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STATISTICAL MODEL FOR THE PREDICTION OF PREFRONTAL CORTEX NEURONAL SIGNALING

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ABSTRACT

Alcohol Use Disorder (AUD) is a public health issue with high costs to the United States. Though no comprehensive model of why individuals develop AUD exist, a combination of genetic, environmental, and dysregulation of neuronal signaling are thought to play a role. In addition to the lack of clear understanding of the causes of AUD, there is a desperate need for targeted treatment through behavioral and pharmaceutical approaches to treat this devastating disorder. The behavioral predictors of neural states in AUD are mostly studied through univariate analysis in varied AUD animal models instead of recognizing the dynamic relationship of behavior and neural signaling through multivariate analysis. Therefore, this project investigated the neurophysiological basis of anxiety-like behavior, via the development of a multivariate statistical and behavioral model capable of predicting the neuronal signaling underlying high and low anxiety behaviors. In future experiments we hope to expand this to high- and low- alcohol binge drinking mice. A particular region of interest for our model is the prefrontal cortex (PFC), in which layers 2/3 pyramidal neurons have reduced GABAergic inputs following varied models of ethanol exposure.

This project was divided into three parts: 1) Behavioral experiments to measure for anxiety-like behavior, 2) Electrophysiological measure of the mPFC, 3) Statistical analysis for correlations between behavior and brain physiology. Behavioral experiments include the use of Elevated Plus Maze (EPM), Open Field (OF), and Sucrose Preference (SP) tests on C57BL/6J male and female mice. Following behavioral assessments, whole cell patch clamp electrophysiology was performed to assess synaptic drive in the prelimbic cortex (a measurement of overall glutamate and γ-aminobutyric acid, GABA, balance). Then, in collaboration with The Department of Human Development and Family Studies, a linear regression was used to identify correlations between behavior and brain physiology, as well as to identify the key behaviors which best predict anxiety-like behavior. The results showed correlations between electrophysiology and behