependency. Interestingly, the term addiction is no longer a category that can be diagnosed using the Diagnostic and Statistical Manual of Mental Disorders (DSM-5., 2013); it is now diagnosed under the term Substance Use Disorder (NIDA., 2018). For this disorder to be diagnosed, the patient must possess two or three of the following criteria for a period of 12 months that can range from “the substance is taken from large amounts or a longer period that was intended for”, “there is a persistent desire or unsuccessful effort to cut down or control the use of the substance” and “a great deal of time is spent in activities regarding that substance”, among others (DSM-5., 2013).

On top of the aforementioned criterias for diagnosing Substance Use Disorder, there are additional signs that the clinician must evaluate when in a session with the patient such as drug cravings, tolerance and/or withdrawal syndrome (DSM-5., 2013). The homeostatic theory of drug abuse is one of the original frameworks that tried to explain the addictive nature behind substance abuse drugs using these concepts. Himmelsbach first proposed it back in 1956 as he observed that the psychological profile of the morphine addict appeared normal as he received an adequate amount of morphine that fitted his addictive needs (Himmelsbach et al., 1941; Himmelsback., 1956). However, during the withdrawal phase, he observed that a range of symptoms arose in the patient that provided a clear sign that the body was going through some period of great distress (Himmelsbach et al., 1941; Himmelsbach et al., 1956). Nevertheless, after a couple of weeks to 6-9 months of total abstinence, the patient

seemed to have recovered from these symptoms (Himmelsbach et al., 1941). As a result of these observations, he developed a system for viewing tolerance and dependence as if it were a seesaw where the first exposure to the drug alters the balance in terms of neural activity. As a result, the brain tries to counteract the initial effect of the drug by creating the opposite effect; this is the stage known as tolerance. However, as the drug is cleared from the system, this counteracting mechanism still persists, creating a withdrawal state that can only be alleviated with more drug use. This leads to the development of dependence, a phenomenon where the organism needs the drug in order to regain the neuronal balance that it once had (Norohna et al, 2014).

Although the homeostatic theory of drug dependence provides many insights into the addictive nature of substance abuse drugs, it falls short when taking some considerations into account. For instance, this theory implies that, for example, if alcohol induces electrochemical reduction, the brain will try to counteract this mechanism by inducing the opposite effect - an electrochemical activation - of the same neuronal system that was affected when the electrochemical reduction occurred (Littleton., 1998). This mechanism of action is what is known as homolog adaptation (Littleton., 1998). Furthermore, other studies have reported that heterologous adaptations could occur where the brain might try to counterbalance the effect of one neuronal system with that of another (Little & Littleton., 1994; Littleton., 1998). Nowadays, one might expect that homolog and heterolog adaptations could occur simultaneously throughout the nervous system, complicating manners even further (Littleton., 1998).

Other theories help explain why drugs are so addictive. These mainly focus on the positive and negative reinforcement effects of the drugs. In the positive reinforcement theory, people are motivated to consume alcohol because it relieves them of stress and produces feelings of pleasure (Conger et al., 1956; Wise et al., 1985). On the other hand, the negative reinforcement theory states that people consume alcohol in order to alleviate symptoms relating to withdrawal (Koob et al., 1994; Koob et al., 2001). Although some may state that these theories are contradictory, they are not mutually exclusive and can work simultaneously in order to motivate individuals to consume drugs in higher quantities.

Finally, another aspect that makes alcohol so addictive is its effect on the reward pathway (Hodge et al., 1977; Gatto et al., 1994; Koob et al., 1994). This pathway is composed of very specific areas of the brain that are associated with memory and behavior that communicate with each other through neurons and chemical signals called neurotransmitters. The major protagonist of the reward pathway in terms of neurotransmission is DOPAMINE (Dahlstrom & Fuxe., 1965; Andén et al., 1966; Berridge & Robinson., 1998; Arias Carrion et al., 2007). The Ventral Tegmental Area (VTA) secretes DOPAMINE that in turns affects the amygdala (Phillips et al., 2010), nucleus accumbens (Cheer et al., 2007) and prefrontal cortex (Hitchcott et al., 2007). The amygdala is the area of the brain associated with emotions (Anderson et al., 2001; Phan et al., 2002), while the nucleus accumbens is involved with emotions and motor function (Sawada et al., 2015) and the prefrontal cortex is involved with decision making (Stus et al., 1986). In addition, the VTA releases DOPAMINE into the hippocampus

(McNamara et al., 2014) as well, which is responsible for memory formation. Studies show that alcohol increases the firing rate of DOPAMINE neurons in organisms (Gessa et al., 1985; Brodie et al.,1990) and increases its release in the amygdala (Funk et al., 2006), nucleus accumbens (Di Chiara et al., 1988; Koob et al., 1993) and prefrontal cortex (Robinson et al., 2009). Even though we've seen that alcohol directly affects the reward pathway, some studies show that the reward pathway can ultimately affect alcohol consumption (Weiss et al., 1993; Rassnick et al., 1992; Hodge et al. 1997; Melis et al., 2005). For example, some studies suggest that alcohol consumption is blocked when a DOPAMINE antagonist is injected into the nucleus accumbens (Hodge et al. 1997; Rassnick et al. 1992). Even more so, other studies show that both alcohol anticipation and alcohol withdrawal increase DOPAMINE, which can lead to more drug use (Weiss et al. 1993; Melis et al. 2005). **Figure 1** shows the theories or factors that have been mentioned that influence drug consumption.

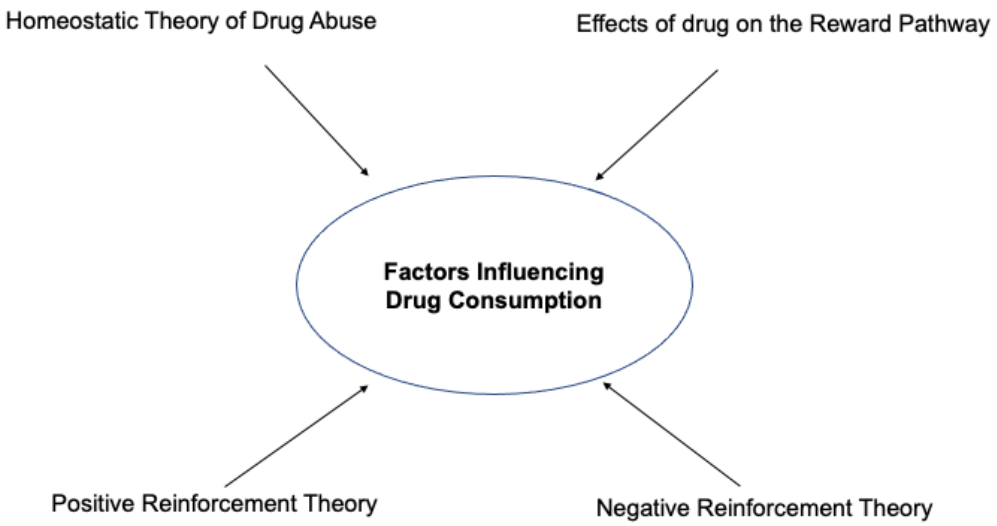


**Figure 1. Factors that influence drug consumption.** Main theories propose to explain the mechanisms operating in drug consumption despite its detrimental consequences. These theories are: the Homeostatic Theory of Drug Abuse, the positive reinforcement theory and the negative reinforcement theory. Another factor that may influence drugs is their effect on the reward pathway.

Taking into consideration the aforementioned studies on how alcohol affects distinct aspects of human behavior, the overall goal of our lab is to understand the molecular mechanisms that underlie drug-induced neuroadaptations that result in substance abuse. Our central hypothesis is that the mechanisms of alcohol neuroadaptation that mediate tolerance and dependence are responsible for a specific set of behavioral disturbances. In this study, we focus our attention on sleep. In particular, we want to look at the neuronal and molecular mechanisms behind alcohol induced sleep dysregulation. In order to do this, it is necessary to understand how alcohol affects the nervous system from a neurobiological perspective.

## 1.3. A closer look at how the nervous system works

How environmental stimuli affect the nervous system in order to influence behavior has been an object of study over the years. One of the theories that described the physiological aspects of brain structure came from Santiago Ramon and Cajal and Camillo Golgi back in the 1800s. Santiago Ramon and Cajal, with a rudimentary microscope by today's standard, created an artistically drawn representation of the brain's neuronal framework and proposed the theory that explains that the brain is composed of individual and segregated units called neurons (Glickstein., 2006). This idea came contrary to what Camillo Golgi proposed that stated that the nervous system was not composed of individual units, but of an interconnected-like web structure. Nowadays, Santiago Ramon and Cajal scientific work set out to be the basis of neuroscience and the neuronal doctrine and it is widely accepted that the brain is composed of individual, independent units called neurons (Golgi., 1885; Golgi., 1886). However, how these individual cells communicate with each other was still not known. In 1906, Sherrington discovered that electrical stimulation in specific areas of the spinal cord of dissected animals generated muscle movements. These experiments became the pioneers in setting forward the notion that electrical signaling is important for the communication between neurons. He also theorized that there was a physical space of separation between neurons—what is now called the synaptic space. Thus, so far, these scientists have gathered evidence that: 1) the brain is composed of discrete units called neurons, 2) these neurons communicate with each other through electrical signals and that 3) there was a space between these neurons that they used to

communicate with each other. Other scientists provided evidence concerning the notion of how a neuron works from an electrical and chemical perspective. Bergstein made important discoveries concerning the resting potential- the electrical potential a neuron has when it is not stimulated. Bergstein reported that the resting potential was due to the neuron's selective permeability to potassium ions. However, it was still unclear how neuronal excitation occurred. Ultimately, through the development of scientific and experimentally based mathematical equations, it was discovered that ionic channels responded to electrical signals and that this response was crucial for the action potential to be generated. These experiments were carried out by Hodgkin and Huxley in 1952 in which they saw how sodium and potassium channels responded to voltage change. Specifically, they establish that in the neuron:

1. An electrical signal causes sodium channels to open up, allowing an inward flow of sodium ions, causing what is known as neuronal depolarization (Hodgkin & Huxley., 1952; Raman et al., 1997). These influx of sodium ions are responsible for generating the action potential.

2. As this influx of sodium ions causes the voltage inside the neuron to rise up to +40 mv, sodium ions close and voltage gated potassium channels open (Hodgkin & Huxley., 1952).

3. This leads to a repolarization stage, where potassium ions flow from the inside to the outside of the cell, causing the voltage inside of the cell to reach -70 mv. Potassium channels are known to take a little longer to close so that a

hyperpolarization state occurs in which the voltage inside of the cell reaches -90mv (Hodgkin & Huxley., 1952; Sakmann et al., 1984; Ginsborg et al., 1991).

In 1998, the sodium potassium pump was discovered by a scientist named Skou. This is a transportation protein that restores the balance of ions generated after the action potential occurs by taking sodium ions out of the cell and potassium ions in (Skou et al., 1998), causing the nerve cell to reach resting potential. After the action potential is generated, the presynaptic neuron sends a chemical signal to the postsynaptic neuron. The firsts experiments that uncovered chemical signaling were conducted by Otto Loewi (1921). Nowadays, we know that these chemical signals are called neurotransmitter and neuropeptides and that they are secreted by one neuron (which we will now call presynaptic neuron) and bind to the receptor protein of another (which we will call post synaptic neuron (Langey et al., 1907; Neher & Sakmann., 1976; Colquhoun.,1998,). Once the neurotransmitter binds to a receptor, the postsynaptic neuron can be either electrically activated or silenced.

## 1.4. Alcohol and its effect on the nervous system

Alcohol is a drug of abuse that affects the nervous system in various ways. It is generally accepted that alcohol is known to have neuronal depressant properties (Pohorecky et al., 1977; Haes et al., 2010). This means that the drug diminishes the ability of the cell to produce an action potential. In order to have its effect, alcohol interacts with various receptors in the nervous system. The following sections will be dedicated to explaining how alcohol interacts with neurotransmitters in the brain.

### 1.4.1. GABAergic Transmission and Alcohol

Gamma-aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters of the nervous system (Bazemore et al., 1956; Kravitz et al., 1963; Kravitz et al., 1965). Through different biological mechanisms, alcohol increases GABAERGIC transmission in the brain, promoting its neuronal depressant properties (Roberto et al., 2003; Roberto et al., 2004). One way it does this is by enhancing GABA receptor function and thereby increasing the inflow of chloride ions into the neuron (Harvey et al., 2002; Kelm et al., 2011). It is important to note that alcohol does not affect all GABA receptors, but a specific subset of them. Specifically, alcohol has been shown to potentiate GABAa receptors (Harvey et al., 2002) and GABAb receptors as well (Kelm et al., 2011). Other studies focus on how alcohol causes an increase in the release of GABA neurotransmitters. For instance, studies show that alcohol causes a dose-dependent increase in the release of GABA in the central nucleus of the amygdala in rats (Roberto et al., 2003; Roberto et al; 2004). In addition, other studies suggest that alcohol increases GABA release in the Ventral Tegmental Area and the reward pathway, ending up increasing the release of DOPAMINE as well. As we know, this release of DOPAMINE affects the nucleus accumbens, which manifests itself in feelings of pleasure (Ding et al., 2015). Ethanol induces GABAERGIC transmission in areas of the cerebellum, possible through mechanisms involving cerebellar granule cells (Carta et al., 2004)

Interestingly enough, not only does alcohol stimulate GABAERGIC transmission, but GABA itself also influences alcohol responses on a behavioral level. For example,

studies show that alcohol consumption is decreased by compounds that block the function of GABA receptors (antagonists) while it increases when administering compounds that stimulate GABA receptors (agonists) (Hyytia & Koobet., 1995; Koob et al., 2004).

### 1.4.2. Glutamatergic transmission and Alcohol

The major excitatory neurotransmitter in the brain is called GLUTAMATE. This neurotransmitter is known to bind to several receptor subtypes, including one called N-methyl-d-aspartate (NMDA) receptor (Ozawa et al., 1998). Contrary to alcohol's effect on GABA, alcohol typically inhibits GLUTAMATE in the brain (Lovinger et al., 1989). Studies show that GLUTAMATE antagonists mimic alcohol's effects (Colombo & Grant, 1992). In addition, it has been found that acute alcohol exposure inhibits NMDA receptor activity in the nucleus accumbens in rats (Carboni et al., 1993). Moreover, in vivo and in vitro studies suggest that ethanol decreases glutamate release into the amygdala (Roberto et al., 2004). Another GLUTAMATE receptor that seems to be affected by alcohol is called mGluR5. Administration of mGluR5 antagonists decreases alcohol-induced withdrawal and increases basal motor activity in rats (Blednov & Harris., 2008). Furthermore, ethanol decreases in a dose dependent manner the ionic current produced by NMDA in voltage clamped studies in areas such as the hippocampus (Lovinger et al., 1989). In addition, alcohol is known to also decrease GLUTAMATERGIC transmission at the prefrontal cortex, which both diminishes overall judgment and enhances the nucleus accumbens to produce feelings of pleasure (Kolpakova et al., 2012; Cheng et al., 2018; Cheng et al., 2018).

Finally, of great importance is the notion that compounds that affect GLUTAMATERGIC transmission are being used as a pharmacological treatment for alcohol dependence. For instance, acamprosate, an antagonist compound that affects NMDA receptors and modulates GLUTAMATERGIC transmission, is being used as a treatment for alcoholism in humans (Littleton., 2007). **Figure 2** summarizes the effect alcohol has on GABAERGIC and GLUTAMATERGIC transmission discussed in the aforementioned studies.

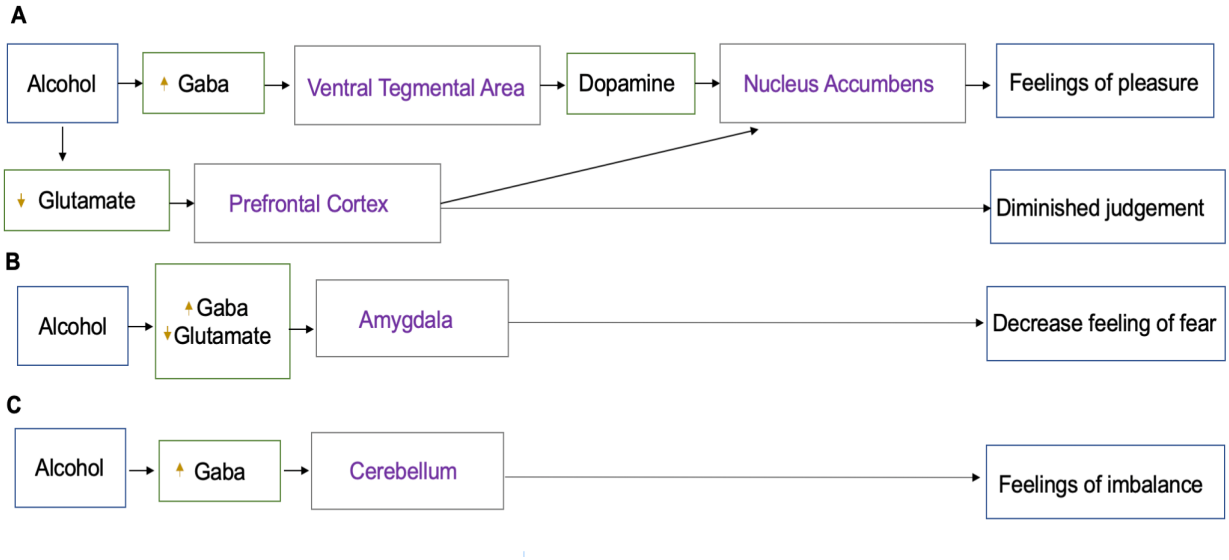


**Figure 2. Effect of Alcohol on the Reward Pathway and other brain structures.** A) Alcohol, through the release of GABA, is known to affect the Ventral Tegmental Area, that in turn releases dopamine into the Nucleus Accumbens in order to cause feelings of pleasure. In addition, alcohol decreases glutamate in the prefrontal cortex that in turns affect that in turn diminishes judgment and affects the nucleus accumbens B) Alcohol increases GABA and decreases glutamate in the amygdala, decreasing feelings of fear. C) Alcohol increases GABA in the cerebellum causing feelings of imbalance.

### 1.4.3. Other neurotransmitters and Alcohol

**Serotonin.** Alcohol is also known to affect other neurotransmitter systems (as reviewed by Banerjee et al, 2014). For example, we have talked before of how alcohol stimulates the reward system by increasing the release of DOPAMINE in the Ventral Tegmental Nucleus (Cheer et al., 2007). Another neurotransmitter important for the modulation of alcohol induced responses is SEROTONIN. Specifically, acute alcohol exposure stimulates the secretion of SEROTONIN, a neurotransmitter that has a huge role in elevating the mood and feelings of well-being in people (LeMarquand et al., 1994). Also, alcohol potentiates the function of SEROTONIN receptors, which include: 5-HT1A, 5-HT1B, 5-HT2, and 5-HT3 (McBride et al., 1993; Lovinger et al., 1997). Moreover, SEROTONIN levels also affect alcohol responses as studies suggest that alcohol preference was decreased or abolished when rats were given an inhibitor that depletes SEROTONIN in the brain (Myers & Veale., 1968).

**Cannabinoids.** From a behavioral perspective, studies have established a link between ethanol and CANNABINOIDS. For example, many studies suggests that both ethanol and cannabis produce feelings of euphoria, intoxication (Jones & Stones., 1970; Heishmann et al., 1997) and affect memory, attention and psychomotor performance (Heishman et al., 1988; Chait & Perry, 1994; Heishman et al., 1997). Moreover, other studies suggest that ethanol cravings and consumption increased during a two week time period abstinence from cannabis (Peters & Hughes., 2010). From a molecular perspective, alcohol dependence is associated with the downregulation of endocannabinoid receptor CB1 (Hirvonen et al., 2012).

## 1.5. Sleep and Circadian Rhythms

### 1.5.1. Fundamental aspects of sleep

Sleep is defined as a prolonged period of behavioral quiescence or immobility, with reduced sensory perception to environmental stimuli, reduced muscle activity and a preferred posture (Schwarz et al., 2012). One must theorize that sleep is important for the maintenance of biological function and survival as it is found in almost every animal that possesses a nervous system (Siegel et al., 2008). Indeed, many studies point out that sleep modulates inflammatory responses (Spiegel et al., 1985), immunity (Kapsimalis et al., 2008; Krueger et al., 2008), biological waste clearance (Xie et al., 2014), metabolism (Benington et al., 1995), learning and memory (Maquet., 2001; Stickgold., 2005) as well as cognitive processes (Roberto et al., 2004). However, the function of sleep itself is still not fully understood and not well known.

In some organisms such as mammals, sleep is divided into different states that have specific wave patterns that are typically analyzed by the use of an electroencephalogram or EEG (Vorster & Born., 2015). There are two stages of sleep called REM and NON-REM. REM (or rapid eye movement) is often called active sleep and its wave activity is very similar to that of wakefulness (Jouvet & Michel., 1959; Jouvet., 1967; Rechtschaffen and Kales., 1968). Waves in REM state are known to be short in amplitude but high in frequency (Jouvet & Michel., 1959; Jouvet., 1967; Rechtschaffen and Kales., 1968; Siegel et al., 2005; Vorster & Born., 2015). Although some muscles are blocked from movement, most specifically those from the neck down,

in REM sleep, some of our muscles can still move, including those of the eyes (Aserinsky & Kleitman., 1953; Dement & Kleitman., 1957). This is also the stage in which dreams are experienced (Dement & Kleitman., 1957).

On the other hand, NON-REM is known as deep sleep and is the stage in which high amplitude but slow frequency waves are present (Rechtschaffen and Kales., 1968; Franken et al., 2009). In this stage, the muscles are completely paralyzed and dreaming is rare (Rechtschaffen & Kales., 1968). During the night, mammals and humans tend to alternate on average 4-6 times between REM and NON-REM, a full cycle being around 90-110 minutes (Rechtschaffen and Kales., 1968).

Borbeley et al., 1982 proposed that sleep is regulated through two different mechanisms—one named Process S that focuses on the homeostatic nature of sleep and another termed Process C that states that sleep is modulated through circadian rhythms (Borbeley., 1982). Indeed, an important aspect of sleep is that it is homeostatically regulated through a process called sleep rebound (Process S), a phenomenon where animals show an increase in baseline sleep when they are sleep deprived (Borbeley., 1982; Machado et al., 2004). Several studies have tried to elucidate how sleep rebound works. Evidence suggests a short period of sleep deprivation increases NON-REM sleep as well as delta waves associated with NON-REM (Tobler et al., 1990; Qui et al., 2015), without necessarily affecting REM sleep. Nevertheless, long periods of sleep deprivation have been found to increase both REM and NON-REM (Tobler et al., 1990). Moreover, it has been found that organisms

experience a state of REM rebound specifically when they are sleep deprived during REM sleep (Grahnstedt & Ursin., 1985; Beersma et al., 1990).

In addition, other studies point out that sleep rebound is affected by neurochemicals. It has been found that the homeostasis regulation of sleep is influenced by adenosine (AD), nitric oxide (NO), prostaglandin D2 (PGD2) and cytokines (Luk et al., 1999; Rosenberger et al., 2000; Hayaishi et al., 2004; Mizoguchi et al., 2001; Porkka et al., 2013). For instance, it has been observed that during waking, neuronal cells that are associated with wake promoting components use high amounts of energy, which leads to a high concentration of extracellular ADENOSINE (Porkka-Heiskanen., 2013). The longer the organism is awake, the greater AD is accumulated, which turns out to increase the need for sleep (Porkka-Heiskanen., 2013). In fact, during sleep deprivation studies, there is an increase in AD in basal forebrain regions which promotes the organism to fall asleep and enhances delta wave activity (Thakkar et al., 2003; Thakkar, et al., 2010). In these cases, sleep rebound was measured through sleep latency and the recovery sleep that followed sleep loss (Borbély, 1982; Porkka-Heiskanen, 2013).

The second regulatory mechanism of sleep focuses on a sleep-independent process that works mainly through the action of circadian rhythms (Borbeley., 1982). The organism can predict and respond to external stimuli through a 24-hour molecular feedback loop called the circadian clock (Hans., 1940; Burning., 1960; De Marian, 1979; Hans, 1988). Through the circadian system, the organism regulates sleep (Xuan et al.,

2011), nutrition & metabolism (Asher et al., 2015), reproduction (Caba et al., 2018) and locomotor activity processes (Hurd., 1998) by responding to light input. In mammals, this molecular feedback loop is composed of key players that regulate the transcription of each other. The key players are CLOCK, BMAL, TIM and PER (Rosebash et al., 1989; King & Sehgal., 2018). During the day, BMAL and CYCLE are known to bind together in order to activate TIM and PER transcription (Reddy et al., 1984; Rosbash et al.,1989; Hardin et al., 1999; Buhr et al., 2015). However, during the night, TIM and PER bind together to inactivate BMAL and CYCLE (Rosbash et al., 1989; Buhr et al., 2015). In mammals, this negative feedback loop is present in neurons that are found in the suprachiasmatic nucleus located in the hypothalamus (Weaver., 1998; Hastings et al., 2008).

### 1.5.2. Neuronal systems associated with sleep

In mammals and higher complex species, a series of brain regions are important for generating sleep/wake cycles (as reviewed by Scammell et al., 2017). The cell bodies of these neurons conglomerate in different brain structures which innervate many parts of the nervous system, giving rise to a complex circadian circuit that regulates distinct aspects of sleep/wake cycles. Experiments concerning the wake promoting component of sleep cycles were first conducted by Economo in 1930. During an epidemic of encephalitis lethargica, he saw that the patients affected with the disease had lesions in both the midbrain and posterior hypothalamus, which caused them to sleep more. Thus, he proposed that these regions acted as wake-promoting components. Other scientists found that when an area called the reticular formation was

electrically activated, cats showed EEG patterns similar to those of waking while they were under the effects of anesthesia (Moruzzi and Magoun., 1949). On the other hand, sleep was altered when lesions were generated in regions relating to the hypothalamus, locus coeruleus, tuberomammillary nucleus, basal forebrain, pedunculopontine tegmental, latero-dorsal tegmental (PPT; LDT) and dorsal median raphe nuclei, indicating that these regions were involved with sleep cycles as well (Webster & Jones., 1988; Shouse & Siegel., 1992; Datta &. Hobson., 1995; Gerashchenko et al., 2004; Blanco-Centurion et al., 2007; Yokogawa et al., 2012).

Nowadays, scientists know that wake-promoting pathways are scattered throughout the brain and are influenced by several neurochemical systems. In this circuit, neurons of the reticular activating formation in the midbrain affect two pathways- the dorsal pathway and the ventral pathway (Nauta & Kuypers, 1958). The ventral pathway itself is composed of MONOAMINERGIC neurons of the locus coeruleus, SEROTONERGIC neurons of the dorsal median raphe nuclei, DOPAMINERGIC neurons of the ventral tegmental area and HISTAMINERGIC neurons of the tuberomammillary nucleus that affect the thalamus, hypothalamus and cortex in order to induce a wake-like state (Gaykema et al., 1996; Guiterrez-Herrera et al., 2016; Kaur et al., 2017; Tyree et al., 2017; Eban-Rothschild., 2020). On the other hand, the dorsal pathway is composed of neurons found in the parabrachial nucleus, pedunculopontine and latero-dorsal tegmental nuclei and basal forebrain that in turn affect the cortex by acting as wake promoting agents (Fulwiler., 1984; Gritti et al., 1997; Kaur et al., 2013; Boucetta et al., 2014).

An area important for the generation of sleep is called the ventro-lateral preoptic nucleus or the VLPO (Economo., 1930; Venner et al., 2015). Indeed, scientists have found that lesions in the VLPO neuronal system induce NON-REM sleep (Luu et al., 2000). Nowadays, scientists know the neuronal mechanisms by which NON-REM is generated by the VLPO. Specifically, through GABAERGIC neurons, the VLPO inhibits tuberomammillary regions of the hypothalamus (Sherin et al., 1996; Sherin et al., 1998;), producing NON-REM sleep. On the other hand, it has been found that tuberomammillary regions of the hypothalamus (such as those in the tuberomammillary nucleus) inhibit VLPO, inducing arousal (Sherin et al., 1996; Sherin et al., 1998).

The median preoptic nucleus is another area that has been associated with NON-REM via two mechanisms. First, evidence suggests that the median preoptic nucleus activates the VLPO, which in turn induces NON-REM sleep (Chou et al., 2002; Uschakov et al., 2007). Second, NON-REM sleep is generated when the median preoptic nucleus inhibits wake promoting areas of the hypothalamus, dorsal raphe nucleus and Locus Coerelus (Uschakov et al., 2007).

In addition, it has been observed that lesions of the pons lead to an decrease in REM sleep, suggesting a role for this region in generating REM (Jouvet., 1962; Webster & Jones., 1988). Furthermore, it was found that prior and during REM sleep, high voltage EEG waves were seen in the pons, lateral geniculate and occipital cortex (Sakai & Jouvet., 1980). This study showed that the PPT and LDT nuclei were important in generating REM sleep as well (Sakai & Jouvet., 1980). This association between PPT

and LTD regions in generating REM sleep have been established in other studies (Van Dort et al., 2014). In contract, neuronal activation of the locus coeruleus, dorsal raphe nucleus and tuberomammillary nucleus stopped REM sleep from occurring (Aston-Jones & Bloom., 1981; Trulson et al., 1981; Steininger et al., 1999; Takahashi et al., 2010).

Finally, the main regulator of circadian rhythms in a variety of species is called the suprachiasmatic nucleus (Hastings et al, 2019). This region modulates circadian rhythms by expressing genes relating to the molecular clock components (TIM, PER, BMAL and CYCLE) that help coordinate circadian rhythms (Yan., 2000; Ono et al., 2015). It is influenced by light stimulus via the retino-hypothalamic pathway and activates or inhibits specific brain areas according to environment cues (Moore & Jenn., 1972; Morin & Allen, 2006). The suprachiasmatic nucleus makes contacts with other areas of the nervous system through two distinct pathways (Aston-Jones et al., 2001; Gonzalez et al., 2006; Riaz et a.l, 2016). One is located in the hippocampus and is known as the dorso-medial nucleus and the other is called the supraventricular zone (Riaz et al, 2016). During the day, the SCN activates the dorsomedial nucleus which in turn activates orexin neurons and the locus coeruleus (Aston-Jones et al., 2001; Gonzalez et al., 2006).

As we have seen, sleep is both regulated and influences a myriad of neurobiological brain regions that are essential for our survival. For this reason, unraveling the mechanisms of how drugs of abuse affect sleep is of great importance.

The next sections will focus on explaining the relationship between alcohol and sleep both from a behavioral, molecular and neurobiological standpoint.

## 1.6. Interactions Between Alcohol and Sleep

### 1.6.1 The behavioral effect of alcohol on sleep

Research concerning the effects of drug abuse on sleep has been a major concern among scientists over many decades. In mammals, the analysis of EEG patterns has proven effective in determining the effect alcohol has on sleep in its different stages. At low doses, alcohol increases the time it takes to fall asleep or sleep latency (Petrucelli et al.,1994). Nevertheless, the effect of low doses of ethanol on REM and NON-REM sleep are still not fully understood (Ebraim et al., 2013; Stein et al., 2015). In contrast, at high and acute doses, alcohol is known to act as a potent sedative and shortens sleep latency, increases delta wave activity associated with NON-REM sleep and decreases REM sleep (Yules et al.,1967; Williams et al., 1983; Roers et al., 1999; Van Reen et al., 2006; Feige et al., 2006; Chan et al., 2014; Colrain et al., 2014). However, this effect is short lived and only lasts a couple of hours as alcohol ends up increasing fragmented sleep at the second half of the night (Bixler et al., 1979).This phenomenon can lead to a vicious cycle as the person overall sleeps poorly after alcohol consumption but ends up drinking alcohol in order to fall asleep faster (Johnson et al., 1998; Roerhs et al., 1999; Arnedt et al., 2000; Stein et al., 2006). This has proven to be a major public health concern as it is estimated that around 20% of Americans use

alcohol as a sleep aid and that the cost of alcohol related sleep problems in the United States exceeds $18 billion (Thakkar., 2015).

A different sleep profile is observed in individuals who are exposed to chronic alcohol use. Most studies suggest that chronic use of alcohol leads to a decrease in REM sleep (Gitlow et al., 1973; Rouhani et al., 1998) and an increase in NON-REM (Veatch., 2006; Mukherjee et al.., 2008; Mukherjee & Simasko., 2009). In terms of sleep disruption manifested after chronic alcohol exposure, usually there is a decrease in total sleep and an increase in sleep latency (Mukherjee et al., 2009; Mukherjee et al., 2009; Britton et al., 2020).

### 1.6.2. The molecular effects of alcohol on sleep

We have talked about how alcohol is a sedative drug that interacts with the nervous system in a unique and complex matter. In this section, we will focus on explaining the effect alcohol has in circadian and wake promoting neurons. Later on, we will talk about how alcohol affects the neuronal circuits involved in producing NON-REM and REM sleep.

It has been well documented that the suprachiasmatic nucleus (SCN) is affected both by glutamatergic and GABAERGIC transmission and that this systems interact with each other in order to regulate SCN activity (Decavel et al., 1990; Van den Pol., 1993; Ding et al., 1994). When it comes to glutamatergic signaling, in vitro and in vivo studies reveal that ethanol inhibits glutamate signaling in the suprachiasmatic nucleus, causing a disruption in circadian clock phase regulation (Prosser et al., 2008; Bragger et al., 2009). Interestingly, results obtained from other studies indicate that the activation of

GABAa receptors by ethanol is what modulates glutamatergic ethanol regulation of circadian clock phases in the suprachiasmatic nucleus (McElroy et al., 2009). All together, these investigations suggest that alcohol inhibits SCN activity disrupting the normal functions of the circadian clock.

Not only does alcohol alter the neuronal systems found in the suprachiasmatic nucleus, but it also affects the core clock genes. For instance, studies show that ethanol exposure during gestational days affects the expression of *per1* and *per2* genes both in the hypothalamus and suprachiasmatic nucleus (Chen et al., 2006). Other studies reveal that both *cry* and *per* genes were altered in neonatal rats exposed to alcohol (Farnell et al., 2009). As a result, this ethanol induced alterations in core clock genes produced long-term changes in free-running period, photoentrainment and phase shifting responses of circadian rhythms (Farnell et al., 2009). Finally, it has also been seen that mutations in *per2* gene lead to an increase in alcohol consumption in humans (Comasco et al., 2010).

In addition to these experiments, evidence suggests that alcohol inhibits specific elements of the wake promoting pathways. For example, West et al., 2015 showed that alcohol suppresses the activity of Locus Coeruleus neurons in a specific type of rat that was selectively bred for susceptibility to stress and that consumed alcohol in large quantities (West et al., 2015). Interestingly enough, this study demonstrated that alcohol's inhibition of Locus Coerelus neurons increases the activity of dopaminergic neurons found in the VTA and the rewarding effects of the drug (West et al., 2015). Although it is still not known how ethanol induced inhibition of Locus Coerelus neurons

might affect sleep/wake cycles, one might hypothesize that it could lead to an increase in sleep as the Locus Coerelus is a region associated with wakefulness. In addition, another wake promoting region that ethanol has been found to inhibit is the tuberomammillary neurons (TMN), possibly by potentiating GABAERGIC transmission in the TMN (Sun et al., 2016).

Finally, we have talked previously about the role the Basal Forebrain has in wakefulness. Several scientists have found that 1) after ethanol exposure, animals with normal Basal Forebrain neuronal function have a decrease in sleep latency accompanied by an increase in NON-REM (Brown et al., 2012; Thakkar et al., 2014; Alam et al., 2017; Sharma et al., 2017) 2) when using an immunotoxin to destroy BF neurons, these alcohol induced sleep promoting effects vanished (Brown et al., 2012; Sharma et al., 2017) 3) significant lower levels of adenosine have been reported on animals with lesions in BF cholinergic levels compared to controls after ethanol administration (Brown et al., 2012; Sharma et al., 2017) 4) a decrease in the sleep promoting effect of alcohol when adenosine receptors were blocked in the Basal Forebrain (Thakkar et al., 2010). Together, these experiments point out that acute alcohol induced sleep responses are mediated through Basal Forebrain neurons and that the buildup of ADENOSINE plays a role in this response.

When it comes to sleep promoting structures, it has been found that an important area in generating NON-REM sleep is called the median preoptic area (Szymusiak & McGinty., 1986). Studies suggest specific administration of ethanol in the preoptic area increases NON-REM sleep (Ticho et al., 1992). Typically, a series of brain regions

communicate with each other in order to generate or inhibit REM; these are called REM ON and REM OFF respectively and they composed what is known as the REM flip-flop switch (McCarley & Hobson., 1975). For instance, REM sleep is produced by LDT/PPT neurons that activate the forebrain to induce muscle atonia (Datta et al., 2000; Pal et al., 2007). On the other hand, the neurons of the locus coeruleus and the Dorsal Raphe Nucleus usually inhibit REM-ON cholinergic systems (Khanday et al., 2016; Swift et al., 2018). Nowadays, it is known that GABAergic transmission plays a role in inducing the REM OFF switch to occur (McCarley et al., 2011). Thus, one can hypothesize that alcohol might inhibit REM sleep by inducing GABA to activate REM OFF neurons.

## 1.7. *Drosophila* as a Model for Neurological Disorders

*Drosophila melanogaster* has been used as a biological model to understand neuronal mechanisms that have intrigued neuroscientists over the years. As a matter of fact, scientists have been awarded with a total of 6 Nobel Prizes for their contributions in science using the fruit flies as their biological models. The first one of these Nobel prizes was awarded to Thomas Morgan, who won the prize by discovering that genes were located on chromosomes and that some genes are inherited together (Morgan., 1910). Nowadays, it is estimated that there is 75% homology of disease-causing genes between the fly and the human (Ugur et al., 2016). One of the most recently won Nobel prizes was awarded to Jeffrey Hall, Michael Rosebach and Michael Young back in 2017 for uncovering the molecular mechanisms we have discussed before relating to the core clock components of circadian rhythms.

One of the reasons why the fruit fly is such an appealing model in research is because it offers a myriad of genetic tools we can use in order to unravel neuronal circuits that affect behaviors. One of these tools is called the UAS-Gal4 system. This technique allows scientists to express genes in a tissue specific manner. In this system, two different transgenic flies are used; one that expresses the yeast transcription factor Gal4 linked to a tissue-specific enhancer and another that expresses a Gal4 responsive sequence (UAS) that has been tethered to a gene of interest (Brand et al., 1993; Brand et al., 1995). Once together, the Gal4 binds to the UAS sequence, allowing for the expression of the gene of interest only in the tissue that corresponds to the enhancer sequence. The *Drosophila* community has generated a collection of hundreds of Gal4 lines, each with a specific tissue specific promoter sequence (Jennet et al., 2012). Similarly, UAS-controlled overexpression and RNAi lines have been produced for almost every fly gene. Nowadays, the UAS-Gal4 system has successfully incorporated a temporal dimension to it as well which can be done so through various methods. For instance, gene expression in the UAS-Gal4 system can be induced via heat shock that can be applied overall to the fly or in the form of a laser microbeam or light (Cambridge et al., 1997; Halfon et al., 1997). Most recently, UAS-Gal4 system can be activated via the use of an estrogen receptor called RU-486 that can be applied to flies in their food (Scialo et al., 2016; Robles-Murgia et al., 2019).

Another technique that has made *Drosophila* research so interesting is the use of optogenetics, which is a non-invasive technique used to temporally activate or inactivate neurons with light (Boyden et al., 2005). For this, an algal protein that functions as a light gated cation channel called Channelrhodopsin-2 is used (Kim et al., 2017). In this

technique, Channelrhodopsin is exposed to a specific wavelength of light in order to depolarize the neuronal membrane of interest (Boyden et al., 2005). Much like the UAS-Gal4 system, the field of optogenetics have diversified greatly over the years, providing opsins that can now even inhibit neurons (Kim et al., 2017).

Finally, studies using electrophysiological methods have provided great insights into neurological conditions. In *Drosophila melanogaster*, this technique mainly focuses on electrical recordings of synaptic transmission using as a model the neuromuscular junction (NMJ), the connection between neurons and muscles (Jan & Jan., 1976). With the use of this technique, scientists have been able to unravel the foundations relating to how neurotransmitters are released and how postsynaptic receptors work (Fatt et al., 1961; Redman et al., 1990).

### 1.7.1. *Drosophila*'s role in Sleep Research.

*Drosophila melanogaster* has been a versatile model to study sleep, specifically because studies show that this invertebrate system possesses a sleep-like state. Indeed, it has been shown that in this species, sleep is characterized by a period of rest that lasts more than 5 minutes (Hendricks et al., 2000). This period of rest has been identified as sleep because it is a moment where the fly has a reduced response to sensory stimuli, has a preferred posture and location for its resting period and produces sleep rebound when it is rest-deprived (Hendricks et al., 2000). Although it has been stipulated that invertebrate systems lack the distinct brain patterns associated with REM and non-REM sleep, sleep in *Drosophila melanogaster* does cycle between light and deep sleep (van Swinderen et al, 2013).

Now that we have established that fruit flies possess a sleep-like state, we must ask ourselves if *Drosophila* sleep has other similarities with mammalian sleep. We have discussed earlier how mammalian sleep is regulated through two processes- Process S that focuses mainly on sleep rebound and the homeostatic mechanisms behind sleep and Process C that focuses on how circadian rhythms regulate sleep (Bobeley et al., 1982). For the first case scenario, studies support the notion that sleep rebound is present in *Drosophila melanogaster*. These studies state that sleep rebound in flies depends, in part, by the intensity and duration of sleep deprivation, same as in mammals, and that sleep rebound is conserved in a variety of wildtype fly lines (Huber et al., 2004). In addition, this study shows that sleep rebound has been shown to affect the fly's performance and overall vigilance (Huber et al., 2004). Moreover, evidence suggests that like in mammals, sleep rebound is independent of the circadian clock. In this study, after 24 hours of sleep deprivation, fruit flies have shown to possess sleep rebound, even after the circadian clock has been destroyed by mutations in the gene cycle period or clock (Shaw et al., 2000).

Now, we will focus on explaining studies concerning Process C- the process relating to circadian rhythms (Bobeley et al., 1982). We have talked about how the Nobel Prize was won last year by scientists who uncovered the molecular mechanisms of circadian rhythm. This prize was won by them through the discovery of the protein PER, the protein encoded by the gene *period*, that oscillates in a 24-hour cycle and effectively accumulates during the night but degrades during the day (Bargiello et al., 1984; Hardin et al., 1990; Liu et al., 1992). This protein interacts with other circadian

oscillators that these scientists discovered as well. The negative feedback loop that regulates circadian rhythms is as follows: during the night, proteins PER and TIM bind together, go into the nucleus and inhibit their own transcription by inactivating genes clock and cycle (Rosbash et al.,1989; Hardin et al., 1999). During the day, the CLOCK and CYCLE bind together in order to promote the transcription of TIM and PER (Reddy et al., 1984; Rosbash et al.,1989; Hardin et al., 1999; Buhr et al., 2015). Another protein, DBT, encoded by the gene *doubletime* reduces the accumulation of PER (Price et al., 1998). In addition, CRY is a protein that is activated by sunlight and binds to TIM, promoting the recruitment of JETLAG that serves as a ubiquitin ligase and degrades TIM (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996; Emery et al., 1998; Yang et al., 1998; Peschel et al., 2009). This in turn leads to the degradation of PER during the day.

In *Drosophila*, a conglomerate of 150 neurons help regulate circadian rhythms (Dubowy et al., 2017). These neurons are influenced by light and communicate with each other via neurotransmitters and neuropeptides (Rieger et al., 2003; Bachleitner et al., 2007; Yoshi et al., 2012). The sleep pattern of *Drosophila* is very specific. The fruit fly tends to sleep more during the night, but it also sleeps during the day (Helfrich-Forster et al., 2004; Stoleru et al., 2004). It has a period of activity just before the morning begins and the evening transitions that are named morning and evening peak, respectively (Helfrich-Forster et al., 2004; Stoleru et al., 2004; Bringmann, 2018). This behavior is controlled by specific neurons. Among them, lateral-ventral neurons, dorsal-lateral neurons and dorsal neurons play a major role (Artiushin et al, 2017; Schubert et

al., 2018). The Lateral Ventral neurons (LNvs) are involved in morning anticipation and, when activated, stimulate wakefulness (Grima et al., 2004; Stoleru et al., 2004; Riegel et al., 2016). The Dorsal Lateral neurons (LNds) are involved in evening anticipation, and when activated, increase sleep (Pittendrigh & Daan., 1976; Yao et al., 2014; Riegel et al., 2016). The two types of neurons communicate with the Dorsal neurons (DNs) that relay information to a variety of sleep and wake promoting brain structures.

Finally, like in mammals, sleep in *Drosophila* is also affected by stimulants and hypnotics. For instance, when given stimulants such as caffeine, modafinil or amphetamines, flies tend to stay awake (Hendricks et al., 2000; Shaw et al.,2000; Hendricks et al., 2003; Andretic et al., 2005) while antihistamines cause flies to fall asleep faster (Shaw et al., 2000). The next chapter will focus specifically on how alcohol affects sleep in *Drosophila melanogaster*.

### 1.7.2. *Drosophila's* role in Alcohol Research

*Drosophila melanogaster* possess key characteristics that make them a versatile and effective biological model to study alcohol responses. First, fruit flies can effectively consume and suffer from the intoxicating effects of alcohol as they encounter it in their natural environment in the form of rotten or fermented fruit (Dudley., 2002). Even larvae are at times exposed and can metabolize alcohol as female flies tend to prefer to ovulate near food that contains 5% ethanol concentrations (McKenzie & Parsons et al., 1972). We have already discussed the various advantages *Drosophila* has in terms of genetic tools, but interestingly enough, another aspect that makes the fly such a versatile model to study alcohol related gene responses is that there is approximately

75% of homology between the fly and the human genome when it comes to disease-causing genes (Berkerley *Drosophila* Genome Project., 1999; Fortini et al., 2000). Although flies possess a relatively small number- around 300,000- of neurons, they show a variety of complex behaviors that include learning and social behaviors (Greenspan & Ferveur et al., 2000; Chen et al., 2002). Moreover, it turns out that humans and flies show similar responses to ethanol in terms of sensitivity and tolerance. At relatively low quantities, alcohol functions as a stimulant while at higher ones it has sedative effects, the same way it does in humans (Herberlein et al., 2002). Tolerance is manifested in flies as well in all of its three stages: acute, rapid and chronic tolerance (Berger et al., 2004).

The response to alcohol in the fly is mediated, in part, by neurons and neurotransmitters that communicate with each other, as it is in mammals. Specifically, in *Drosophila*, alcohol is known to induce changes in neurotransmitter release to provoke distinct behavioral responses. For instance, in *Drosophila*, alcohol increases the release of DOPAMINE throughout the nervous system (Ojelade et al., 2019). This increase of DOPAMINE caused by in the ellipsoid bodies leads to a reduction in locomotor activity (Kong et al., 2010). In addition, alcohol induced increase in DOPAMINE levels in the mushroom bodies significantly alters learning and memory (Honjo & Furukubo-Tokunaga., 2009; Peru y Colon de Portugal et al., 2014; Cognigni et al., 2019). SEROTONIN also plays a role in alcohol responses and in the fly, it is involved with olfactory attraction to alcohol, reduced locomotion after drug exposure and mediate alcohol induced changes in sleep (Ellen & Mercer., 2012; Majeed et al., 2016; Ries et al., 2017; Liu et al., 2019). Finally, GABA plays a major role in mediating alcohol related

changes in learning and memory, sleep, locomotion and tolerance (Dzitoyeva et al., 2003; Lear et al., 2004; Liu et al., 2007; Agosto et al., 2008; Pitman et al., 2011).

Finally, as in humans, alcohol also affects different aspects of circadian rhythms in the fruit fly. For instance, alcohol sensitivity is mediated, in part, by circadian rhythms, with studies reporting that the greatest sensitivity to the drug occurs from mid to late night or the flies inactive phase (Van der Linde & Lyons., 2010). In addition, circadian genes mediate alcohol responses, specifically, in the case of tolerance. Studies show that flies with mutations in the circadian core clock genes- *per, tim* and *cyc* seem to lack the ability to gain tolerance (Pohl et al., 2013).

This proposal will focus on understanding the role lateral-ventral neurons have on alcohol-induced sleep behaviors. These neurons are known to have a wake promoting effect and mediate their response through the secretion of a neuropeptide called PDF or Pigment Dispersing Factor (Parisky et al, 2008). In flies, the PDF-releasing LNv neurons are located in a region called the accessory medulla and innervate the optic lobe and the dorsal proto-cerebellum (Yao et al, 2014). It resides in four to five lateral ventral neurons that have a unique role in regulating sleep. In mammals, PDF has a human homolog termed VIP (VasoIntestinal Peptide) and is found on the suprachiasmatic nucleus of the hypothalamus and is also involved with circadian rhythms (Colwell et al., 2003; Aton et al., 2005; Yao et al., 2014). In this thesis, in Chapter 2, we genetically manipulated LNvs to observe their overall effect in alcohol-induced sleep behaviors. Later on, in Chapter 3, we exposed flies to ethanol vapor in order to study alcohol-induced sleep behaviors. In Chapter 4, we genetically

manipulated LNvs in order to elucidate the role of these neurons in alcohol-induced sleep responses. The evidence that is described on Chapter 1-3 has been submitted for publication and is currently under revision. Lastly, we finished this thesis by looking at how PDF gene expression changes after ethanol exposure and how alcohol affects sleep related genes.

# Chapter 2: Effect of Lateral Ventral Neurons on Alcohol Sensitivity and Tolerance

## 2.1. Introduction

The addiction to alcohol is a neurobiological complex and long-term disease that is characterized by an uncontrollable and increasing urge to use alcohol, despite the detrimental consequences it brings (NIDA., 2018). Some scientists argue that addictive behaviors towards drugs originate, at least partially, through a series of drug-induced neuro-adaptive changes that effectively oppose the effects of the drug (Himmelsbach., 1964; Ritzmann & Tabakoff., 1976; Miller et al., 1987; Clapp et al., 2008; Kalivas & O'Brien., 2008). Two important terms that encompass these adaptations and that give rise to alcohol dependence are called sensitivity and tolerance (Miller et al., 1987; Di Chiara & North., 1991; Rosetti et al., 1992; Allan., 2020). On one hand, alcohol sensitivity is described as the organisms first response to the drug, while tolerance is the change in that response induced by a prior exposure (Norohna et al., 2014).

A number of studies in *Drosophila melanogaster* have uncovered that alcohol sensitivity and tolerance are influenced by neuronal activity and synaptic transmission (reviewed in Park et al., 2017). Specifically, the foundation for the mechanisms that affect neuronal synaptic transmission and alcohol responses are believed to reside in the modulation of transcription of a series of genes that encode ion channels, synaptic proteins and signaling molecules (Ghezzi et al., 2013). Moreover, many of these alcohol-responsive genes are also involved in the regulation of sleep or are well known

core circadian genes Ghezzi et al., 2013; Pohl et al., 2013). One interesting example is the BK type calcium activated potassium channel gene called *slowpoke* (Atkinson et al., 1991), whose function is to regulate neuronal activity in both flies and humans (Vergara et al., 1998). Evidence suggests that alcohol exposure increases the expression of *slowpoke* (Cowmeadow et al., 2006). Moreover, evidence points out that the upregulation of *slowpoke* gene expression induces flies to develop alcohol tolerance, possibly by potentiating neuronal excitability (Ghezzi et al., 2004). This increase in *slowpoke* expression still persists after alcohol clearance, leading to an increase in seizures, one of the characteristics of the withdrawal state of physiological alcohol dependence (Cowmeadow et al., 2006; Ghezzi et al., 2014). Even more so, BK channels also appear to be important in the regulation of circadian rhythms. In flies, a mutation of the *slowpoke* gene induces circadian arrhythmia (Ceriani et al., 2002). In addition, it has been seen that *slowpoke* in flies directly affects the synaptic release of a neuropeptide called Pigmented Dispersing Factor by small lateral ventral neurons (Fernandez et al., 2007).

In mammals, it has been found that BK channels are directly controlled by the intrinsic circadian clock located at the suprachiasmatic nucleus (Meredith et al., 2006; Kent et al., 2008). Indeed, it was found that mice that lacked BK channels have an increased firing rate at the suprachiasmatic during the night (Meredith et al., 2006). Interestingly, these mice showed normal BMAL1 gene expression, suggesting that BK channels might be important in regulating suprachiasmatic pacemaker output instead of modulating core clock gene expression (Meredith et al., 2006). Moreover, studies

performed in rats also highlight that BK channels directly modulate the development of acute ethanol tolerance by the expression of its B4 subunit (Martin et al., 2008).

A previous study exploring the neural substrates of alcohol tolerance in *Drosophila* has found sleep regulating structures that affect alcohol sensitivity directly through *slowpoke* gene expression (Ghezzi et al., 2013). In this study, *slowpoke* expression was enhanced in brain regions that regulate circadian rhythms such as the mushroom bodies, ellipsoid bodies and lateral ventral neurons, among others. Out of all these structures, a more robust change in alcohol resistance was found when the *slowpoke* gene was overexpressed in LNvs, a specific set of neurons that are involved in sleep regulation. For this reason, we decided to characterize the effect LNvs have in alcohol responses using *Drosophila melanogaster* as a biological model.

## 2.2. Methods

### 2.2.1. Fly Maintenance

Flies were maintained in a 25.9ºC, 80% relative humidity and a 12 hour light:12 hour dark cycle incubator. They were fed with a corn-based diet. When performing the ethanol tolerance assay and preparing the food for the monitor tubes, we mixed 50 ml of food with 10 ml of water. We used 3-5 year old female virgins for alcohol sensitivity and tolerance assays.

### 2.2.2. UAS-Gal4 system

For these experiments, flies were collected from a 2-day interval and used for experiments 3-5 days after collection. All fly strains were obtained from Bloomington Stock Center, unless otherwise specified. For one set of the experiments, we used *Pdf*$^{01}$, a 2nd chromosome mutant transgenic fly strain that lacked the PDF neuropeptide and was backcrossed for 6 generation into the CS background in order to decrease the effect of genetic background (Pohl et al., 2013). Dr. Nigel Atkinson from the University of Texas provided us with these flies. For the other experiments, we used the Pdf-GAL4 line (stock # 80939); the UAS-Kir2.1 line (stock # 6595); the UAS-TeTxLC line (stock # 28837); and the UAS-NaChBac line (stock # 9469). We crossed UAS-Kir2.1, UAS-TetxLC & UAS-NachBach male flies with female virgin pdf-gal4 flies to electrically manipulate the activity of pdf neurons. For the control lines for transgenic experiments, we crossed the Pdf-Gal4 driver line with the UAS-responder likes with the wild-type strain CS.

### 2.2.3. Alcohol exposure

We exposed flies to alcohol using a custom built alcohol delivery system **(Figure 3A).** This system is composed of two air pumps that were attached to two 30ml midget bubblers (Ace Glass inc.; Vineland, NJ) which contained 10 ml of water or 10 ml 95% ethanol. Each bubbler was in turn connected to airflow regulators so we could control the flow of air independently for each other. The bubblers were also connected to 15 ml or 50 ml conical tubes (depending on the number of flies being treated). **Figure 3** shows a representation of the alcohol delivery system.

### 2.2.4. Alcohol Sensitivity and Tolerance Experiments

We used a two day time period to measure alcohol sensitivity and tolerance. On the first day, we exposed one group of flies to alcohol (experimental group) and another to humidified air (control group). In total, we used 16 flies, 8 for the experimental group and another 8 for the control group, which we placed in 2- 15 ml conical tubes. Flies were exposed for five minutes to a stream of air vapor (1.5 LPM H20/ 0 LPM ETOH) so that they can acclimate to the chamber. Later on, they were exposed to ethanol vapor (1.5 LPM ETOH/0 LPM H20) until all flies lost postural control. For the control groups, flies were simultaneously exposed to water vapor (1.5 LPM H20/0 LPM ETOH). For the next day, all flies were exposed to ethanol vapor (1.5 LPM ETOH/0 LPM H20) on *Drosophila* Activity Monitors. Sedation and sedation recovery will be used as proxies for alcohol tolerance. The control group was simultaneously exposed to humidified air for the entire time period of the experiment (1.5 LPM H20/0 LPM ETOH). All alcohol exposures were performed at ZT-9 or approximately 3 hours before lights off.

On a second day, individual flies were placed in *Drosophila* Activity Monitors (DAM2); this allowed us to track the locomotor activity of flies as they passed through an infrared sensor **(Figure 3B).** In turn, the DAM system was connected to a gas manifold (MAN2) so that we could expose these flies to ethanol saturated air (Trikinetics; Waltham, MA). During this day, flies from both groups were exposed to ethanol vapor (1.5 LPM ETOH/0LPM H20) until all flies reached sedation. The activity of the flies was recorded using the DAM system every minute of the experiment. We used the time of the last movement of the fly recorded by the monitor in order to calculate the time the fly took to

sedate. We calculated the average sedation time for each group for our statistical analysis. We removed those outliers whose average deviated more than two standard deviations. Alcohol sensitivity was calculated by comparing the time of sedation in flies during the first exposure to ethanol **(Figure 3C).** On the other hand, tolerance to alcohol was determined by comparing the time of sedation between the control and alcohol group at the second exposure **(Figure 3C).** We used a Student's t test to calculate for the analysis of significance.

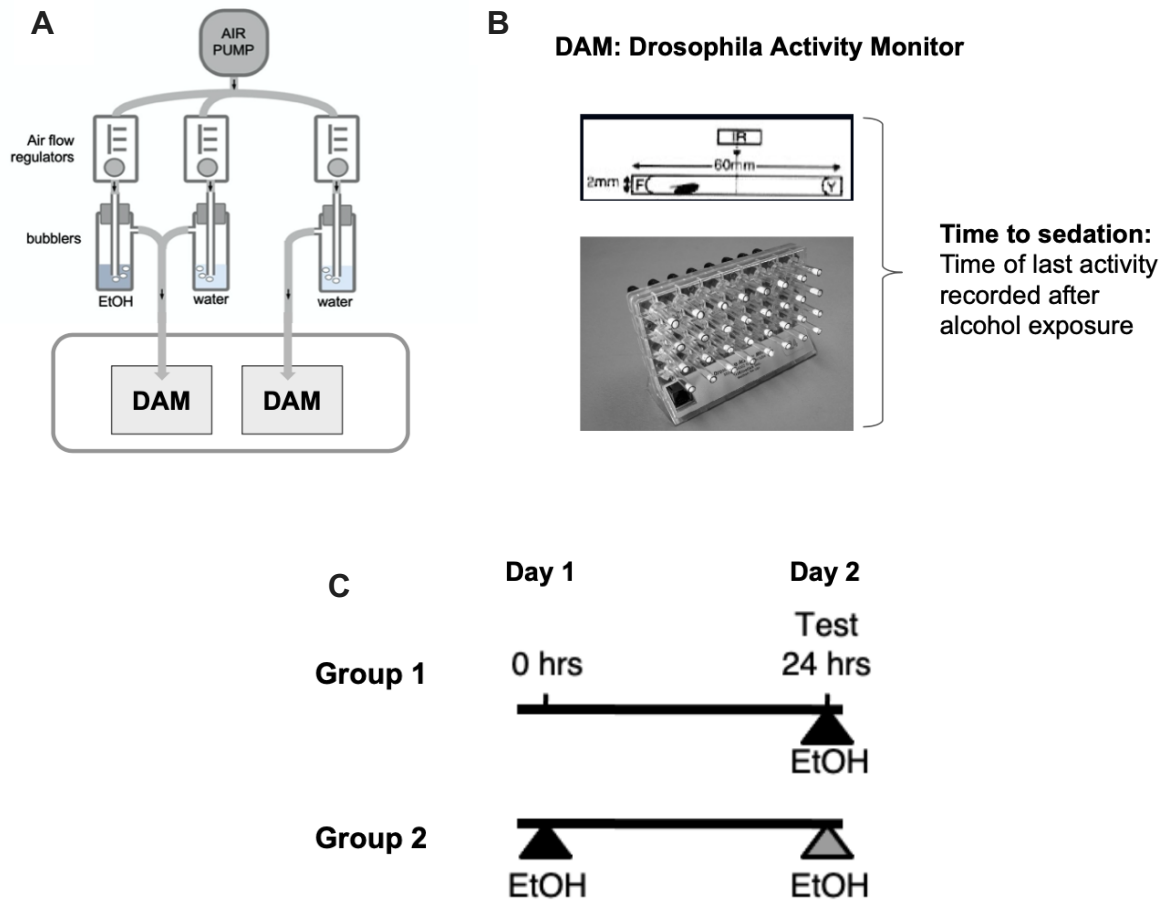


**Figure 3. Alcohol Sensitivity and Tolerance Assay.** A) The alcohol delivery system is composed of an air pump, air flow regulators that regulate the flow of air, bubblers that contain the liquid ethanol or water that are in turn connected to falcon tubes. B) For alcohol sensitivity assay, we exposed fies to ethanol vapor until all of them were sedated, the time to sedation was using the time of the last activity recorded after alcohol exposure using the DAM system. C) For alcohol tolerance assay, the flies were divided into two groups, one was exposed to air on Day 1 and again to ethanol on Day 2. The second group was exposed to ethanol on both day 1 and day 2. For alcohol tolerance, we compared the average time to sedation of the second and the first groups.

## 2.3. Results

### 2.3.1. PDF neurons affect sensitivity and tolerance

We began our experiments by studying the effect LNvs neurons- and most particularly PDF release- have on alcohol sensitivity and tolerance. To measure these two alcohol responses, we used a 2-day alcohol exposure assay system. On day 1, each genotype was divided into the experimental and a control group. The experimental group will be the one exposed to alcohol vapor while the control group will be exposed to humidified air. On day 2, both the experimental and control groups will be exposed to ethanol vapor simultaneously as we monitored the time the flies took to sedation independently for each group using the *Drosophila* Activity Monitor. In essence, the flies that belong to the control group received their first dose of ethanol on day 2 while the flies that belong to the experimental group received their second dose. Alcohol sensitivity between genotypes was quantified by comparing the time it took for flies to sedate after their first ethanol exposure, while tolerance was calculated by comparing time to sedation between the first and second alcohol exposure.

For our first set of experiments, we used female wild-type CS flies and a $Pdf^{01}$ fly line that possesses a null mutant gene that codes for the PDF neuropeptide **(Figure 4).** Scientific evidence points out that PDF release by LNvs neurons is important for the regulation of sleep in flies (Park & Hall., 1998). This mutant has been genetically backcrossed for 6 generations to the CS background, an effort made in order to reduce

the effects of genetic background (Pohl et al., 2013). Specifically, we saw that while CS flies took approximately 20 minutes to knock-down during their first exposure to ethanol, *Pdf*$^{01}$ flies took around 40 minutes. A student's t-test reveals that the difference was, indeed, significant, suggesting that *Pdf*$^{01}$ mutants showed less sensitivity to the effects of the drugs. Taking this evidence into consideration, we wanted to see how neuronal activity of lateral ventral neurons affected alcohol sensitivity.

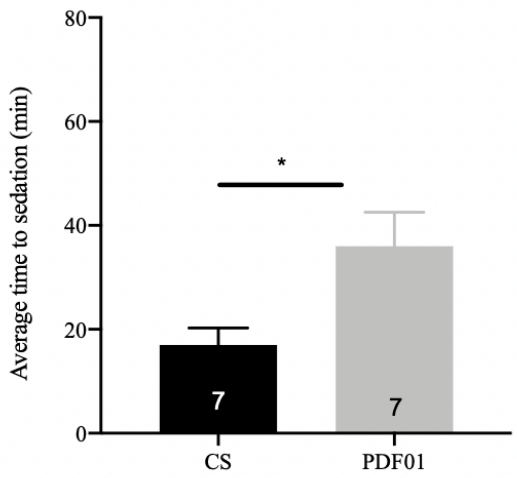


**Figure 4. Alcohol sensitivity is affected in flies that possess a mutation in the PDF neuropeptide gene.** A null mutation in the *Pdf* gene (*Pdf* $^{01}$) increases the average time to sedation after the 1st exposure to alcohol and blocks the capacity to acquire alcohol tolerance (increased time sedation upon a second exposure). The average time to sedation upon first or second sedation to alcohol is shown for wild-type Canton-S (CS) control flies and the *Pdf* $^{01}$ mutants.

We used the versatile UAS-Gal4 technique in order to study more in detail the effect LNvs neuronal activity have on alcohol responses such as sensitivity and tolerance. With this technique, we genetically change the neuronal activity of LNvs using a transgenic construct containing the yeast transcription factor Gal4 (in this case Pdf-GAL4) that binded to a Gal4-responsive sequence (UAS) that was tethered to a

gene of interest. Because the Gal4 and UAS constructs were originally present in different flies, the system only became activated in the progeny of a cross between the two. Specifically, we chose three proteins; two were used to silence neuronal activity of PDF cells and one was used to potentiate it. The first approach consisted of overexpressing tetanus toxin light chain (TeTxLC) protease in LNvs. The TeTxLC is a drug that inhibits vesicles from fusing with the neuronal membrane and releasing its neurotransmitters, specifically by interfering with synaptobrevin, syntaxin or SNAP-25 (Sweeney et al., 1995; Ding et al., 2019). The second approach focused on overexpressing the potassium channel Kir2.1 to electrically suppress neuronal excitability. It has been shown that in both mammals and *Drosophila*, Kir2.1 has the capacity to diminish the activity of excited neurons (Johns et al., 1999; Baines et al., 2001; White et al., 2001). The last approach consisted of overexpressing sodium channel NaCHBac on PDF neurons. This sodium channel has been shown to enhance neuronal activity and excite neurons in *Drosophila* melanogaster (Luan et al., 2006; Nitabach et al., 2006; Sheeba et al., 2008).

When we inactivated PDF neurons using TeTxLC, we saw that the alcohol sensitivity remained unaffected when compared to the controls **(Figure 5A).** However, it is important to note that our controls displayed variability when it came to alcohol sensitivity. Thus, we decided to use a second method of inactivating PDF neurons using potassium channel Kir2 **(Figure 5B).** Here, we observed that there was no change in alcohol sensitivity when inactivating PDF neurons using this potassium channel. We also activated PDF neurons using NaChBac **(Figure 5C).** Here, we observed that flies who had PDF neurons activated by NaChBac were more sensitive than controls.

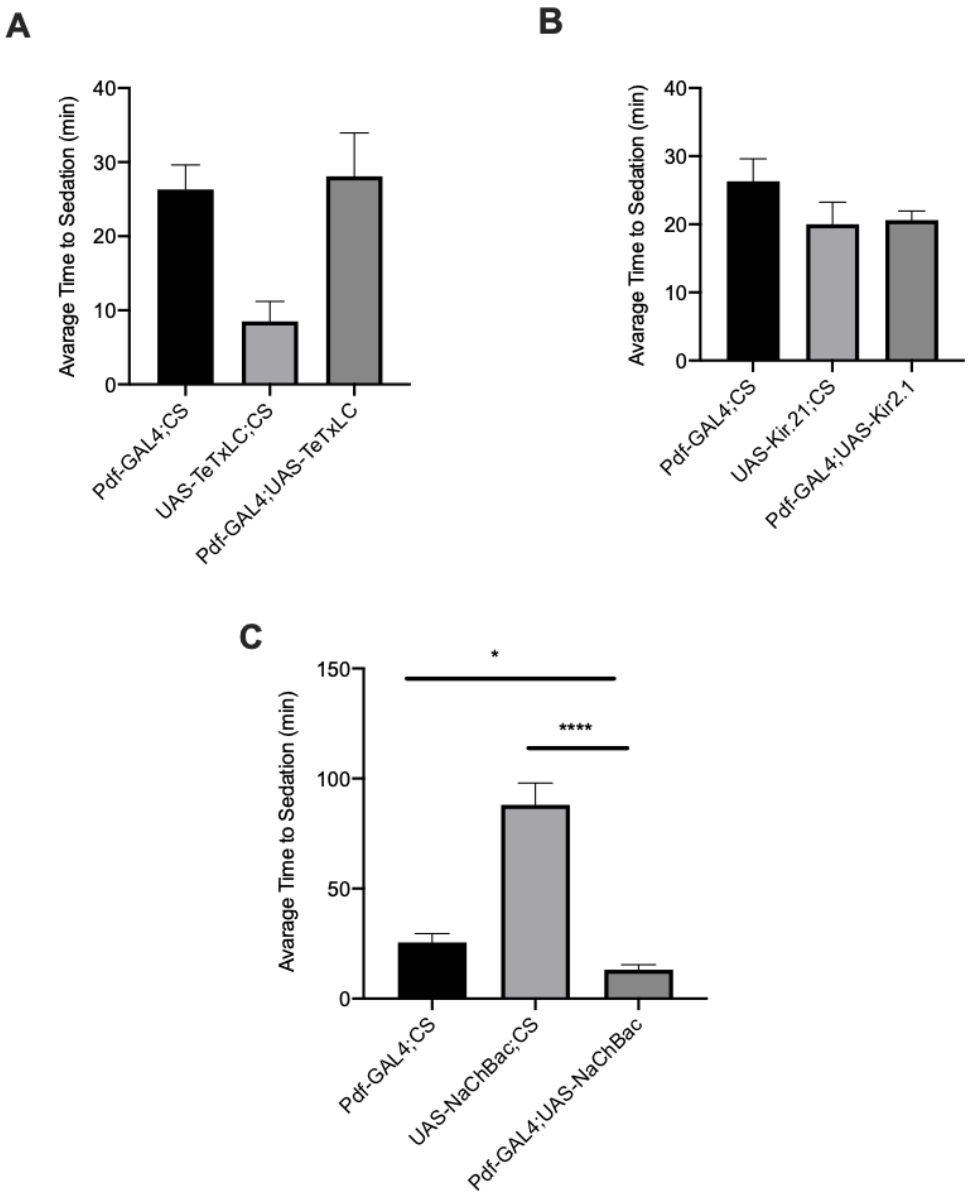


**Figure 5. Effect of inactivating PDF neurons in alcohol sensitivity/resistance** When we inactivated PDF neurons using TetxLC (A) or Kir2.1 (B) there was no change in alcohol sensitivity/resistance. When we activated PDF neurons using NaChBac (C), there was a decrease in resistance.

When we looked at alcohol tolerance- by comparing the time to sedation of the first exposure with that of the second- all genotypes developed the ability to gain tolerance except for the mutant *Pdf*$^{01}$ flies and the transgenic flies where we used TeTxLC to inhibit synaptic transmission of LNvs **(Figure 6).** This includes the group in which we electrically activate LNvs using sodium channel NaChBac. Together, these data point out that synaptic neuropeptide release of PDF, rather than the electrical activity of LNVs, is important in the development of alcohol tolerance.

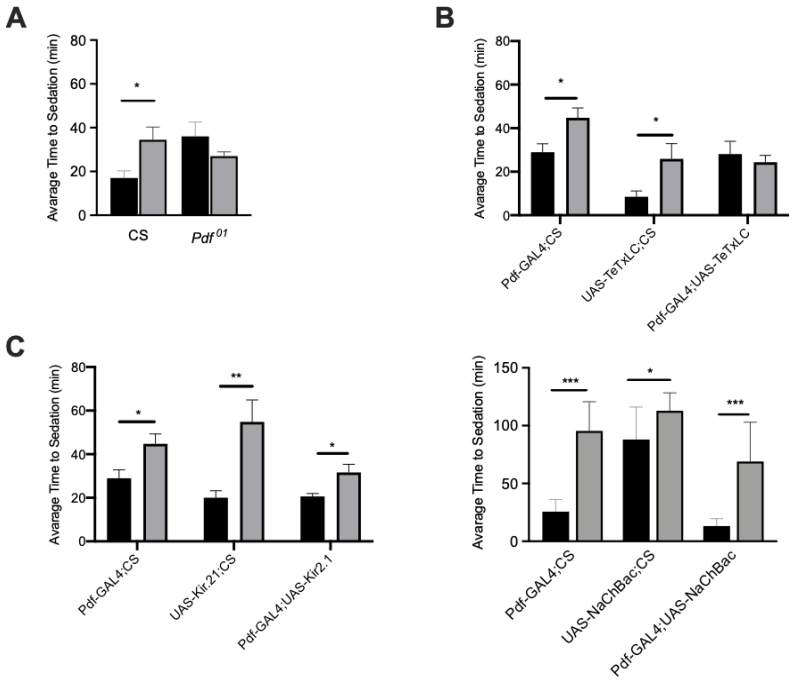


**Figure 6. Alcohol tolerance is dependent on the pigment dispersing factor (PDF).** Inhibition of PDF-expressing neurons by expression of the tetanus toxin light chain (TeTxLC) (A) protease or overexpression of a heterologous inward rectifying potassium channel (Kir2.1) (B) differentially affects the capacity to acquire alcohol tolerance (increased time sedation upon a second exposure). The average time to sedation upon first or second sedation to alcohol is shown for parental controls flies (Pdf-GAL4/CS, UAS-kir2.1/CS, UAS-TeTxLC/CS) and the genetically inhibited lines (Pdf-GAL4/UAS-Kir2.1 and Pdf-GAL4/UAS-TeTxLC). C) Activation of PDF expressing neurons by expression of the heterologous sodium channel construct NaChBac augments the capacity to acquire alcohol tolerance (increased time sedation upon a second exposure). The average time to sedation upon first or second sedation to alcohol is shown for parental controls flies (Pdf-GAL4/CS, UAS-NaChBac/CS) and the genetically activated lines (Pdf-GAL4/UAS-NaChBac). Error bars represent SEM. Statistically significant differences determined by Student's t-test: * denotes $P < 0.05$; * denotes $P < 0.01$; *** denotes $P < 0.001$.

## 2.4. Discussion

Alcohol is known as a suppressor of neuronal activity and has the ability to set off a series of homeostatic neuroadaptations that arise in order to counteract the effect of the drug (Pohorecky et al., 1977; Haes et al., 2010). It is known that these neuroadaptations enhance the development of alcohol tolerance and dependency, as well as affect various neuronal processes (Littleton., 1998; Koob & Le Moal, 2001; Ghezzi & Atkinson., 2011). Sleep has proven to be a biological process that is extremely sensitive to both internal and external factors and can be modulated to potentiate the adaptability of the organism to its environment, overall enhancing it chance of survival (Okamoto-Mizuno & Mizuno., 2012; Allada et al., 2017). Thus, it is of no surprise that these two processes might interact with each other. We used a set of techniques to genetically manipulate the neuronal activity of a specific set of neurons that regulate sleep and arousal states and that have also been shown to be involved in alcohol induced homeostatic responses.

The fruit fly, *Drosophila melanogaster*, is composed of approximately 150 neurons that regulate circadian rhythms through the expression of the molecular core components (Kaneko et al., 2000; Forster et al., 2007; Schubert et al., 2018; King & Sehgal., 2020). They have a similar function to what it is known as the suprachiasmatic nucleus (SCN). These neurons possess different functional roles, express a variety of neurotransmitter, neuropeptide and receptors and are located throughout the *Drosophila* brain (Reviewed in Dubowy & Sehgal., 2017). The *Drosophila* Ventral Lateral Neurons are one of the cells that regulate circadian rhythms, are located at the

accessory medulla and innervate the optic lobe and proto-cerebellum (Shafer & Yao., 2014). They are also known to be composed of four to five cells- four which release the neuropeptide PDF and functions to control various characteristics of circadian rhythms and sleep (Parisky et al., 2008; Sheeba et al., 2008; Chung et al., 2009; Parisky et al., 2008). Specifically, PDF neuropeptide is a signaling molecule that modulates behavioral activity by synchronizing clock cells with one another. VIP (Vaso-Intestinal Peptide) is the mammalian homolog of PDF (Shafer & Yao., 2014), it is located at the suprachiasmatic nucleus and also has a major role in regulating circadian rhythms (Todd et al., 2020).

In our first set of experiments, we wanted to see how LNvs, through PDF release, mediated alcohol sensitivity/resistance **(Figure 7A).** We found alterations in baseline sensitivity to alcohol in a null mutant fly line called $Pdf^{01}$ when compared to wild-type CS flies **(Figure 7B).** Specifically, $Pdf^{01}$ mutants had less sensitivity and enhanced resistance to alcohol when compared to wild-type CS flies. When we electrically silenced LNvs using Kir 2.1, there was no effect in resistance. **(Figure 7B)** It is important to state that Kir2.1 has been shown to have somal release of PDF, therefore in theory, these flies did not lose the capacity to release this neuropeptide (Klose et al., 2021). When we electrically activated LNvs using PDF, there was a decrease in resistance **(Figure 7B)**. Thus, we think it is through PDF release, rather than the electrical activity of the neuron, that alcohol is increasing resistance **(Figure 7C).**

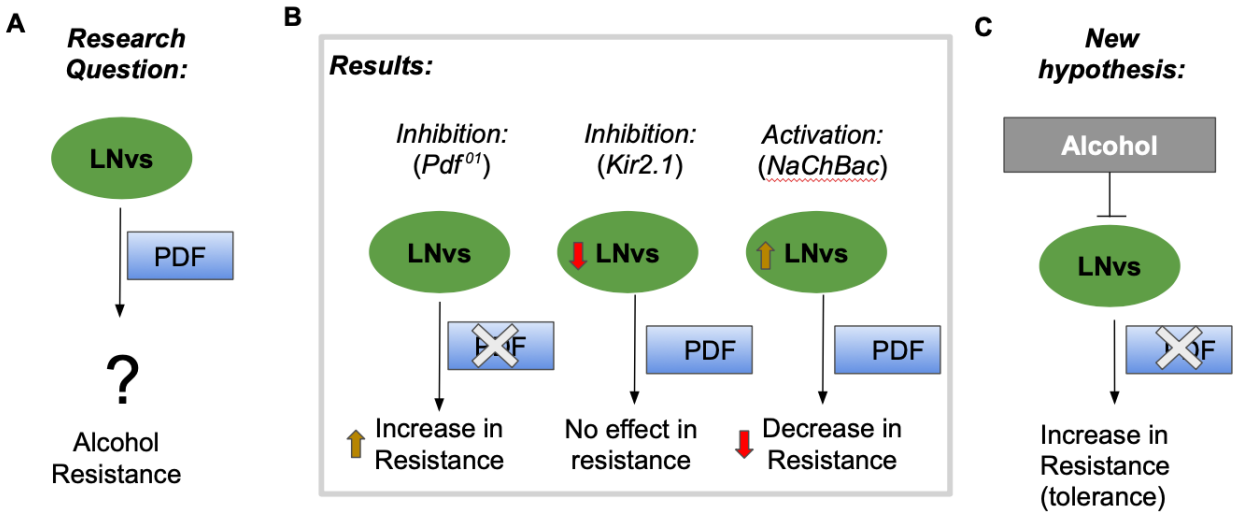


**Figure 7. Alcohol resistance is mediated by PDF release** A) We wanted to understand how LNvs, through PDF release, affected alcohol resistance. B) When we blocked the release of PDF, we saw that there was an increase in resistance. When we electrically inactivated LNvs using Kir2.1 we saw that this produced no effect in resistance. When we electrically activated LNvs, we saw a decrease in resistance. C) We think that it is through PDF release that alcohol affects resistance, thus we hypothesize that PDF release will affect tolerance.

With these sets of experiments in mind, we decided to look at how PDF release is affecting tolerance **(Figure 8A).** First, we saw that when we inactivated PDF release by using TeTxLC and *Pdf*$^{01}$ mutants, flies did not possess the capacity to develop alcohol tolerance **(Figure 8B).** When we electrically silence and/or activate LNvs using Kir2.1 and NaChBac, flies have the ability to develop tolerance **(Figure 8C).** Thus, we think that alcohol is creating tolerance through the release of PDF rather than the electrical activity of the neuron.

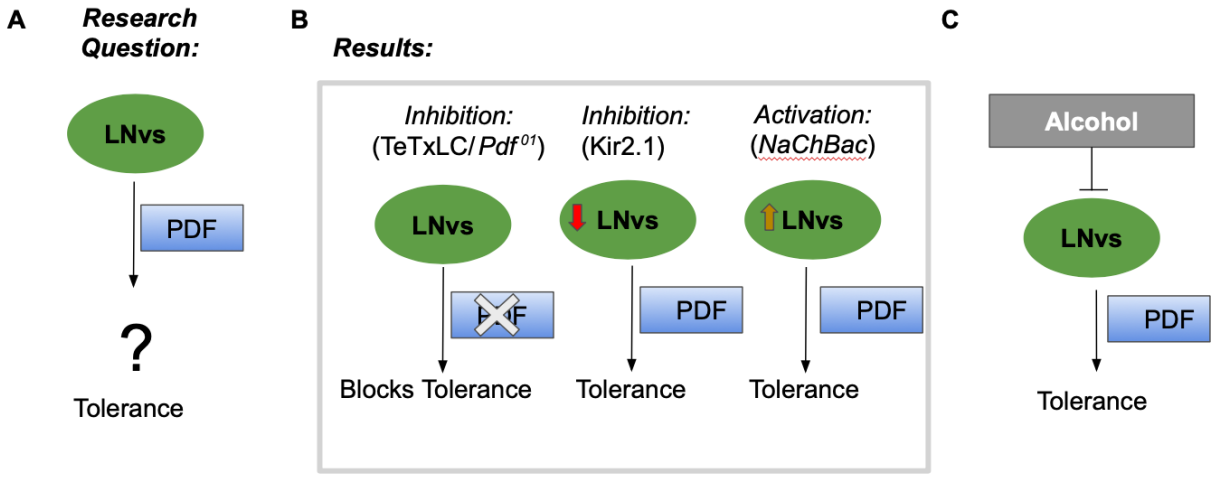


**Figure 8. Alcohol tolerance is mediated by PDF release** A) We wanted to understand how LNvs, through PDF release, affected alcohol tolerance. B) When we blocked the release of PDF, we saw that flies lacked the ability to develop tolerance. When we electrically inactivated and activated LNvs using Kir2.1 and NaChBac, we saw that flies developed tolerance. C) We think that it is through PDF release that alcohol affects tolerance, rather than the electrical activity of the neuron

Curiously, we obtained different results concerning alcohol tolerance when we inhibit neuronal activity of LNvs using the potassium channel Kir2.1 and inhibit synaptic fusion using TeTxLC. We saw that flies maintained the ability to develop alcohol tolerance when we silence neuronal activity of LNvs using Kir2.1. Nevertheless, we saw that flies lacked the ability to develop tolerance when we used TeTxLC. Although these two results contradict each other, they are not surprising. As we have explained, these two methods of neuronal silencing work through two different mechanisms; one where we block synaptic transmission using TeTxLC (Sweeney et al., 1995) and, therefore, neuropeptide release (Ding et al., 2019) and another when we electrically reduce neuronal excitability using Kir 2.1 (Baines et al., 2001). Previous studies suggests that using TeTxLC to inhibit synaptic transmission of LNv influences modestly locomotor rhythmicity, but severely affects morning anticipation in a way similar to *Pdf*$^{01}$ flies

(Jaumouillé et al., 2021; Kaneko et al., 2000). On the other hand, evidence suggests that reducing neuronal activity via Kir2.1 in LNvs produces both behavioral and molecular arrhythmicity (Nitabach et al., 2002; Nitabach et al., 2005). Finally, we also enhance electrical activity of LNvs using sodium NaChBac. Although previous experiments point out that this genetic manipulation significantly alters rhythmicity (Harrisingh et al., 2016), this group of flies maintained their ability to develop tolerance.

It is important to note that most of these manipulations could be affecting various biological processes that could be interfering with our results (Hodge et al., 2009). In addition, these manipulations could produce a series of homeostatic side-effects where a decrease in one channel can be countered by an increase of another (Hodge et al., 2009). For these reasons, for future investigations, scientists could attempt to temporally alter the neuronal activity of the neuron using techniques such as gene-switch. Another interesting aspect scientists could study would be to physically observe through a confocal microscope or electrophysiological studies if alcohol is preventing PDF release. All together though, these results encourage the notion that alcohol responses could be chemically dependent on PDF release by LNvs rather than by interfering with electrical signals.

# Chapter 3: Effect of alcohol on sleep in *Drosophila melanogaster*

## 3.1. Introduction

In humans, studies point out that alcohol's effect on sleep is, in part, dependent on the dose or whether the person has consumed alcohol in an acute or chronic manner. For instance, when taken at high and acute doses, alcohol works as a sedative, shortening sleep latency or the time it takes to fall asleep (Yules et al.,1967; Williams et al., 1983; Roers et al., 1999; Van Reen et al., 2006; Feige et al., 2006; Chan et al., 2013; Colrain et al., 2014). However, as alcohol leaves the system, sleep becomes highly fragmented, reducing total sleep overall (Bixler et al., 1979). On the other hand, as the brain creates counteractive measures to mitigate the effect of the drug at a chronic dose and at the withdrawal stage, alcohol has the opposite effect as it ends up increasing sleep latency but diminishing total sleep (Mello & Mendelson., 1970; Baekeland et al., 1974; Foster et al., 2000; Britton et al., 2020).

In addition, alcohol has been found to affect circadian rhythms as well. In mammals such as rats, it was observed that alcohol increases *clock* and *per2* gene expression in different parts of the organism, including the nervous system (Guo et al., 2016; Filiano et al., 2016). We have observed how temperature and biochemical substances are modulated through the action of circadian rhythms. It turns out that chronic alcohol exposure leads to alterations in body temperature, locomotor activity and biochemical rhythms in glucose, potassium, lactic acid and even cholesterol (Rajakrishnan et al., 1999; Guo et al., 2016). In addition, not only does alcohol

consumption affect circadian rhythms, but circadian rhythms can significantly influence alcohol use. In humans, it has been found that disruption in circadian rhythms and sleep during adolescence increases the risk for consuming alcohol (De Nobrega et al., 2017; Hasler et al., 2013).

Previous studies have used the fruit fly, *Drosophila melanogaster*, as a biological model to unravel the neuronal and molecular mechanisms behind both alcohol dependence and sleep. Studies suggest that the sleep in fruit flies have the basic characteristics of sleep in mammals which are: sustained period of quiescence, increased arousal threshold and homeostatic rebound seen after the fly has been deprived of sleep (Hendricks et al., 2000; Shaw et al., 2000). As the fly has a myriad of genetic tools we can use to manipulate the activity of genes, we decided to investigate the relationship alcohol has on sleep using this model. Our central hypothesis is that the mechanisms of alcohol neuroadaptation that mediate sensitivity and tolerance are responsible for the physiological disruption of sleep.

Studies concerning *Drosophila melanogaster* and the relationship alcohol has on sleep has been performed over the years. For instance, it has been reported that alcohol sensitivity is regulated by the circadian clock and changes according to the time of day of ethanol administration as studies show that the greatest sensitivity to alcohol occurs during the mid-to late subjective night, which corresponds to the inactive phase of the fly (Van der Linde., 2011; De Nobrega et al., 2016). In addition, it has been reported that circadian core clock gene mutants have different responses to alcohol in

terms of sensitivity and tolerance. For example, flies with mutations in genes *per*, *tim* and *cyc* significantly increase alcohol sensitivity but have no effect on tolerance (Pohl et al., 2013; De Nobrega et al., 2016).

Considering all the aforementioned reasons, we decided to investigate the effect alcohol has on sleep, using *Drosophila melanogaster* as a biological model. In these studies, we exposed flies to a single stream of ethanol vapor and observed their overall sleep pattern for five days. Our results suggest that alcohol influences sleep and we think this is due through mechanisms underlying stress. We hope to continue our research focusing on the role LNvs play in alcohol induced sleep behaviors.

## 3.2. Methods

### 3.2.1. Fly Maintenance

Flies were maintained in a 25.9 ºC, 80% relative humidity and a 12 hour light: 12 hour dark cycle incubator. They were fed with a corn-based diet. When performing the ethanol tolerance assay and preparing the food for the monitor tubes, we mixed 50 ml of food with 10 ml of water. We used 3-5 year old female virgins for alcohol-sleep assay. CS wild-type flies were obtained from the *Drosophila* Bloomington Center

### 3.2.2. Alcohol exposure

We exposed flies to alcohol using a custom built alcohol delivery system **(Figure 7).** This system is composed of two air pumps that were attached to two 30ml midget bubblers (Ace Glass inc.; Vineland, NJ) which contained 10 ml of water or 10 ml 95%

ethanol. Each bubbler was in turn connected to airflow regulators so we could control the flow of air independently for each other. The bubblers were also connected to 15 ml or 50 ml conical tubes (depending on the number of flies being treated).

### 3.2.3. Alcohol-Sleep Analysis

We separated female virgin flies into two groups- one that was exposed to alcohol-saturated air and another to humidified air. **Figure 9** describes the methodology used for our alcohol-exposure experiment. We used the same ethanol chamber described in Chapter 2, only that this time the chamber was connected with 2 perforated 15 ml conical tubes where we placed around 32 flies for each group. For the experimental group, flies were given a 5 minute exposure to humidified air (1.5LPM H20/0 LPM ETOH) so that they could acclimate to the chamber. Later on, they were given alcohol until all flies were knocked down (1.5LPM ETOH/1.5LPM H20) **(Figure 9A).** Afterwards, flies were given humidified air (1.5LPM H20/0 LPM ETOH) for about 10% of the time the flies took to knock down after ethanol exposure. The control group was simultaneously exposed to humidified air (1.5LPM H20/0 LPM ETOH) for the entire experiment using an identical experimental set up, but with no alcohol **(Figure 9B).** After flies were treated, we transferred them to 65 mm X 5 mm glass tubes that contained fly food (Trikinetics, Waltham, MA) containing fly food. Afterwards, we place the tubes in *Drosophila* Activity Monitors (DAM2) with an incubator set to the parameters explained above. We used the Trikinetics monitoring system (Waltham, MA) for a period of 5-9 days to record locomotor activity in 1 min bins (Agosto et al., 2008) **(Figure 9C).** On the other hand, sleep was identified as a period of uninterrupted

inactivity that lasted for 5 minutes. We used MATLAB software (Natick, MA) to calculate daily sleep patterns as described in (Parisky et al., 2008). We calculated sleep parameters such as total sleep duration, sleep latency, number of sleep episodes, mean sleep episode duration, and total locomotor activity for each 12 hr period of the light/dark protocol per day. Two-way Anova was used to calculate levels of significance. We used Rethomics framework in R (Geissmann et al., 2019) to analyze morning and evening anticipation peaks and night sleep ratios as described in Harrisingh et al. (2007). We divided the sum of activity for each fly between ZT21 and ZT24 by the sum of activities between ZT18-24 for each day in order to calculate the morning anticipation index. On the other hand, we divided the sum of activity for each fly between ZT9 and ZT12 by the sum of activities between ZT6-12 for each day in order to determine evening anticipation. Finally, we used the sum of total sleep for each fly between ZT18 and ZT24 divided by the sum of activities between ZT12-18 for each day analyzed in order to calculate night sleep ratios. Two-way Anova-mixed effects analysis were used to calculate statistically significance levels between alcohol and control groups per day.

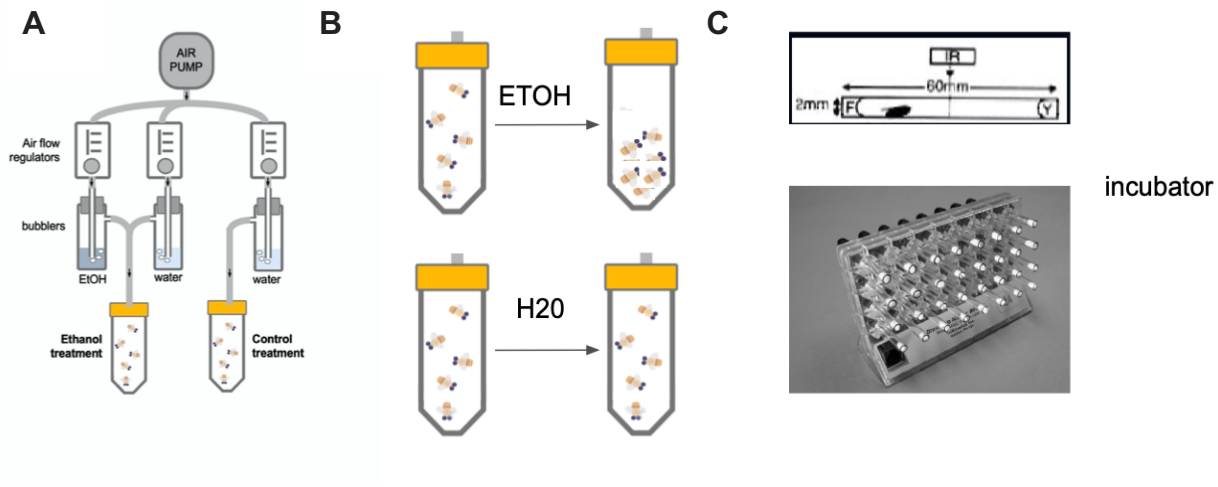


**Figure 9. Schematics of Alcohol-Sleep Experiments.** A) Flies are placed in falcon tubes connected with the Ethanol Chamber that turns liquid ethanol into air. B) All flies are exposed to ethanol until they are knocked down. The control group is simultaneously exposed to water. C) Afterwards, in order to measure locomotor activity of the fly as it passess through an infrared sensor, they are placed in the *Drosophila* Activity Monitor system and an incubator for a period of 9 days.

## 3.3. Results

### 3.3.1. Alcohol promotes daytime sleep in wild-type flies

Keeping in mind the aforementioned studies relating to LNvs and alcohol sensitivity and tolerance, we decided to investigate the effect of alcohol on sleep focusing on lateral ventral neurons. However, first, we needed to characterize the effect alcohol had on sleep itself. For these experiments, we administered a single dose of ethanol vapor to flies and analyzed their overall sleep profile for a period of nine consecutive days using the *Drosophila* Activity Monitors (DAM2). In *Drosophila*, sleep is defined as a prolonged period of rest that lasts for at least five minutes (Hendricks et al., 2000; Shaw et al., 2000). One of the characteristics that are displayed in this period is that flies possess an increase in arousal threshold when using mild mechanical

stimulation (Hendricks et al., 2000; Shaw et al., 2000). In addition, during this period flies choose to stay in rest in a preferred location and possess a specific sleep posture (Hendricks et al., 2000; Shaw et al., 2000). If flies are deprived of these periods of quiescence, they will display symptoms of sleep rebound, a phenomenon observed when the organism sleeps more in order to compensate for lack of sleep (Hendricks et al., 2000; Shaw et al., 2000). In **Figure 10A,** we can observe the percentage of time sleeping wild-type CS flies possessed during a 12 hour light and a 12 hour dark period for nine consecutive days after ethanol exposure. The black line represents untreated controls while the blue lines represent ethanol exposed flies. It can be seen that during the first two days, alcohol exposure leads to a significant increase in daytime sleep. Nevertheless, sleep decreases to normal levels as the days post experiment pass by. At night, both alcohol and untreated flies seem to be sleeping, on average, for about the same amount of time.

In order to understand more in detail the effects alcohol has on overall sleep architecture, we sought out to quantify different sleep parameters as well. Overall, we analyzed the following sleep parameters: (i) the total sleep, which is defined as the total amount of sleep flies experienced during day or night time; (ii) the sleep latency, which is defined as the time it takes for the flies to fall asleep after the transitions of lights-on or lights off; (iii) the number of sleep episodes, which is determined by the number of sleep intervals flies have during a specific time period and (iv) the mean sleep episode, calculated by adding the amount of time flies spent sleeping in each sleep episode divided by the number of sleep episodes themselves.

This quantitative analysis reveals that: (i) total sleep significantly increases during the first two days after ethanol exposure during day-time **(Figure 10B).** (ii) The sleep latency during the day significantly decreases in ethanol exposed flies compared to control on day 1 but did not change during the rest of the days. No differences in sleep latency were observed during the night-time **(Figure 10C).** (ii) the number of sleep episodes significantly increases during the first three days after ethanol exposure. No significant differences were observed in the number of sleep episodes at night **(Figure 10D).** (iv) the mean sleep episode duration increases during the day-time on various days post ethanol exposure. No significant difference in mean sleep episode duration was observed at night (**Figure 10E).** This decrease in the number of sleep episodes and increase in the mean sleep episode duration suggests that alcohol exposed flies experience a more consolidated sleep pattern than untreated controls.

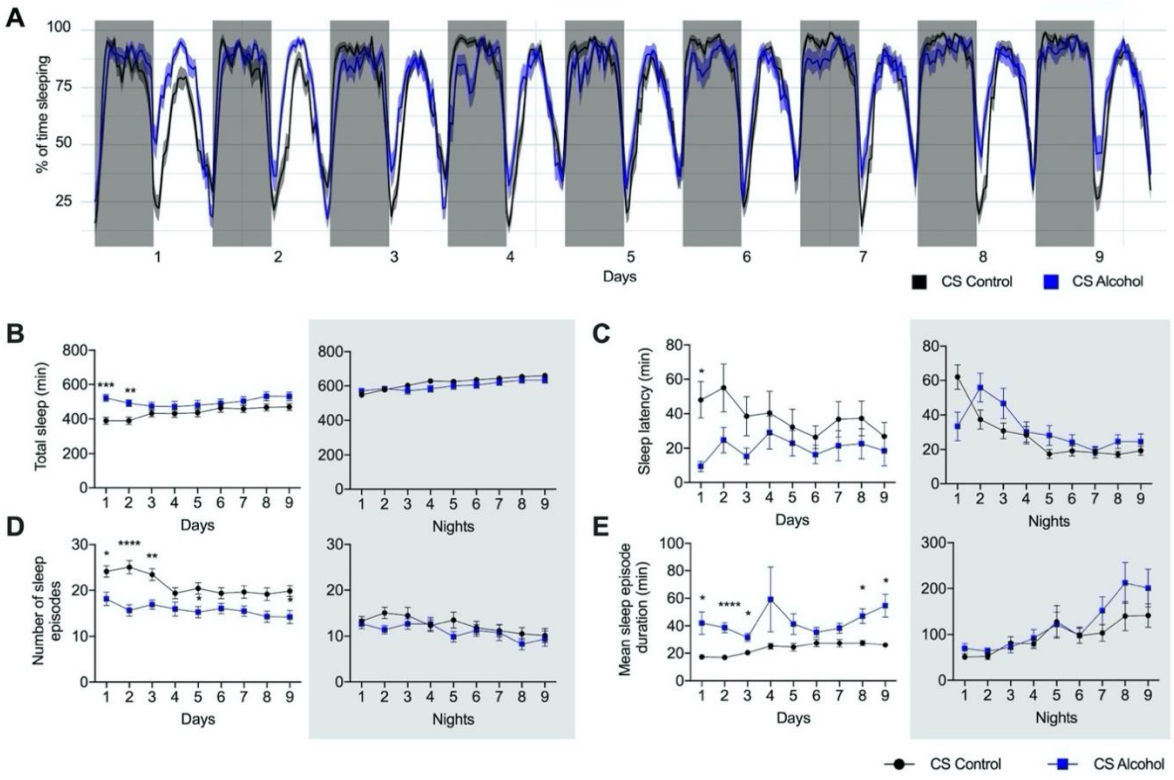


**Figure 10. A single alcohol exposure alters the activity pattern and sleep architecture of wild-type flies.** A) The percentage of time sleeping (in 30-minute bins) of control (black line) and alcohol-exposed (blue line) of wild-type flies is displayed over a period of 9 consecutive days after alcohol exposure. C-E) A diverse set of sleep parameters is displayed for every daytime period (left) and night-time period (right) after alcohol exposure. Sleep parameters displayed are as follows: B) total time sleeping, C) sleep latency, D) number of sleep episodes, and E) mean sleep episode duration. Averages per day are displayed for alcohol-treated flies (blue) and untreated controls (black). Error bars are SEM. Statistically significant differences between alcohol and control groups per day were determined by two-way Analysis of Variance (ANOVA): * denotes P < 0.05; ** denotes P < 0.01; *** denotes P

### 3.3.2. Alcohol exposure alters night sleep architecture

Although no significant ethanol induced sleep patterns were observed in terms of total sleep during the night period, closer inspection of night sleep reveals that alcohol administration does lead to a series of changes in sleep when it comes to its structure. Interestingly, we saw that the sleep of wild-type untreated control flies was consistent throughout the night while alcohol exposed flies slept significantly less during the first half of the night and more during the second half. In addition, we also observed that alcohol-exposed flies slept more than untreated wildtype controls in the last six hour of the night, which caused them to wake up approximately 3 hour before the lights-on transition. This difference in sleep was observed consistently over the nine days after flies had been administered alcohol **(Figure 11A).** We sought to quantify this effect by calculating the night sleep ratio or the ratio of sleep during the first 6 hours of night divided by the sleep in the last 6 hours of the night **(Figure 11B).** We saw that alcohol treated flies had an increased ratio in night sleep than untreated controls for over 8 days after ethanol exposure.

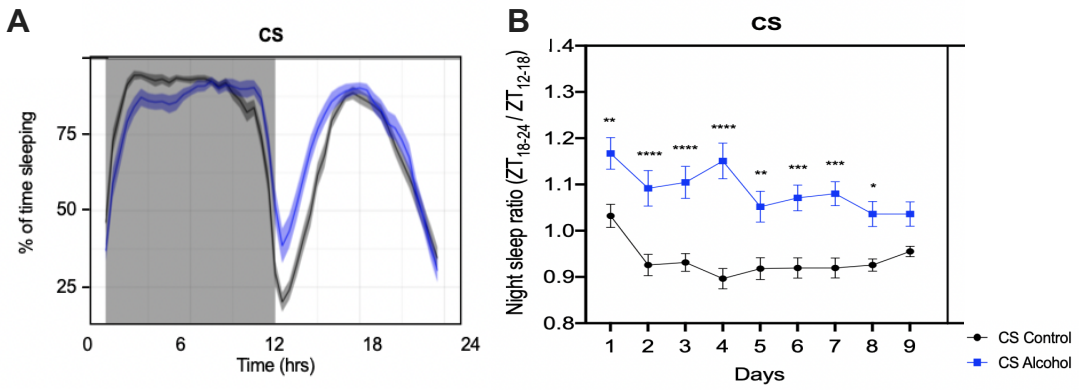


**Figure 11. Alcohol exposure alters night sleep architecture.** A) The average percentage of time sleeping (in 30-minute bins) of control (black line) and alcohol-exposed (blue line) of wild-type CS flies over a nine consecutive day period after alcohol exposure is displayed. B) The night sleep ratio of wild-type CS flies for a nine consecutive day period after alcohol exposure. Error bars are SEM. Statistically significant differences between alcohol and control groups per day were determined by two-way Analysis of Variance (ANOVA): * denotes $P < 0.05$; * denotes $P < 0.01$; *** denotes $P < 0.001$; **** denotes $P < 0.0001$. Periods shaded in gray denote night-time, unshaded periods denote daytime.

### 3.3.3. Alcohol exposure disrupts morning anticipation

Normally, *Drosophila melanogaster* displays anticipation of morning and evening time through an increase in locomotor activity just before the transitions of lights-on and lights-off; this is known as morning and evening peaks respectively (Dubowy and Sehgal., 2017). We decided to look closely at the effect of alcohol on morning and evening peaks because we saw in our data that alcohol-treated flies slept more than untreated controls during the last hours of the night. We can see in **Figure 12A** a double-plotted actogram where we observe a graphic representation of the locomotor activity of flies during a 12 hour light and a 12 hour dark period for every day after ethanol exposure and the respective controls. In untreated wild-type CS flies, we can see visually that the activity of flies gradually increases several hours before the lights

transition in anticipation of morning and evening peaks (red and gray arrows respectively). We performed a quantitative analysis of morning **(Figure 12B)** and evening peaks **(Figure 12C),** specifically using the last three hours before the transition over the activity level of the six hours before transition for individual alcohol treated and untreated controls (Harrisingh et al., 2007). This analysis reveals that alcohol exposure greatly reduced morning anticipation in wild-type CS flies for a prolonged period of time. On the other hand, alcohol increased evening anticipation in wild-type CS flies for at least 2 days.

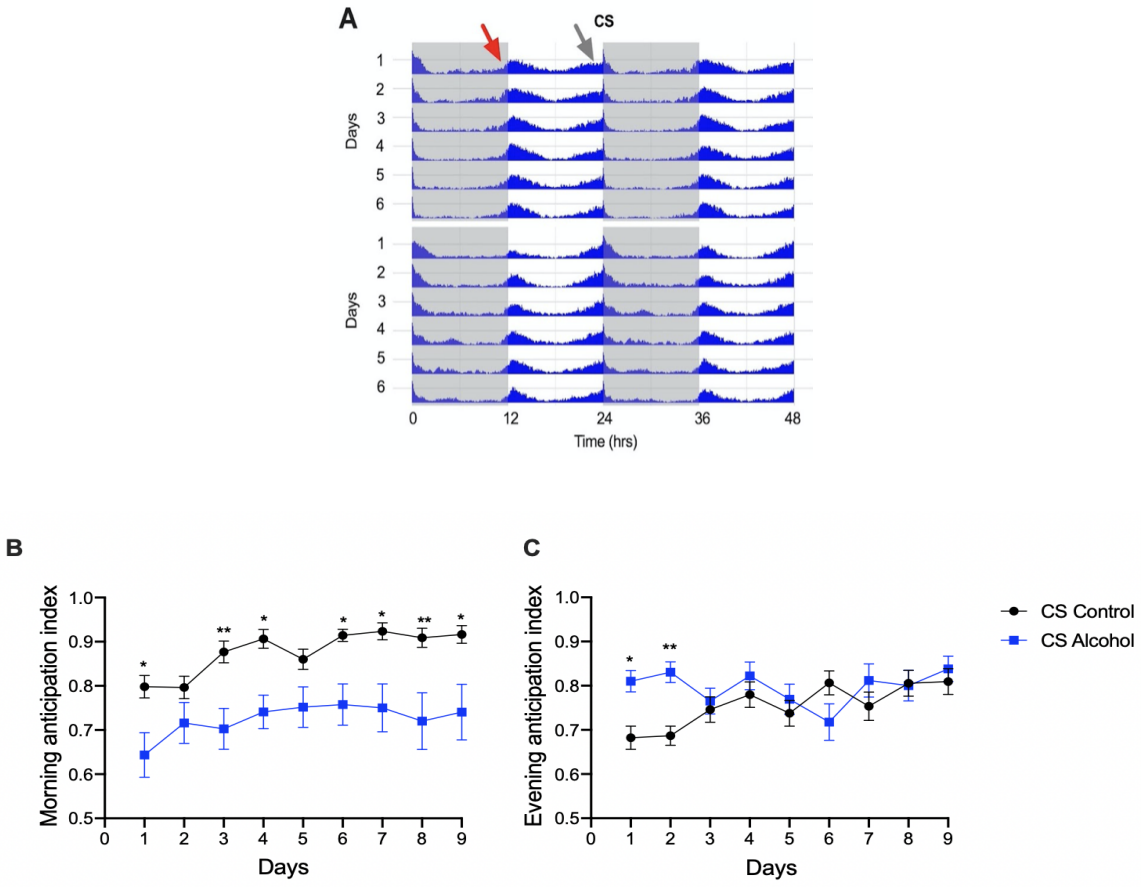


**Figure 12. Alcohol exposure disrupts morning anticipation**. A) Double-plotted actograms for wild-type CS (left), of control (top) or alcohol-treated (bottom) flies are shown. Each row represents a different day after alcohol (or mock) exposure. Red arrows point at morning anticipatory activity. Gray arrows point at evening anticipatory activity. Periods shaded in gray denote night-time, unshaded periods denote day-time. B) The morning anticipation index is shown for CS (left). Averages per day are displayed for alcohol treated flies (blue) and untreated-controls (black). Error bars are SEM. Statistically significant differences between alcohol and control groups per day were determined by two-way Analysis of Variance (ANOVA): * denotes $P < 0.05$; ** denotes $P < 0.01$.

### 3.4. Discussion

In our analysis, we characterized the effect alcohol has on sleep in wild-type CS flies. Specifically, we found that alcohol affects 3 important aspects of sleep: (1) Alcohol exposure increases day-time sleep. This effect is achieved by consolidating sleep, which is represented by a decrease in the number of sleep episodes and an increase in the mean sleep episode duration. (2) Although it does not affect total sleep, alcohol influences the architecture of night-time sleep. We saw in our experiments that alcohol increases total sleep during the first half of the night and increases it during the second half. This evidence suggests that alcohol might be influencing sleep rebound, a phenomenon observed where organisms sleep more in order to compensate for lack of sleep (Allada et al., 2017). (3) Alcohol affects morning anticipation. Considering these results it seems that the effect alcohol has on morning anticipation is related with its effect on sleep architecture as the longer the flies slept in the night, the lower the activity during the morning. Together, these results are of extreme interest to scientists as they are the firsts ones using *Drosophila melanogaster* to effectively describe the effect ethanol has on sleep. We hope that they serve as a basis for future scientific research concerning other biological processes that could be mediating alcohol induced sleep behaviors.

Of particular interest to us is to determine the effect alcohol has on the morning anticipation of flies. It is known that specific neurons of the circadian circuit regulate morning anticipatory peak; they are called lateral ventral neurons or LNvs. These neurons do so through the secretion of a neuropeptide called pigment dispersing factor

or PDF. It has been reported that in *Drosophila*, animals that had the PDF neuropeptide gene mutated or animals whose PDF neurons were ablated were arrhythmic under constant conditions (Renn et al., 1999). In addition, evidence suggests that loss of PDF or its receptor (PDFR) decreases morning anticipation (Renn et al., 1999) while using expression of *per* in order to restore clock function in LNv mutant restores morning activity (Grima et al., 2004). Considering this evidence, we think that LNvs are solid molecular candidates that could be mediating alcohol induced sleep behaviors.

It is important to note that it has been observed in humans that sleep decreases significantly when located in a novel in a novel or unfamiliar environment; this is called the first night effect (Agnew et al., 1966). Some hypothesized that this effect is mainly due to the stress felt by organisms when being in a new environment as they have to be vigilant for novel predators (Agnew et al., 1966; Tamaki et al., 2016; Tamaki et al., 2019). We saw in our experiments with untreated wildtype- CS flies a gradual increase in night-time total sleep after they have been placed in activity monitors. We think this is in part due to the first night effect as the experimental procedures require that flies are isolated and transferred to tiny tubes. In addition, we also think that the first night effect is linked with stress related mechanisms, as flies had to be tapped various times in order to transfer them. Curiously, it is well known that alcohol and sleep interact with each other through stress related mechanisms. For instance, in humans, alcohol dependence in adulthood has been linked with social stress in earlier stages of life (Dube et al., 2006). Furthermore, stress and circadian rhythms are both regulated by the hypothalamic-pituitary adrenal axis, the extra-hypothalamic brain stress axis (Nader

et al., 2010; Herman et al., 2016). Interestingly, alcohol seems to affect these regions as well (Stephens & Wand., 2012). From a molecular perspective, CLOCK and CRY influence the HPA axis to secrete hormones and neuropeptides associated with stress (Nader et al., 2010; Koch et al., 2017).

In addition, LNvs also seemed to be involved in regulating stress related responses **(Figure 13).** One molecular target of LNv neurons are the DN1 neurons (Fuji et al., 2010; Zang et al., 2010; Schubert et al., 2018). These neurons, while also part of the core clock cells in the *Drosophilae* brain, have recently been associated with the control of stress and anxiety through the release of another peptide: the Diuretic Hormone 31 (DH31) (Shafer et al., 2008; Goda et al., 2016; Goda et al., 2019). It is believed that through PDF neuropeptide, LNv neurons communicate with DN1 neurons to modulate the secretion of DH31, a homolog of the vertebrate neuropeptide calcitonin gene related peptide (CGRP) (Johnson et al., 2005; Goda et al., 2016). In addition, this protein is a known to be a vasodilator associated with anxiety behavior as it increases neuronal activity in anxiety related brain structures (Furuya et al., 2000; Sink et al., 2011). In *Drosophila*, as the DN1 neurons release DH31, a pair of three bilateral pairs of cells in a region called the Pars Intercerebralis (PPI) produce the Diuretic Hormone 44 (DH44), the mammalian counterpart of the stress hormone known as corticotropin releasing factor (Cabrero et al., 2002; Johnson et al., 2005; Cavanaugh et al., 2014; Barber et al., 2021). It has been reported that DH31 loss of function mutants display elevated sleep levels, specifically during the last 6 hours of the night while sleep was reduced when DH31 was overexpressed in pan-neuronal neurons (Kunst et al., 2014).

Interestingly, this effect is similar to the one we observed when we exposed flies to alcohol. All together, we propose that there might be a strong relationship between alcohol and sleep through LNvs neurons. In our next set of experiments, we plan to activate and inactivate LNvs neurons in order to study the overall effect these manipulations have in alcohol induced sleep behaviors.

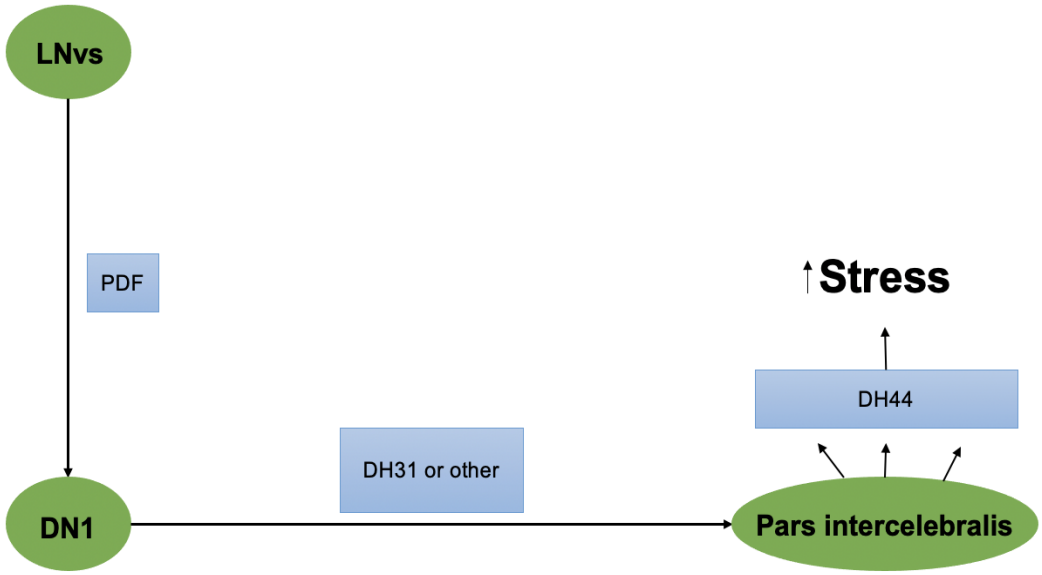


**Figure 13. Pathway associating LNvs with Stress.** LNvs, through PDF neuropeptide, promote the DN1 neurons to release chemical substances that end up affecting the Pars Intercelebralis in producing DH44, a hormone involved in increasing stress

# Chapter 4: Role of the Lateral Ventral Neurons on Alcohol-Induced Sleep Behaviors

## 4.1. Introduction

Homeostatic adaptations in the brain are triggered by alcohol administration and play an important role in the development of tolerance and dependence (Himmelsbach., 1964; Ritzmann & Tabakoff., 1976; Miller et al., 1987; Clapp et al., 2008; Kalivas & O'Brien., 2008). A series of sleep disturbances arise through these adaptations and manifest themselves during the development of alcoholism (Yules et al.,1967; Mello & Mendelson., 1970; Baekeland et al., 1974; Williams et al., 1983; Roers et al., 1999; Foster et al., 2000; Van Reen et al., 2006; Feige et al., 2006; Chan et al., 2014; Colrain et al., 2014; Britton et al., 2020). Nevertheless, the neuronal and molecular mechanisms that are behind this alcohol induced behaviors remain unknown. This limits our efforts on finding pharmaceutical treatment for mental diseases such as addiction. Taking into consideration the evidence we found on Chapter 3, we decided to explore the relationship alcohol has on sleep using the *Drosophila* model system and focusing on a specific set of neurons known as LNvs that influence sleep and affect alcohol sensitivity and tolerance in the fruit fly. We hypothesize that alcohol-induced sleep behaviors arise from changes in the activity of LNVs through the PDF neuropeptide and that these adaptations are also involved in the development of alcohol tolerance.

We have discussed previously that in both invertebrates and mammalian species, sleep is a modulated process established by a circadian clock - that controls rest/activity periods - and a homeostatic process called sleep rebound - a phenomenon where more sleep is needed in order to compensate for lack of it (Borbeley., 1982). In terms of circadian rhythms, although fruit flies tend to sleep more at night, they also sleep during the day and present high periods of locomotor activity specifically during dawn and dusk- these periods are termed morning and evening peaks (Vanin et al., 2012; Green et al., 2015). They typically occur just before lights-on and lights-off transitions under a 12:12 hr light/dark (LD) cycle. In addition, we have previously mentioned regarding the composition of the *Drosophila* circadian circuit that is made of approximately 150 neurons that modulate circadian rhythms as well as sleep (King & Sehgal., 2020). Specifically, this circadian circuit consists of the Lateral Neurons and Dorsal Neurons (DN1, DN2 and DN3) (Kaneko et al., 2000; Schubert et al., 2018; King & Sehgal., 2020). In turn, the Lateral Neurons are composed of the small and large ventral neurons, the dorsolateral neurons and the lateral posterior neurons (Kaneko et al., 2000; Helfrich-Forster et al., 2007; Schubert et al., 2018; King & Sehgal., 2020). These cells express different neurotransmitters and neuropeptides that grant them different functional abilities. For instance, all lateral ventral neurons except one known as the “5th sLNvs” express the neuropeptide PDF (Schubert et al., 2018). Scientists have found that PDF plays a major role in adjusting the circadian clock of clock neurons (Klarsfeld et al., 2004; Lin et al. 2004; Yoshii et al. 2009; Zhang et al. 2010). In addition, flies that lack PDF or the PDF receptor have no morning activity peaks in LD (Renn et al. 1999; Lin et al. 2004; Hyun et al. 2005; Lear et al. 2005; Mertens et al. 2005).

In mammals, VIP is the homolog for Pigment Dispersing Factor or PDF. It is found in the suprachiasmatic nucleus (SCN) of the hypothalamus- the main center that regulates circadian and biological clocks in mammalian species and entrainment to environmental dark-light cycles (Moore., 1983). It does so through the retino-hypothalamic tract (RHT)- a pathway that links the retinal ganglion cells with the SCN through fibers that contain neurotransmitters and neuropeptides- one of them is the Vasoactive Intestinal Peptide or (VIP) that is release in the SCN during the light period (Pickard., 1982; Moore., 1983; Ibata et al., 1989). Interestingly, VIP has been involved in modulating circadian rhythms (Albers et al., 1984; Piggins et al., 1995). Moreover, there is evidence that directly links VIP with alcohol responses. For instance, it has been reported in previous studies that in rats VIP and its receptor -VIPR1- are downregulated after chronic alcohol exposure and that this effect was dose dependent (Feng & Liu et al., 2015). In addition, evidence suggests that intraperitoneal injection of 100 micrograms of VIP increases ethanol consumption in rats (Kulkosky et al., 1989).

In *Drosophila*, it was shown that over-expression of the BK channel named *slowpoke* in LNv neurons significantly affects alcohol resistance (Ghezzi et al., 2013). This evidence directly establishes a link between alcohol responses and neuronal circuits involved in regulating sleep. Thus, in this study, we have decided to investigate the role PDF releasing LNv neurons have in alcohol induced sleep responses using *Drosophila melanogaster* as a biological model.

## 4.2. Methods

### 4.2.1. Fly Maintenance

Flies were maintained in a 25.9∘ $C$, 80% relative humidity and a 12 hour light: 12 hour dark cycle incubator. They were fed with a corn-based diet. When performing the ethanol tolerance assay and preparing the food for the monitor tubes, we mixed 50 ml of food with 10 ml of water. We used 3-5 year old female virgins for alcohol sensitivity and tolerance assays. We used this fly lines for our experiments: wild-type CS, *Pdf*$^{01}$ mutant line, a fly line that was backcrossed for six generations to the CS background in order to reduce the effects of genetic background as described in Pohl et al. (2013).

### 4.2.2. Alcohol exposure

We exposed flies to alcohol using a custom built alcohol delivery system. This system is composed of two air pumps that were attached to two 30ml midget bubblers (Ace Glass inc.; Vineland, NJ) which contained 10 ml of water or 10 ml 95% ethanol. Each bubbler was in turn connected to airflow regulators so we could control the flow of air independently for each other. The bubblers were also connected to 15 ml or 50 ml conical tubes (depending on the number of flies being treated).

### 4.2.3. Alcohol-Sleep Analysis

We separated female virgin flies into two groups- one that was exposed to alcohol-saturated air and another to humidified air. For alcohol-exposure experiments, we placed around 32 flies for each group into 2 perforated 15ml conical tubes. For the experimental group, flies were given a 5 minute exposure to humidified air (1.5LPM

H20/0 LPM ETOH) so that they could acclimate to the chamber. Later on, they were given alcohol until all flies were knocked down (1.5 LPM ETOH/0 LPM H20). Afterwards, flies were given humidified air (1.5LPM H20/0 LPM ETOH) for about 10% of the time the flies took to knock down after ethanol exposure. The control group was simultaneously exposed to humidified air (1.5LPM H20/0 LPM ETOH) for the entire experiment using an identical experimental set up, but with no alcohol. After flies were treated, we transferred them to 65 mm X 5 mm glass tubes that contained fly food (Trikinetics, Waltham, MA) containing fly food. Afterwards, we place the tubes in *Drosophila* Activity Monitors (DAM2) with an incubator set to the parameters explained above. We used the Trikinetics monitoring system (Waltham, MA) for a period of 5-9 days to record locomotor activity in 1 min bins (Agosto et al., 2008). On the other hand, sleep was identified as a period of uninterrupted inactivity that lasted for 5 minutes. We used MATLAB software (Natick, MA) to calculate daily sleep patterns as described in (Parisky et al., 2008). We calculated sleep parameters such as total sleep duration, sleep latency, number of sleep episodes, mean sleep episode duration, and total locomotor activity for each 12 hr period of the light/dark protocol per day. Two-way Anova was used to calculate levels of significance. We used Rethomics framework in R (Geissmann et al., 2019) to analyze morning and evening anticipation peaks and night sleep ratios as described in Harrisingh et al. (2007). We divided the sum of activity for each fly between ZT21 and ZT24 by the sum of activities between ZT18-24 for each day in order to calculate the morning anticipation index. On the other hand, we divided the sum of activity for each fly between ZT9 and ZT12 by the sum of activities between ZT6-12 for each day in order to determine evening anticipation. Finally, we used the

sum of total sleep for each fly between ZT18 and ZT24 divided by the sum of activities between ZT12-18 for each day analyzed in order to calculate night sleep ratios. Two-way Anova-mixed effects analysis were used to calculate statistically significance levels between alcohol and control groups per day.

## 4.3. Results

### 4.3.1. Effects of alcohol on sleep are dependent on the pigment dispersing factor (PDF)

In Chapter 3, we looked at the effect alcohol had on sleep in wild-type CS flies in a 12 hour light and a 12 hour dark period for 9 consecutive days. We saw that alcohol exposure leads to an increase in daytime sleep that slowly comes back to normal levels as the days post-experiment pass by. In addition, our study suggests that alcohol plays a major role in consolidating sleep in flies by decreasing the number of sleep episodes and increasing the mean sleep episode duration. In order to investigate how PDF-expressing neurons affect alcohol-induced sleep behaviors, we decided to look at sleep and locomotor activity patterns of $Pdf^{01}$ mutant flies for a period of 9 consecutive days **(Figure 14).** Here, we observe that untreated $Pdf^{01}$ flies sleep more during the night than during the day and seem to possess no morning activity peak from a visual perspective **(Figure 14A)**. Alcohol seems to persistently increase the percent of time sleeping during the day. On the other hand, during the night, alcohol increases sleep on night 1 but decreases sleep after.

A closer quantitative inspection of sleep parameters reveals that *Pdf*$^{01}$ flies slept significantly more during the first two days after ethanol exposure than controls **(Figure 14B).** This effect wears off the rest of the days. This was a very similar effect to that observed in ethanol exposed CS flies. However, in this specific case, there was a more robust difference in total sleep in *Pdf*$^{01}$ flies due possibly by the mutation. Interestingly, during the night period, *Pdf*$^{01}$ ethanol exposed flies slept more than controls on day 1 but switched to sleeping less after day 2. They slept less for about three days post ethanol exposure but normalized to control levels afterwards. This effect on total night sleep of ethanol on *Pdf*$^{01}$ flies was different from that observed in wild-type flies where alcohol had no effect in total sleep during the night. Although no effect was observed in sleep latency after ethanol administration, we saw that *Pdf*$^{01}$ flies had a decreased sleep latency compared to wild-type CS flies themselves **(Figure 14C).** Finally, no significant differences were observed in the number of sleep episodes **(Figure 14D)** and the mean sleep episode duration **(Figure 14E)** between ethanol treated *Pdf*$^{01}$ flies and controls. This is different from what we saw in wild-type CS flies where they experience a more consolidated sleep pattern portrayed by an increase in the number of sleep episodes and a decrease in their duration.

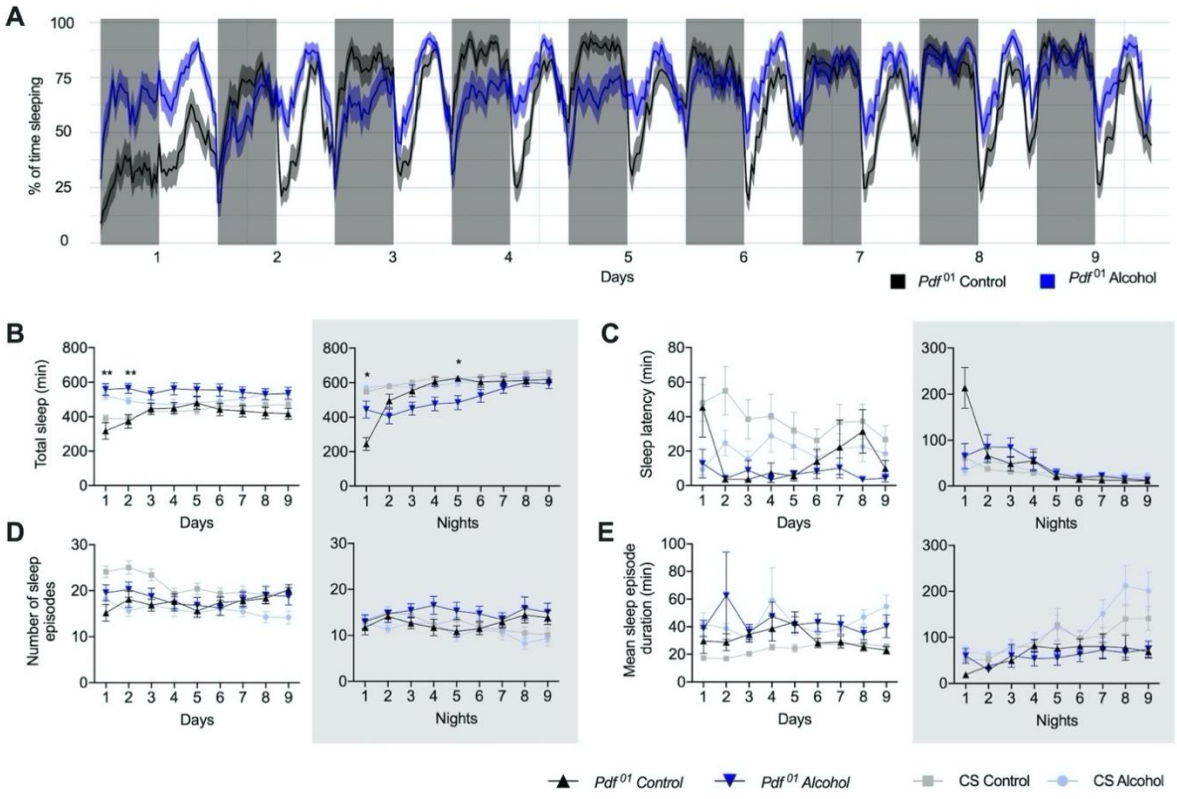


**Figure 14. Alcohol-induced sleep disruption is dependent on the pigment dispersing factor (PDF).** A) The percentage of time sleeping (in 30-minute bins) of control (black line) and alcohol-exposed (blue line) of *Pdf*$^{01}$ flies is displayed over a period of 9 consecutive days after alcohol exposure. C-E) A diverse set of sleep parameters is displayed for every daytime period (left) and night-time period (right) after alcohol exposure. Sleep parameters are displayed as follows: B) total time sleeping, C) sleep latency, D) number of sleep episodes, and E) mean sleep episode duration. Averages per day are displayed for alcohol-treated *Pdf*$^{01}$ flies (blue) and untreated controls (black). Additionally, averages per day of wild-type flies are displayed for convenience: alcohol-treated CS flies (light blue) and untreated controls (gray). Error bars are SEM. Statistically significant differences between alcohol and control groups per day were determined by two-way Analysis of Variance (ANOVA): * denotes $P < 0.05$; ** denotes $P < 0.01$. Periods shaded in gray denote night-time, unshaded periods denote daytime.

### 4.3.2. Alcohol exposure alters night sleep architecture

We observed in wild-type CS flies that sleep architecture of night-time sleep was severely disrupted after ethanol exposure as flies slept less during the beginning of the night and more at the end of it. This effect was not seen in *Pdf*$^{01}$ flies as evidence suggests that both ethanol exposed flies and untreated controls slept well during the night. **(Figure 15A).** When we calculated the ratio of sleep displayed at the first 6 hours of the night divided by the sleep displayed during the last 6 hours of the night, we saw in wild-type CS flies this ratio was higher in alcohol treated flies than controls even after 8 days post-ethanol exposure. On the other hand, *Pdf*$^{01}$ flies display no differences in this ratio **(Figure 15B-C).** This evidence suggests that this effect might be, at least partially, dependent on PDF neuropeptide.

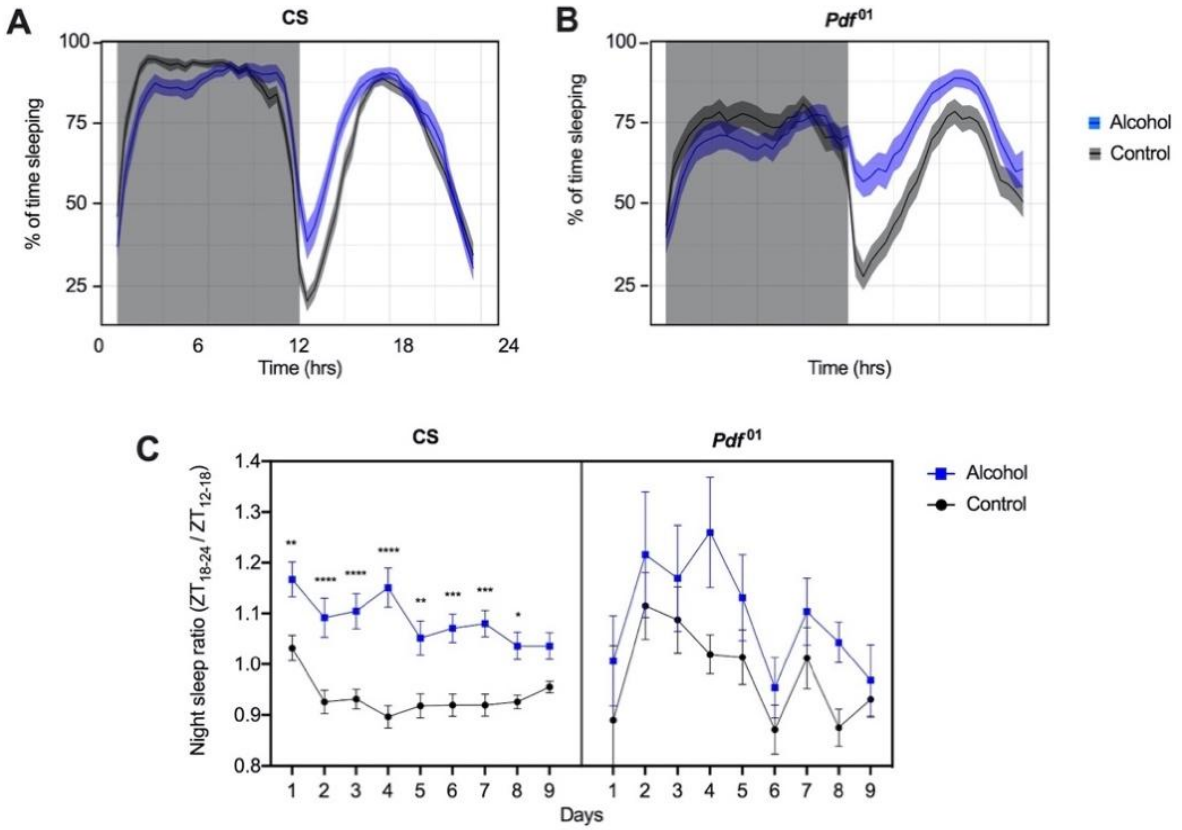


**Figure 15. Effect of alcohol exposure on night sleep architecture in *Pdf$^{01}$* flies.** A) The average percentage of time sleeping (in 30-minute bins) of control (black line) and alcohol-exposed (blue line) of wild-type CS flies over a nine consecutive day period after alcohol exposure is displayed. B) The average percentage of time sleeping (in 30-minute bins) of control (black line) and alcohol-exposed (blue line) of Pdf 01 flies over a nine consecutive day period after alcohol exposure is displayed. C) The ratio of sleep displayed during the first 6 hours of the night over the sleep displayed in the last 6 hours of the night is shown for CS (left) and Pdf 01 mutant flies (right). Averages per day are displayed for alcohol-treated flies (blue) and untreated controls (black). Error bars are SEM. Statistically significant differences between alcohol and control groups per day were determined by two-way Analysis of Variance (ANOVA): * denotes P < 0.05; * denotes P < 0.01; *** denotes P < 0.001; **** denotes P < 0.0001. Periods shaded in gray denote night-time, unshaded periods denote daytime.

### 4.3.3. Alcohol exposure disrupts morning anticipation

In **Figure 16**, we see in a double-plotted actogram that wild-type CS flies display a robust morning and evening peaks of activities during the transitions from lights on and off respectively (red and gray arrows, respectively). In addition, this anticipation became evident every single day of the experiment. Interestingly, *Pdf*$^{01}$ mutant flies displayed no morning and evening peak **(Figure 16A).** Quantitative analysis reveals that ethanol significantly reduces wild-type CS morning anticipation index **(Figure 16B-C).** On the other hand, morning anticipation remained unaffected in *Pdf*$^{01}$ mutant flies after ethanol exposure. Alcohol increases evening anticipation peaks in wild-type CS flies. However, no significant differences were observed in ethanol exposed *Pdf*$^{01}$ mutant flies.

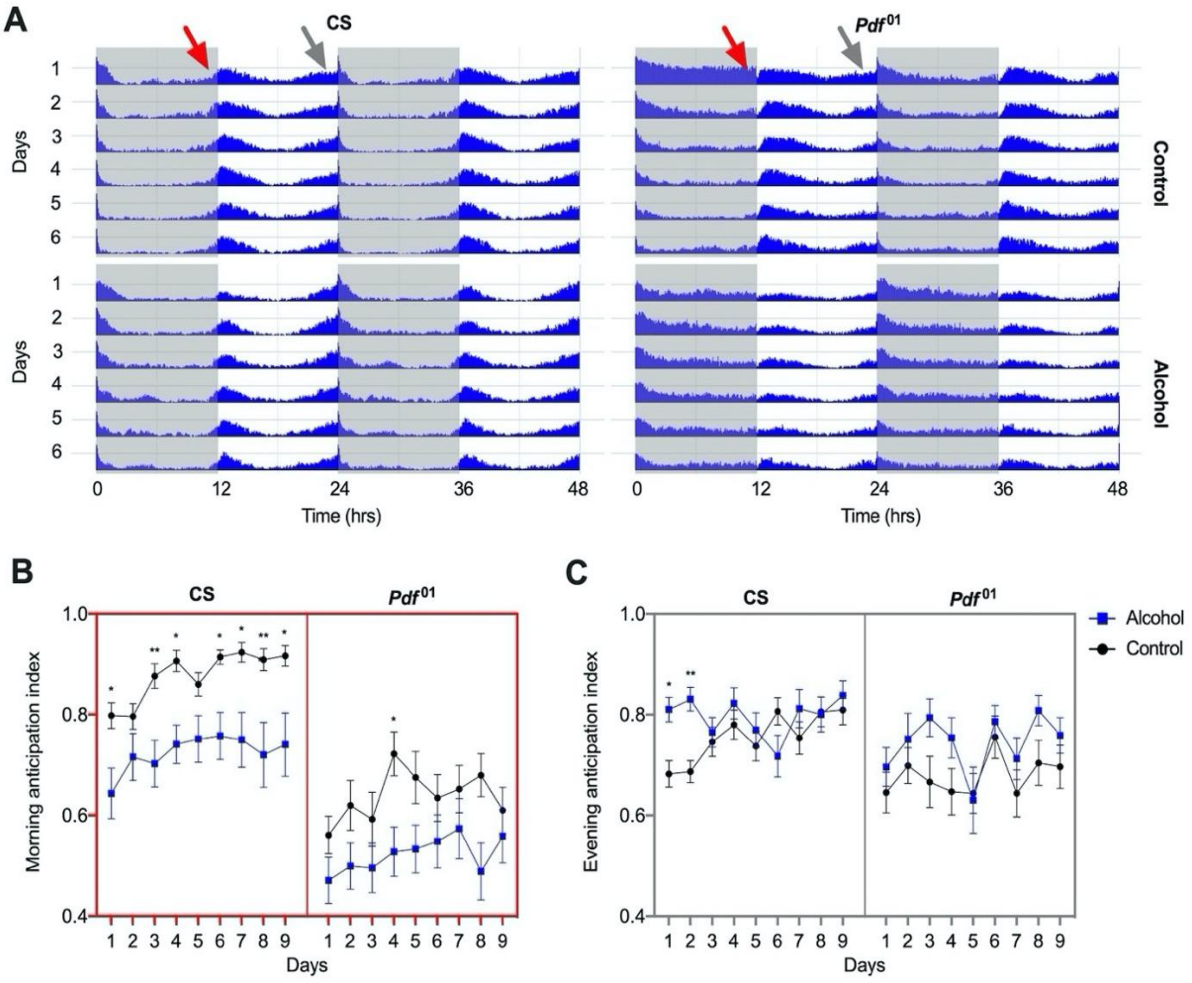


**Figure 16. Effect of alcohol exposure on morning anticipation in *Pdf*$^{01}$ flies.** A) Double-plotted actograms for wild-type CS (left) and *Pdf*$^{01}$ (right), of control (top) or alcohol-treated (bottom) flies are shown. Each row represents a different day after alcohol (or mock) exposure. Red arrows point at morning anticipatory activity. Gray arrows point at evening anticipatory activity. Periods shaded in gray denote night-time, unshaded periods denote daytime. B) The morning anticipation index is shown for CS (left) and *Pdf*$^{01}$ mutant flies (right). Averages per day are displayed for alcohol-treated flies (blue) and untreated controls (black). C) The evening anticipation index is shown for CS (left) and *Pdf*$^{01}$ mutant flies (right). Averages per day are displayed for alcohol-treated flies (blue) and untreated controls (black). Error bars are SEM. Statistically significant differences between alcohol and control groups per day were determined by two-way Analysis of Variance (ANOVA): * denotes $P < 0.05$; ** denotes $P < 0.01$.

## 4.4. Discussion

Alcohol has been successfully described as a suppressor of neuronal activity that triggers a myriad of neuronal responses that stem from homeostasic neuroadaptations (Littleton., 1998; Koob & Le Moal., 2001; Ghezzi & Atkinson., 2011). On the other hand, sleep is modulated through internal and external factors that adjust accordingly in order for the organism to successfully adapt to its environment (Okamoto-Mizuno & Mizuno., 2012; Allada et al., 2017). We think that it is of no surprise to find that these two processes interact with each other. We used *Drosophila* melanogaster in order to manipulate a specific set of neurons- called Lateral Ventral neurons that secrete the neuropeptide PDF- in order to investigate how they are involved in regulating alcohol induced sleep behaviors.

Our results obtained from these sets of experiments suggest that PDF is involved in alcohol responses. While ethanol-exposed $Pdf^{01}$ mutants had an increase in sleep during the first two days after the experiment, the same as in wild-type CS flies, we observed that alcohol did affect the number of sleep episodes and the mean sleep episode duration in $Pdf^{01}$ flies. This evidence points out that there is only a partial involvement of PDF in the effect alcohol has on daytime sleep. On the other hand, $Pdf^{01}$ mutant flies possessed no morning anticipation peak, suggesting that PDF neuropeptide itself might be playing an important role in regulating this sleep behavior. Curiously, one behavior that presented itself more robustly on untreated $Pdf^{01}$ mutants than in wild-type CS flies was that they showed a gradual increase in total night-time sleep after ethanol administration. We think that this increase is due, in part, to the anxiety or stress

manifested in flies after being placed in the novel or perhaps uncomfortable environment of the activity monitor tube. On the other hand, alcohol exposed *Pdf*$^{01}$ mutants appeared to sleep normally throughout the nights, suggesting that alcohol, in part, rescues this effect.

In humans, a reduction in sleep when sleeping in a new environment is rather common and typically known as the first-night effect (Agnew et al., 1966). We have potential evidence that suggests that this effect is also observed in flies in the form of the increase in total night time sleep. While it is still not fully understood the mechanisms behind how PDF is involved in this phenomenon, a well studied neuronal circuit might shed some light on this issue. In this circuit, lateral ventral neurons interact with DN1 neurons through PDF (Cabrero et al., 2002; Johnson et al., 2005; Hector et al., 2009; Cavanaugh et al., 2014; Cannell et al., 2016; Goda et al., 2019; Barber et al., 2021). In turn, DN1 neurons secrete DH31 into the pars intercerebralis to aid in the production of DH44, the fly homolog of the stress related hormone called corticotropin (Cabrero et al., 2002; Johnson et al., 2005). While we still don't fully understand how PDF neuropeptide is related to alcohol and sleep, our results shed light on a potential interaction through the aforementioned stress related pathway. Taking all this evidence into account, we think that alcohol is suppressing the release of PDF by LNVs, which in turn results in a myriad of events that ends up affecting sleep **(Figure 17).** This suppression in PDF release could result in a decreased release of DH44 by the Pars Intercelebralis, overall decreasing stress. Thus, overall, one possible effect of alcohol on this model system is that alcohol is decreasing PDF release, which in turn leads to a

decreased release of DH44 that results in stress reduction. For future studies, it would be interesting to observe how stress is affected by our alcohol-sleep assay. Another interesting study is to inactivate DH44 release in the Pars Intercelebralis and observe its overall effect in alcohol induced sleep behaviors.

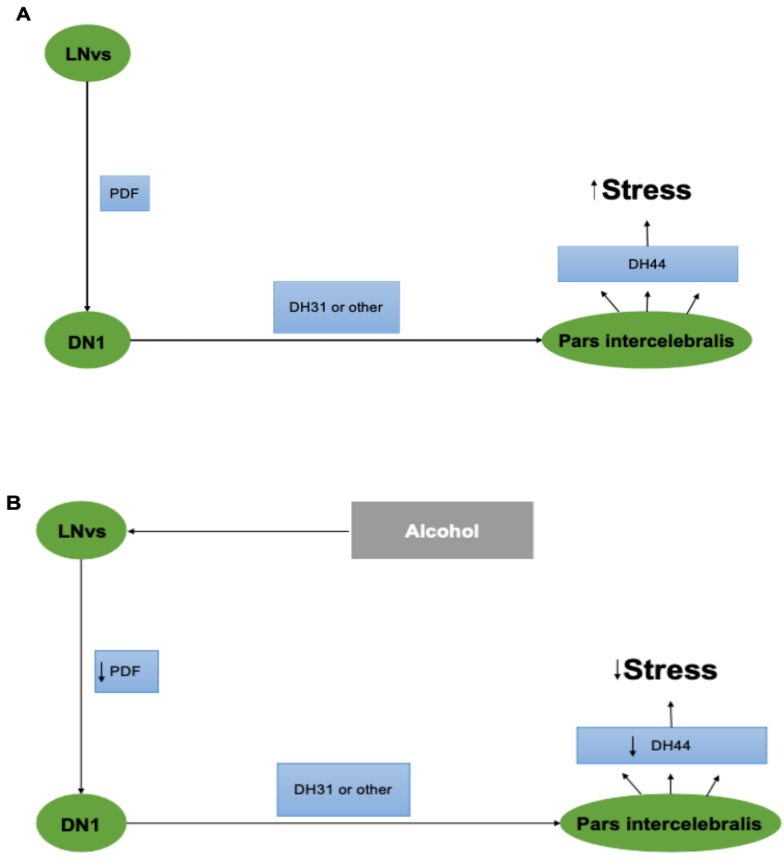


**Figure 17. LNvs are associated with stress through an established molecular mechanism.** A) Here, LNvs, through the secretion of PDF, induce DN1s to produce neurotransmitters and neuropeptides to potentiate the Pars Intercerebralis in releasing DH44, the mammalian counterpart of corticotropin stress releasing factor. B) We think that alcohol is reducing PDF release, affecting the release of DH44 by the Pars Intercelebralis, overall causing a decrease in stress

# Chapter 5: Effect of alcohol on *Drosophila melanogaster* sleep related gene expression

## 5.1. Introduction

Alcohol dependence is a chronic disease that is characterized by a compulsive need to consume ethanol despite the negative consequences it can potentially have (DSM-IV., 2013). Alcohol is known to do this by directly affecting transcription factors and microRNAs involved in the regulation of gene transcription. Evidence of alcohol induced changes in gene expression have been found in mammalian brain regions such as the hippocampus (Farris et al., 2015), central amygdala (Ponomarev et al., 2012), nucleus accumbens (Mamdani et al., 2015) and frontal cortex (Iwamoto et al., 2004; Alexander-Kaufman et al., 2006; Barbier et al., 2015). These changes in gene transcription, together with alcohol's effect on epigenetic systems, end up influencing translation and protein synthesis as well as many biological processes.

Studies point out that alcoholism tends to run in families and that genetic factors tend to play a predominant role when it comes to developing alcoholism (Amark., 1951; Cotton et al., 1979). In humans, studies concerning alcoholism and genetics have been performed through multiple channels. One approach to address this question is through the conduction of adoption studies that allows scientists to evaluate the role both genetics and environmental risk factors have in alcohol dependency. The data from these studies suggest that the risk of alcoholism in adoptees is more highly correlated

with their biological families (or genetic factors) rather than their adopted ones (Bohman et al., 1981; Cloninger et al., 1981; Cloninger et al., 1985). Another approach that has helped unravel the genetic mechanisms behind alcoholism have been conducted using twins. These studies have reported that there is a 45-65% genetic liability when it comes to developing alcoholism (Pickens et al., 1991; Heath et al., 1995; Edenberg et al., 2013). All together, studies report that genetic elements are associated with more than 50% of the variance for developing alcoholism (Ducci et al., 2008).

It is highly relevant for our research project to study how alcohol influences sleep related gene expression in order to understand its effect on sleep behavior. From a behavioral perspective, alcohol and circadian rhythms highly interact with each other in a bidirectional and complex manner. For example, studies show that alcohol preference is modulated by time of day and that disruption of circadian rhythms seen in, for example, frequent travelers or rotating shift workers, ultimately results in increased alcohol use (Trinkoff & Storr., 1998; Spanagel et al., 2005; Rosenwasser et al., 2010). Alcohol also ends up affecting the circadian secretion of melatonin (Danel et al., 2009), leading to severe disruptions in temperature (Devaney et al., 2003) and blood pressure rhythms (Nakashita et al., 2009).

Moreover, alcohol is known to severely disrupt the expression of circadian genes. Studies in rats point out that alcohol can severely disrupt *per1* and *per2* gene expression in the hypothalamus (Chen et al., 2006). In addition, neonatal alcohol exposure significantly causes a reduction in *cry1* gene expression in the

suprachiasmatic nucleus while it advances *per2* rhythms at both the cerebellum and liver, leaving long term changes in the fetus (Farnell et al., 2008). Another circadian gene that has been associated with alcohol responses is the BK channel coded by the gene called *slowpoke*. In *Drosophila melanogaster*, a single exposure to alcohol upregulates this gene that in turn was proven to be necessary for the development of tolerance. This gene also plays a major role in the manifestation of alcohol related withdrawal symptoms such as seizures (Cowmeadow et al., 2005). Finally, Ghezzi et al., 2013 uncovered several genes that are affected by alcohol exposure through histone acetylations. Although not all of them are circadian core clock genes, some of them are important for the regulation of sleep such as *pum*, *brp* and *para*.

Although the aforementioned evidence points out that alcohol directly targets core clock and sleep related gene expression, more in depth analysis is needed in order to elucidate the molecular mechanisms by which alcohol affects sleep from a genetics perspective. In order to investigate the genetic mechanisms that underlie this process, we use *Drosophila* melanogaster as a biological model. The fruitfly is particularly useful for performing genomic studies because most of its genome has already been sequenced and a reference genome of the fly already exists (Adam et al., 2000). In addition, the fruit fly, as humans, show signs of alcohol tolerance, dependence and withdrawal, making it a unique organism to study these behavioral phenomena (Scholz et al., 2000; Berger et al., 2004; Bhandari et al., 2009;). Using this animal model, we found that alcohol influences PDF and DH44 gene expression and that the expression

pattern of those genes change across time. In addition, cluster analysis reveals that sleep is among the top categories that have been influenced by alcohol exposure.

## 5.2. Methods

### 5.2.1. Fly Maintenance

Fly strains were kept in an incubator under a temperature of 25.9 ºC, 80% relative humidity and a 12 hour light: 12 hour dark cycle. Their nutrition consisted of a cornmeal-based diet. For these experiments, 3-5 year old Canton S male and female wild type flies were used. All fly strains were obtained from Bloomington Stock Center.

### 5.2.2. Ethanol Administration Assay

For ethanol exposure, flies are placed inside falcon tubes that are connected to the ethanol chamber, an instrument that generates vapor from liquid ethanol. The ethanol chamber itself is composed of an air pump connected to regulators that are attached to a set of bubblers that contain liquid ethanol and water. When the air pump is turned on, the liquid turns into air and travels to the falcon tubes, where flies are located. During the first five minutes of the experiment, flies are exposed to water vapor (1.5 LPM H20/0 LPM ETOH) so that they can acclimate to the chamber. Later on, they are exposed to ethanol vapor (1.5 LPM ETOH/0LPM H20) until they are sedated. The control group is exposed to air simultaneously. The experiment is finished by exposing all flies to water vapor (1.5 LPM H20/0 LPM ETOH) (for 10% of the amount of time flies

took to knock-down. A post-doc named Jenny Acevedo was responsible for this part of the experiment.

### 5.2.3. RNA extraction, sequencing & analysis

RNA samples were obtained for each day for four days after alcohol exposure. Each sample collection consisted of four replicates belonging to the control group and four to the experimental group. The EZ1 RNA Tissue mini kit (EZ1 RNA Tissue Mini Kit no. 95034) was used to extract the RNA of the fly's brain of 150 flies for each replicate. The samples underwent a quality assessment of their RNA content using Nanodrop2000c. After obtaining these readings, Poly-A selection was used to isolate the mRNAs for each sample and sequenced using the Ilumina HiSeq 2000 500 via paired-end sequencing. Fastqc files were generated as a result of the sequencing.

Verification of the quality of the raw sequence data was performed using Fastqc 0.11.9. Afterwards, adaptor sequences and low quality reads were removed with Trimmomatic. We created an index with the reference genome of the fly obtained from Flybase and aligned the reads using STAR algorithm. This algorithm generates the number of reads per Kilobase per Million reads (RKPM) that were used for the differential expression analysis. The latter was obtained using the DESEQ library package found in R. In addition, DESEQ library analysis calculates the p-value via the Benjamini-Hochberg False Discovery Rate method.

### 5.2.4. Functional Annotation and Cluster Analysis

David Bioinformatics was used to analyze the most represented gene categories or annotations. Four gene lists were provided to DAVID Bioinformatic using the top thousand most differentially expressed genes for each day after ethanol exposure, when sorting them by increasing p-value. The site generated the “Official Gene Symbol” for the identification of each gene and the Biological Process option was used to generate the analysis. To obtain the gene annotations most affected by alcohol for each day, we used the “Functional Annotation Chart section” of the DAVID Bioinformatic tool box, which revealed the fold enrichment and p values for each cluster.

## 5.3 Results

### 5.3.1. Differential Expression Analysis

We quantified those genes whose p-values were less than 0.005 in our differential expression analysis. For the first day, 3,315 genes had a p-value less than 0.005, for second day, 4,896 genes had a p-value less than 0.005, for the third day a total of 4,244 genes had this p-value and for the fourth day a total of 3,315 genes had a p-value less than 0.005.

### 5.3.2. Alcohol Changes PDF and DH44 expression overtime

We used the differential expression analysis to quantitatively study how alcohol affects PDF and DH44 gene expression overtime **(Figure 14).** For this, we used the counts per read, which is a measure of how many reads have been mapped to each

gene in order to investigate changes in gene expression (Koch et al., 2018). We saw that PDF gene expression decreased during day 1 and day 2 after ethanol exposure but increased during day 3 and day 4 **(Figure 14A).** In addition, we saw that alcohol decreased DH44 expression Day 1, Day 2 and Day 3 post ethanol exposure and increased it during day 4 **(Figure 14B).** Together, these results that alcohol could indeed be causing a reduction in PDF and DH44 release, at least during the beginning of the experiment, which could be resulting in a decreased stress phenotype.

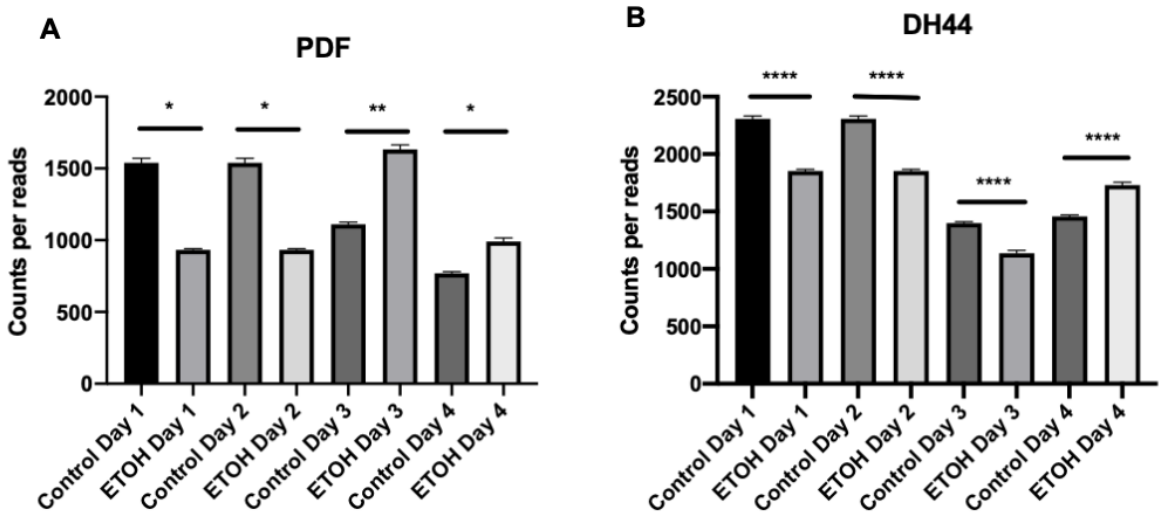


**Figure 18. Alcohol is affecting PDF and DH44 expression overtime.** A) PDF gene expression is decreases during the first 2 days after ethanol exposure after it increases during the last 2 days. B) DH44 gene expression decreases during the first 3 days after ethanol exposure but increases on day 4.

### 5.3.3 Database for Annotation, Visualization, and Integrated Discovery (David) Bioinformatic Analysis

We provided DAVID Bioinformatics the top thousand genes whose expression changed significantly after ethanol exposure for each day. Our analysis reveals that genes related to cytoplasmic translation are among the most affected during all days post-drug administration. These include the RLP family of genes, which codify for proteins that makeup the ribosome. During the first day, genes related to the sleep biological process occupy the second category of the list, according to descending p values. These sleep genes are composed of calcium or calcium regulating channels such as *nacp60e, calcineurin A, pp2b-14d, cana-14f, rut, carl, quiver, shaker & shaw.* In addition, ethanol influences DOPAMINE transporter genes, as well as genes associated with Mushroom Body regions (*mub*) and Tim neurons. Finally, genes that are necessary for the maintenance of circadian rhythms such as *dco* and *acer* were affected by drug exposure. This data is summarized in **Table 1**, that presents the top 10 functional annotations provided by DAVID Bioinformatics for the data corresponding to Day 1.

| Term | Genes | P-Value | Fold Enrichment |
|---|---|---|---|
| cytoplasmic translation | RPL4, STA, RPL5, RPL30, RPL3, RPL32, RPL31, RPLP1, RPLP0, RPL34A, RPL34B, RPL8, RPL9, RPL6, RPL7, RPS15, RPL7A, RPS17, RPS16, RPL18A, RPS18 | 2.7E-59 | 9.4287833 |
| sleep | MND, KTL, REPTOR, DNR1, QVR, CHER, NACP60E, CANB, SH, LK6, BUN, COX1, RDL, MUB, PP2B-14D, NACHRALPHA4, SPEN, DRAT, CANA-14F, SHEP, BGM, SHAW, DCO, KIS, GLOB1, DAT, MEN, TIM, AAY, HSC70-3, CALR, ATT | 1.2E-12 | 3.8642554 |
| centrosome duplication | RPL4, INVADOLYSIN, RPL30, RPL3, RPL10AB, RPL11 | 2.2E-9 | 3.5393330 |
| long-term memory | DDC, HN, TOB, CASK, CHER, G9A, STAT92E, PST, CAMKII, MGLUR, MURA, DBR, ECR, APPL, ORB2, HIW, NF1, BSK, KRA, GCLM, PKC98E | 3.8E-7 | 3.7162997 |
| chemical synaptic transmission | NACHRALPHA7, SYT1, CAC, PARA, ACSL, IPP, FRQ1, SHAB, CAMKII, ATPALPHA, UNC-13, SHI, SAP47, PGK, PABP, CALS, LAP, GAT, CSAS, VACHT | 4.1E-7 | 3.8610906 |
| proton transport | ATPSYNC, ATPSYND, ATPSYNGAMMA, ATPSYNG, VHA36-1, ATP6, ATPSYNCF6, BLW, VHA13, ATPSYNBETA, ATPSYNB, ATPSYNO | 1.7E-6 | 5.8807380 |
| imaginal disc-derived wing morphogenesis | SBB, CROL, UNK, MBL, PKA-C1, EMC, BUN, IF, CG5966, CG, GOT1, SMOX, TMOD, L(3)NEO38, PSQ, CG6767, XRP1, KIS, ECR, RL, CALR, MTD, PAN, HTH, CTP, ZFH2 | 2.0E-6 | 2.2899648 |
| axon guidance | ALK, PROS, LANA, INR, MICAL, MSPS, ROBO1, SLI, SBB, AP-1SIGMA, BETA-SPEC, FAS1, HRB27C, SM, BSK, LOLA, TRX, 14-3-3EPSILON, PTP69D, IF, EPHRIN, TUTL, SPEN, PKA-R2, ACSL, SMOX, JEB, CKIIALPHA, KIS, HSC70-4, CADN, PLEXA, AAY, PLEXB, DAW | 2.2E-6 | 2.4105727 |
| neurotransmitter secretion | SYT4, SNAP25, SYT1, CAC, RAB3, CADPS, CASK, SYN, NSYB, AP-2ALPHA, FRQ1, CANB, SYT7, HDAC6, NEJ, UNC-13, HSC70-4, AP-1SIGMA, GAMMASNAP1, DAP160, AMPH, LAP | 3.6E-6 | 3.1496087 |
| behavioral response to ethanol | BACC, VAP, PKA-R2, CASK, SYN, CHER, SIRT1, FU12, MURA, CG3011, GBS-70E, PKA-C1, AAY, RL, ACCOAS, KRA, GCLM | 4.8E-6 | 3.8001260 |

**Table 1. Top 10 Functional Annotation provided by DAVID Bioinformatics for the data corresponding to Day 1, when organized according to descending p-values for each annotation.** Table presents the term, genes, p-value and fold enrichment for the category.

On day 2, sleep is the seventh annotation most represented in our data. Genes corresponding to cytoplasmic translation, chemical synapse transmission, visual perception, centrosome duplication, determination of adult lifespan and synaptic transmission from cholinergic neurons are among the top six. Among the sleep genes affected on day 2 post ethanol administration were: *quiver*, *nacp60e*, *acer, sh, rdl, mub, nachralpha4, cana-14f*, *shaw, calr* and *atta*. **Table 2** presents these gene annotations in order of descending p-value, along with the genes, p-values and fold enrichment of each category.

| Term | Genes | P-value | Fold Enrichment |
|---|---|---|---|
| cytoplasmic translation | RPL4, STA, RPL5, RPL30, RPL3, RPL32, RPL31, RPLP1, RPLP0, RPL34A, RPL34B, RPL8, RPL9, RPL6, RPL7, RPS15, RPL7A, RPS17, RPS16, RPL18A, RPS18, RPL35, RPL38, RPS11, RPL39, RPS13, RPS12, RPS9, RPS7, RPL21, RPS8, RPL23, RPS6, RPL13A, RPL37A, RPL27, RPL26, RPL28, RPL12, RPL10AB, RPL11, RPL36A, RPS3, | 4.31E-47 | 8.508809 |
| chemical synaptic transmission | BRP, VMAT, CPO, PARA, IPP, SHAB, CAMKII, ATPALPHA, VACHT, ACE, NACHRALPHA7, SYT1, CAC, EAAT1, KCC, FRQ2, FRQ1, DLG1, ROP, UNC-13, SAP47, PGK, LAP, CALS, GAT, CSAS, CHC | 5.26E-13 | 5.355194 |
| visual perception | RH6, GALPHAQ, INAC, GGAMMA30A, RDGC, GYCALPHA99B, CHP, RDGA, RDGB, ARR1, ARR2, NONA, SOL, NORPA, NINAC, NINAE, RH2, RH5, RH4 | 8.508E-8 | 4.441411 |
| centrosome duplication | RPL4, RPL30, RPL3, RPL10AB, RPL11, RPL8, RPL9, RPL6, RPL7, RPL7A, RPL18A, RPL14, RPL13, RPL35, RPS4, RPL17, RPL19, RPL21, RPS6, RPL13A, RPL23A, BETATUB56D, RPL27A, RPL27, HSC70-3, RPL28, RPS23 | 9.89E-8 | 3.272619 |
| determination of adult lifespan | F1ALPHA48D, ATPSYND, ATP6, LT, COMT, TPI, EGFR, ALPHA-MAN-IA, THIOLASE, CLN3, CALPA, ATPALPHA, BABO, JAFRAC1, MTPALPHA, WDB, SM, ALPHA-EST7, GLYP, TRXR-1, IH, DOR, ILP2, EAAT1, MIPLE1, GNMT, THOR, KIS, ECR, MEN, AGBE, NF1, UBA1, RL, DAW | 1.53E-7 | 2.695098 |
| synaptic transmission, cholinergic | NACHRALPHA4, CG1909, NACHRALPHA5, NACHRALPHA6, NACHRALPHA7, NACHRBETA1, DOPECR, NACHRBETA2, NACHRALPHA1, NTR | 2.76E-7 | 8.726984 |
| sleep | AMY-P, QVR, NACP60E, ACER, SH, DNASEII, COX1, RDL, MUB, SODH-1, NACHRALPHA4, SPN88EB, SPEN, CANA-14F, SHEP, ILP2, PPO1, BGM, SHAW, IRIS, KIS, MEN, HSC70-3, CALR, ATTA | 3.55E-7 | 3.004371 |
| antibacterial humoral response | THOR, CECA1, CECA2, DRS, DEF, IM23, ATTC, CECC, IM3, ATTA, ATTB | 2.89E-6 | 6.260662 |
| deactivation of rhodopsin mediated signaling | NORPA, INAC, RDGC, NINAC, RDGA, RDGB, ARR1, CAM, ARR2 | 4.40E-6 | 7.854285 |
| humoral immune response | CECA1, IM4, CECA2, DEF, SPE, DPTB, ATTC, NPLP2, CECC, ATTA, ATTB | 4.631E-6 | 5.999801 |

**Table 2. Top 10 Functional Annotation provided by DAVID Bioinformatics for the data corresponding to Day 2, when organized according to descending p-values for each annotation.** Table presents the term, genes, p-value and fold enrichment for the category.

On day 3 **(Table 3),** we can observe that genes associated with sleep are among the 9th most affected after drug administration. Genes involved in cytoplasmic translation, chemical synaptic transmission, neurotransmitter secretion, locomotor rhythms, centrosome duplication, neuromuscular synaptic transmission, synaptic transmission from cholinergic neurons & muscle cell cellular homeostasis are among the top 8. Sleep genes such as *quiver*, *nacp60e, acer, sh, rdl, mub, nachralpha4, shaw, rut, tim* and *atta* showed a change in differential expression the third day after ethanol administration.

| Term | Genes | P-value | Fold Enrichment |
|---|---|---|---|
| cytoplasmic translation | RPL4, STA, RPL5, RPL30, RPL3, RPL32, RPL31, RPLP1, RPLP0, RPL34A, RPL34B, RPL8, RPL9, RPL6, RPL7, RPS15, RPL7A, RPS17, RPS16, RPL18A, RPS18, RPL35, RPL38, RPS11, RPL39, RPS13, RPS12, RPS9, RPS7, RPL21, RPS8, RPL23, RPS6, RPL13A, RPL37A, RPL27, RPL26, RPL28, RPL12, RPL10AB, RPL11, RPL36A, RPS3, | 4.31E-47 | 8.50880 |
| chemical synaptic transmission | BRP, VMAT, CPO, PARA, IPP, SHAB, CAMKII, ATPALPHA, VACHT, ACE, NACHRALPHA7, SYT1, CAC, EAAT1, KCC, FRQ2, FRQ1, DLG1, ROP, UNC-13, SAP47, PGK, LAP, CALS, GAT, CSAS, CHC | 5.26E-13 | 5.35519 |
| visual perception | RH6, GALPHAQ, INAC, GGAMMA30A, RDGC, GYCALPHA99B, CHP, RDGA, RDGB, ARR1, ARR2, NONA, SOL, NORPA, NINAC, NINAE, RH2, RH5, RH4 | 8.50E-8 | 4.441411 |
| centrosome duplication | RPL4, RPL30, RPL3, RPL10AB, RPL11, RPL8, RPL9, RPL6, RPL7, RPL7A, RPL18A, RPL14, RPL13, RPL35, RPS4, RPL17, RPL19, RPL21, RPS6, RPL13A, RPL23A, BETATUB56D, RPL27A, RPL27, HSC70-3, RPL28, RPS23 | 9.89E-8 | 3.272619 |
| determination of adult lifespan | F1ALPHA48D, ATPSYND, ATP6, LT, COMT, TPI, EGFR, ALPHA-MAN-IA, THIOLASE, CLN3, CALPA, ATPALPHA, BABO, JAFRAC1, MTPALPHA, WDB, SM, ALPHA-EST7, GLYP, TRXR-1, IH, DOR, ILP2, EAAT1, MIPLE1, GNMT, THOR, KIS, ECR, MEN, AGBE, NF1, UBA1, RL, DAW | 1.53E-7 | 2.695098 |
| synaptic transmission, cholinergic | NACHRALPHA4, CG1909, NACHRALPHA5, NACHRALPHA6, NACHRALPHA7, NACHRBETA1, DOPECR, NACHRBETA2, NACHRALPHA1, NTR | 2.76E-7 | 8.726984 |
| sleep | AMY-P, QVR, NACP60E, ACER, SH, DNASEII, COX1, RDL, MUB, SODH-1, NACHRALPHA4, SPN88EB, SPEN, CANA-14F, SHEP, ILP2, PPO1, BGM, CG42613, SHAW, IRIS, KIS, MEN, HSC70-3, CALR, ATTA | 3.55E-7 | 3.004371 |
| antibacterial humoral response | THOR, CECA1, CECA2, DRS, DEF, IM23, ATTC, CECC, IM3, ATTA, ATTB | 2.89E-6 | 6.260662 |
| deactivation of rhodopsin mediated signaling | NORPA, INAC, RDGC, NINAC, RDGA, RDGB, ARR1, CAM, ARR2 | 4.40E-6 | 7.854285 |
| humoral immune response | CECA1, IM4, CECA2, DEF, SPE, DPTB, ATTC, NPLP2, CECC, ATTA, ATTB | 4.63E-6 | 5.999801 |

**Table 3 Top 10 Functional Annotation provided by DAVID Bioinformatics for the data corresponding to Day 3, when organized according to descending p-values for each annotation.** Table presents the term, genes, p-value and fold enrichment for the category.

Finally, **Table 4** summarizes the functional annotations most represented in our data on day 4. Among these categories, cytoplasmic translation, synaptic vesicle exocytosis, neurotransmitter secretion, response to sucrose, translation, sleep, axon guidance, courtship behavior, chemical synaptic transmission & regulation of circadian sleep/wake cycles seem to be affected by ethanol. Sleep genes most significantly affected were: *drat, sh, rdl and quiver.*

| Term | Genes | P-Value | Fold Enrichment |
|---|---|---|---|
| cytoplasmic translation | RPL3, RPS8, RPL10AB, RPL12, RPL7, RPS15, RPS16, RPS18, RPL36, RPS3, RPS4, RPS2, RPS30, RPS23, RPS12 | 3.84E-14 | 18.53258 |
| synaptic vesicle exocytosis | UNC-13, SYT1, CADPS, CPX, SYT7 | 3.60E-05 | 25.73970 |
| neurotransmitter secretion | SNAP25, UNC-13, HSC70-4, SYT1, CAC, CADPS, SYT7 | 7.55E-05 | 9.717459 |
| response to sucrose | DESAT1, ACC, FASN1, PYK | 1.69E-04 | 35.30016 |
| translation | RPL3, RPS8, RPL10AB, RPL12, RPS15, EF2, RPS16, RPS18, RPL36, RPS3, RPS4, RPS2, RPS30, RPS23, RPS12 | 2.75E-04 | 3.083624 |
| sleep | DRAT, KIS, SH, MEN, AAY, RDL, QVR | 4.26E-04 | 7.088966 |
| axon guidance | PROS, HSC70-4, KIS, BETA-SPEC, PLEXA, FAS1, AAY, DAW | 6.86E-04 | 5.342727 |
| courtship behavior | CAMKII, PROS, CAC, SH | 0.001127 | 19.00777 |
| chemical synaptic transmission | CAMKII, UNC-13, SYT1, CAC, CALS | 0.001891 | 9.359891 |
| regulation of circadian sleep/wake cycle, sleep | SH, QVR, HSP83 | 0.007965 | 21.80304 |

**Table 4. Top 10 Functional Annotation provided by DAVID Bioinformatics for the data corresponding to Day 4, when organized according to descending p-values for each annotation.** Table presents the term, genes, p-value and fold enrichment for the category.

## 5.4. Discussion

In this study, we conducted an analysis of how PDF and DH44 gene expression changed after ethanol exposure. We saw that alcohol decreases both PDF and DH44 gene expression at the beginning of the experiment. This evidence points out that alcohol could be decreasing stress by affecting LNv release of PDF and the Pars Intercerebralis release of DH44 **(Figure 13).** However, more experiments are needed in order to elucidate the changes in PDF and DH44 across time after ethanol exposure. I suggest that confocal experiments where PDF and DH44 release are quantitatively measured after ethanol exposure could be performed in the future.

Our bioinformatic analysis reveals that sleep is among the most affected categories for four days after ethanol exposure according to DAVID. Most of these genes code for calcium channels, potassium channels and sodium channels that are found in the brain and are vital in producing the action potential. Among the sleep genes observed, a specific set was studied based on their functional role in sleep. We dedicated this section to reviewing the function of these genes in order to try and elucidate their role in alcohol induced sleep behaviors.

We decided to look for information regarding the sleep related genes that were most repeated throughout the 4 days. One of these genes in our list is *quiver* (*qvr*), which in *Drosophila* is an allele of the gene *sleepless* and its mutation is known to disrupt currents generated by potassium channel Sh also known as Shaker (Humphreys., 1966; Wang., 2000). *Sleepless* is a gene that codes for a glycosyl-

phosphatidylinositol-anchored protein that is important for the generation of sleep (Koh et al., 2008). Loss of the gene *sleepless* has been associated before with both a significant reduction in sleep and sleep rebound (Koh et al., 2008). Interestingly enough, Sh mutants also have a reduced-sleep phenotype (Cirelli et al., 2005). Another potassium channel that regulates neuronal firing and appeared in our list of genes is called *Shaw*. SHAW is known to play an important role in generating circadian rhythms (Gasque et al., 2005), even more so, when it is overexpressed in LNvs (Hodge et al., 2019).

Other genes from this set code for proteins that are involved in calcium-signaling transduction pathways. For example, CALCINEURIN B is a phosphatase stimulated by calcium and calmodulin. CALCINEURINS are proteins that react to and are activated by calcium and they serve a role in relaying signals to other proteins, enzymes and/or ion channels once they detect changes in calcium levels (Klee et al., 1979; Stewart et al., 1982). In this specific case, this protein activates a phosphatase that removes phosphate groups from serine and threonine residues. This protein is mostly expressed in the brain but it can be found on non-neuronal tissues as well (Kincaid, 1993). It is typically known to be involved in intracellular calcium signaling, gene regulation and other biological responses (Crabtree., 1999). CANA-14F is a subunit of CALCINEURIN while RUT (Nakai et al., 2011; Diesel et al., 2015). Interestingly, CALCINEURIN B is involved with sleep, as pan-neuronal knockdown of this gene has been shown to effectively reduce sleep as well (Tomita et al., 2011). CANA-14F is another gene that is

important in increasing sleep (Nakai et al., 2011) while RUT seems to be involved in sleep processes related to visual attention (Kirszenblat et al., 2018).

In addition, other genes that showed up in our lists are called: *dar, drat, dco, rdl and nachralpha4*. *Dat* codes for a DOPAMINE transporter that decreases sleep in flies when it is knocked-down (Ueno et al., 2012). On the other hand, *drat* codes for a protein that is an effector of alcohol apoptosis (Chen et al., 2012). In addition, *dco* is a gene that codes for the catalytic subunit of cAMP protein kinase pathway (PKA). Studies show that DCO binds with period and plays a major role in generating circadian rhythms (Kalderon et al., 1997). *Rdl* is a gene that codes for a GABA receptor that has been located on PDF neurons and RNAi knockdown of this gene in LNvs have reduced sleep (Agosto et al., 2008). Lastly, nachralpha4 codes for a subunit of the nicotinic acetylcholine receptor, which plays a role in restoring sleep (Wu et al., 2015).

The last set of genes play a role in sleep fragmentation; these genes code for proteins CALR and ACER. *Carl* codes for a calcium binding chaperone that in mice has been associated with neuroprotection of, most specifically, dopaminergic neurons (Dukes et al., 2008; Lessner et al., 2010). This gene seems to increase when flies experience fragmented sleep (Williams et al., 2016). Finally, ACER is an angiotensin converting enzyme that plays a role in the disruption of night-time sleep (Carhan et al., 2011).

The aforementioned evidence suggests that most of these genes are important for inducing sleep. We think that alcohol is probably increasing the expression of these

genes as data from Chapter 2 suggest that alcohol increases sleep during the day. In addition, it is of no surprise to see that some genes are involved with generating circadian rhythms as we have previously discussed evidence that suggest that alcohol severely disrupts this biological phenomenon (Chen et al., 2006; Prosser et al., 2008; Bragger et al., 2009; Farnell et al., 2009; Comasco et al., 2010). Finally, even more intriguing is the identification of genes that disrupt specifically night time sleep as our evidence from Chapter 2 suggest that alcohol causes a disruption in night-sleep. In the future, scientists can ultimately activate or inactivate these genes on different neuronal circuits to uncover more in-depth how they can affect alcohol-induced sleep behaviors. As our alcohol-sleep studies indicate that the drug is increasing and consolidating total daytime sleep and affect night sleep architecture, I would begin my experiments with those genes that are important for generating sleep and disrupting night-time sleep.

# Chapter 6. Discussion of Results

Alcohol is a drug of abuse that is known to elicit a variety of neurobiological changes that occur in order to mitigate the initial effects of the drug. It has been documented that these adaptations lead to the development of tolerance and dependence (Himmelsbach., 1964; Ritzmann & Tabakoff., 1976; Miller et al., 1987; Clapp et al., 2008; Kalivas & O'Brien., 2008). On the other hand, sleep is a biological process that can be affected by internal and external factors that influence the survival of the organism (Altun et al., 2012; Yilmaz et al., 2017). Thus, we think that there might be a link between alcohol and sleep where alcohol can serve as an external factor that affects sleep. In order to prove this concept, we used *Drosophila melanogaster* to uncover the relationship between alcohol and sleep, amplifying the scientific use of this animal model to explore the underlying molecular and neuronal mechanisms behind alcohol induced sleep dysregulation. We used a combination of genetic tools to electrically manipulate the activity of a specific set of cells in the *Drosophila* brain known as the Lateral Ventral Neurons (LNvs), that have been shown to be involved in sleep regulation, the arousal state and alcohol responses.

Interestingly, *Drosophila melanogaster* offers a unique biological model that can effectively be used to study sleep. The circadian circuit of *Drosophila melanogaster* has been well studied over the years. As we have described before, the circadian circuit is composed of approximately 150 neurons that express the core molecular clock components that secrete a wide range of neurotransmitters and neuropeptides that

have a myriad of functional roles (Kaneko et al., 2000; Forster et al., 2007; Schubert et al., 2018; King & Sehgal., 2020). These neurons have a similar biological function to the mammalian suprachiasmatic nucleus (SCN), the main mammalian regulator of circadian rhythms of behavioral activity. The lateral ventral neurons are of particular interest for our study; they are made of 4-5 small cells that are found in the accessory medulla and innervate regions such as the optic lobe and proto cerebellum (Yao & Shafer., 2014; Schubert et al., 2018). An important neuropeptide that they secrete is called PDF, which has been well documented to regulate circadian rhythms and sleep (Helfrich-Förster., 1998; Renn et al., 1999). Specifically, PDF is found in all *Drosophila* lateral ventral neurons, except in the 5th small LNv (Helfrich-Förster., 1998; Schubert et al., 1998). Lateral Ventral Neurons communicate with various neurons, but of most importance to us is their interaction with Dorsal Neurons. Dorsal Neurons are made of three cells titled DN1, DN2 and DN3 (Rieger et al., 2006; Yao & Shafer., 2014; Liang et al., 2017). They serve as a link between the circadian clock neurons and other brain structures to regulate biological processes (Schubert et al., 2018; Reinhard et al., 2022). VIP (VasoIntestinal Peptide) is the mammalian homolog of PDF.

In our first set of experiments, we focus on understanding the role of LNvs in alcohol sensitivity and tolerance. We saw that baseline alcohol sensitivity is influenced by a mutation in the gene that codes for PDF. Specifically, we found that flies that lack PDF neuropeptide were more resistant to alcohol than controls. In addition, interestingly enough, we saw that flies that lack the PDF neuropeptide lack the ability to acquire alcohol tolerance. When we silenced LNv using potassium channel Kir2.1, we saw that

flies had the ability to develop tolerance. On the other hand, flies whose PDF neurons were silenced using tetanus toxin or TeTxLC were unable to develop this alcohol response. Finally, when activating PDF neurons by overexpressing sodium channel NaChBac, we saw that flies still had the ability to develop tolerance. Although contradictory, these results are of no surprise to us as the two methods we used for inactivating Lateral Ventral Neurons can influence the neurophysiology and activity of the neuron in very different ways. In humans, tetanus toxin is a chemical produced by the anaerobic bacillus' Clostridium tetani and is the primary cause for tetanus symptoms such as muscular rigidity and spasms (Mendell et al., 2005). From a molecular level, tetanus toxin directly interferes with neuronal vesicle fusion and both neurotransmitter and neuropeptide release by cutting synaptobrevin, an important protein of the SNARE complex. Research in *Drosophila* indicates that expressing tetanus toxin in embryonic neurons significantly removes synaptobrevin, directly reduces synaptic activity as well as vesicle release (Sweeney et al., 1995). On the other hand, Kir 2.1 is a potassium channel that effectively produces a filter for potassium and thus allows for the movement of potassium ions across the neuronal membrane (Hibino et al., 2010). It is composed of a tetrameric structure of Kir2.1 subunits and possesses a two-transmembrane domain (de Boer et al., 2010). In *Drosophila*, expression of Kir2.1 suppresses action potential on embryonic neurons, thereby influencing neurotransmitter, however, not necessarily neuropeptide release as these chemicals do not need the presence of the action potential to be released l (Baines et al., 2001). Lastly, NaChBac is a sodium channel of the bacteria Bacillus halodurans. Structurally, this channel only has one domain that possesses six transmembrane segments that

connect with each other via extra-and intracellular loops, resembling the structure of voltage gated potassium channels rather than sodium or calcium channels (Ren et al., 2001). NaChbac is a channel that is selective for sodium ions and activates by voltage (Ren et al., 2011) In addition, structurally speaking, NaChBac monomers are known to organize themselves into tetrameric structures to form channels (Ren et al., 2001).

It is important to state that PDF is not the only chemical substance released by LNvs. Thus, these manipulations can interfere with the LNvs ability to release a wide variety of neurotransmitters and neuropeptide. In a review written by Nassel., 2018, it was stated that LNvs allow for the release of neurotransmitters such as glycine and neuropeptides such as NPF, sNPF and ITP. And although *Pdf*$^{01}$ flies do not have the gene that codes for the PDF neuropeptide, thus cannot release PDF, they can release other neurotransmitters and neuropeptides such as NPF, sNPF, ITP and glycine. Manipulations with Tetanus Toxin interferes with specifically synaptic vesicle fusion, which can affect both the release of neurotransmitters and neuropeptides. On the other hand, Kir 2.1 electrically silences the neurons, but does not prevent PDF or other neuropeptides from being released in the soma (Klose et al., 2021). However, it may block neurotransmitter such as glycine release. Lastly, in theory, NaChbac allows for the release of all chemical substances produced by LNvs, including neurotransmitters and neuropeptides. **Table 5** summarizes this information.

| Chemical Substance | Neurotransmitter/ Neuropeptide | *Pdf*$^{01}$(mutant fly line that lacks the PDF neuropeptide) | Manipulation with UAS-TeTxLC (prevents vesicle from fusing and the release of both neurotransmitters and neuropeptides) | Manipulation with Kir 2.1 (electrically silence neurons) | Manipulation with NaChBac |
|---|---|---|---|---|---|
| PDF | Neuropeptide | Blocks release of PDF | Blocks release of PDF | Can still release PDF | Can still release PDF |
| NPF | Neuropeptide | Can still release NPF | Blocks release of NPF | Can still release NPF | Can still release NPF |
| sNPF | Neuropeptide | Can still release sNPF | Blocks release of sNPF | Can still release sNPF | Can still release sNPF |
| ITP | Neuropeptide | Can still release ITP | Blocks release of ITP | Can still release ITP | Can still release ITP |
| Glycine | Neurotransmitter | Can still release glycine | Blocks release of glycine | Blocks release of glycine | Can still release glycine |

**Table 5. Effect of Manipulations on Neurotransmitters and Neuropeptides secreted by LNvs.** Chemical substance secreted by the LNv neuron, followed by classification of chemical substance (neuropeptide/neurotransmitter) and effect of the genetic manipulations on release of said neurotransmitter and neuropeptide.

Considering the results obtained with alcohol sensitivity and tolerance, we sought to understand how alcohol affects sleep through lateral ventral neurons as possible neuronal candidates that mediate this process. But first, we described the effect an acute dose of alcohol has on sleep in *Drosophila*. We found that i) Daytime sleep is increased by alcohol exposure. Specifically, as alcohol increased mean sleep episode duration and decreased number of sleep episodes, we think that alcohol is important in consolidating daytime sleep ii) Although in total, sleep at night remained unaffected after ethanol exposure, we found that the architecture of Nighttime sleep was affected as alcohol exposed flies slept more during the beginning of the night and less at the end iii) We found that alcohol plays an important role in morning anticipation. Moreover, it has been well documented that LNvs are involved with this biological phenomena in *Drosophila*, implicating that they could be involved in alcohol responses as well.

Thus, we decided to first explore the effect alcohol has on sleep by using the transgenic mutant *Pdf*$^{01}$; a mutant that lacks the ability to secrete the PDF neuropeptide. When we look at how alcohol affects sleep in *Pdf*$^{01}$ flies, we saw that i) alcohol induced sleep behaviors as described in wild-type CS flies are only partially affected by PDF. Specifically, we found no significant difference on day-time sleep after ethanol exposure in wild-type CS and *Pdf*$^{01}$ mutants. However, the number of sleep episodes and mean sleep episodes remained unaffected by ethanol on *Pdf*$^{01}$ flies ii) alcohol does not seem to affect night-time sleep architecture on *Pdf*$^{01}$ flies iii) *Pdf*$^{01}$ mutants lack morning anticipation, which still remain unaffected after ethanol exposure.

We also saw a very interesting behavior in sleep architecture that manifested itself more robustly in untreated *Pdf*$^{01}$ mutants rather than wild-type CS flies. We saw that there was a significant increase in night-time sleep after performing the experiment in untreated *Pdf*$^{01}$ controls. We think that this increase in sleep is tightly associated with what we know as the first-night effect. In humans, it has been well documented that sleep tends to decrease when the organism is found in a novel and unfamiliar environment (Agnew et al., 1966; Tamaki et al., 2016; Tamaki et al., 2019). It is produced because half of the brain is more vigilant than the other when the organism is sleeping in a new place, which is probably a protective mechanism derived from staying guard for predators (Tamaki et al., 2016; Tamaki et al., 2019). It is one of the primary reasons as to why we usually feel tired after sleeping in a novel environment (Tamaki et al., 2016). Although the scientific literature regarding the first night effect in *Drosophila melanogaster* is scarce, the first night effect has been reported in other mammals such

as cows (Ternman et al., 2018) and dogs (Reicher et al., 2020). During our experiments, flies are taken out of their vials where they are housed with other flies and placed individually in small tubes. And although we have seen that total sleep itself is increased during the first two days after ethanol exposure, in the specific case of untreated wild-type CS flies, sleep tends to increase during the night after the experiment pass by as well. Even though the molecular mechanisms behind the first night effect have not been studied, curiously enough, our research suggests that the first night sleep effect seems to be more evident in *Pdf*$^{01}$ mutants.

Scientific evidence indicates that there is a link between alcohol and sleep through mechanisms related to stress. Studies have found an association between social stress in the early stages of life and an increase in alcohol consumption later on (Dube et al., 2006). In humans, the hypothalamic-pituitary adrenal axis and the hyopthalamic brain stress axis modulate both circadian and stress responses (Nader et al., 2010; Herman et al., 2016). Interestingly, studies report that these regions are influenced by alcohol exposure as well (Stephens & Wand., 2012). From a molecular perspective, circadian genes clock and cry modulate the secretion of stress related hormones and neuropeptides through the HPA axis (Nader et al., 2010; Koch et al., 2017).

In *Drosophila*, studies have found a direct connection between PDF release and stress (Furuya et al., 2000; Cabrero et al., 2002; Johnson et al., 2005; Hector et al., 2009; Cavanaugh et al., 2014; Cannell et al., 2016; Goda et al., 2019; Barber et al.,

2021). Specifically, they point out that LNvs, through the secretion of PDF, activate the release of DH31 by DN1 neurons, the homolog for the vertebrate neuropeptide calcitonin gene-related peptide (CGRP) (Johnson et al., 2005; Goda et al., 2016). It has been documented that in mammals, this peptide is a vasodilator, involved in increasing neuronal activity specifically in brain structures associated with anxiety (Furuya et al., 2000; Sink et al., 2011). In the fly, DH31 activates three pairs of cells that are located in the Pars Intercerebralis, that in turn activates the secretion of Diuretic Hormone 44 (DH44), the mammalian counterpart of the stress hormone corticotropin releasing factor, which has been well known to be involved with stress (Cabrero et al., 2002; Johnson et al., 2005; Hector et al., 2009; Cavanaugh et al., 2014; Cannell et al., 2016; Goda et al., 2019; Barber et al., 2021). In addition, a recent study suggests that loss of function DH31 mutants possess a high amount of sleep in the lasts 6 hours of the night, while, on the other hand, flies where DH31 was expressed pan-neuronally possessed reduced sleep during that same time period (Kunst et al., 2014). Interestingly, this effect is consistent with our results relating to alcohol and PDF. All together, this evidence points out that there might be a link between alcohol, LNv neurons and DN1 neurons. This stress related neuronal pathway might constitute a possible modulator of the effect alcohol has on sleep. **Figure 19** is a representation of the stress pathway that could be affected by alcohol via LNvs. Interestingly enough, our RNA sequencing data suggest that alcohol is decreasing PDF and DH44 gene expression after ethanol exposure.

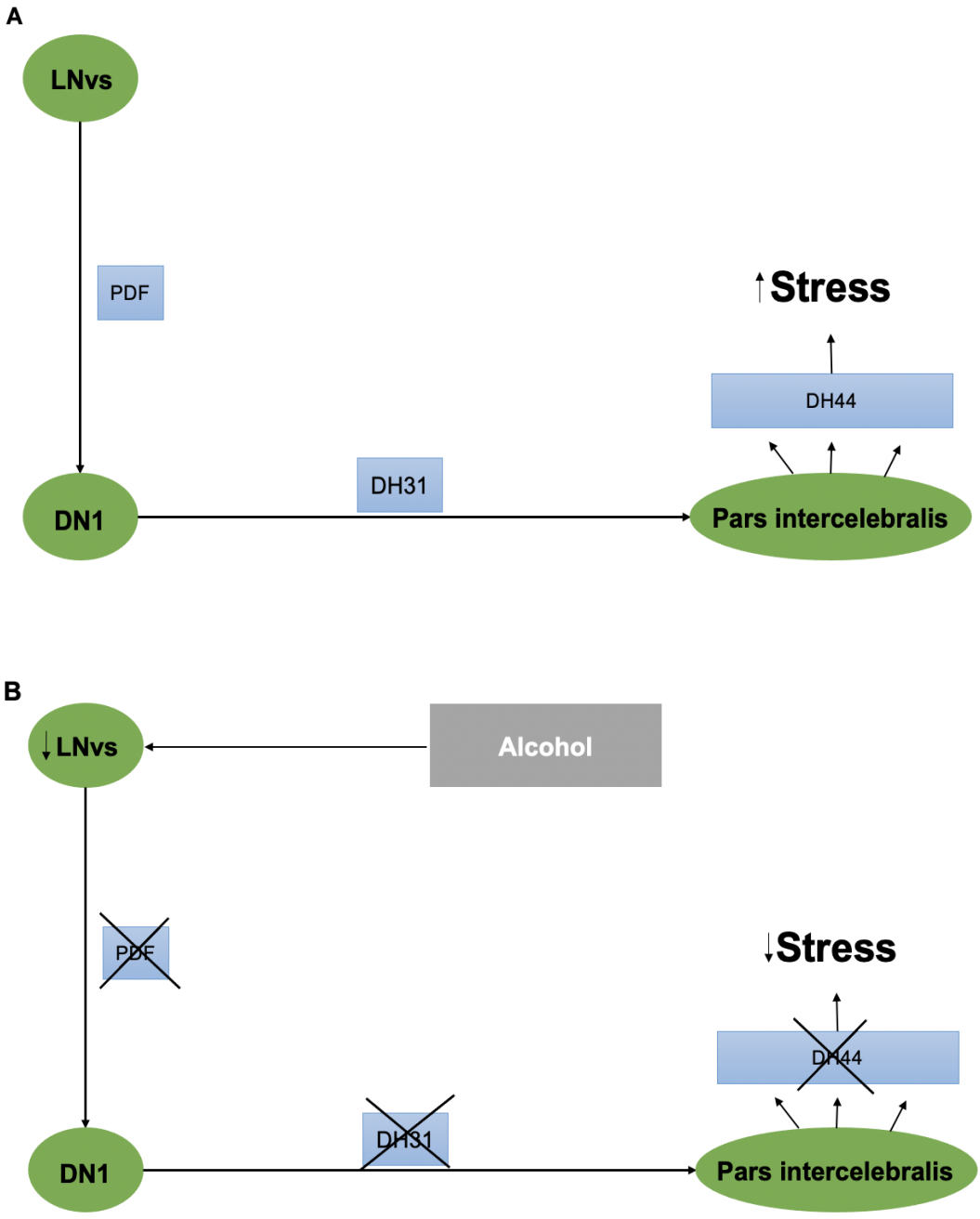


**Figure 19. Alcohol Effect on Lateral Ventral Neurons via Pathway Related to Stress** A) LNvs potentiate secretion of DH31 by DN1 through the PDF neuropeptide. DN1 secretion of DH31 enhances the Pars Intercelebralis to produce DH44, which in mammals is known as corticotropin-stress releasing factor and increases stress. B) Alcohol diminishes the activities of LNvs, which in turn affects PDF release and DH31 release by DN1s. At the end, the Pars Intercelebralis does not release DH44, diminishing stress overall.

While there are still many questions regarding the role of LNvs in alcohol induced responses, our results suggest that this interaction might potentially be through the aforementioned proposed mechanism. We think that PDF release is suppressed by alcohol exposure. In turn, DH31 release by DN1s neurons is affected, causing a disruption in the release of DH44, which could be affecting stress. Nevertheless, more studies are needed in which PDF and DH44 release is quantitatively measured in order to elucidate its role on alcohol-induced sleep behaviors. However, all together, this thesis provides the scientific community with novel research. First, it tries to uncover the role of LNvs in alcohol responses, such as sensitivity and tolerance as well as alcohol induced sleep behaviors, an area where very little research has been conducted in the past. Lastly, to our knowledge this is the first study to effectively characterize the effect alcohol has on sleep in *Drosophila melanogaster*. This investigation can serve as a foundation for future studies that try to elucidate the neuronal and molecular mechanisms behind these behaviors.

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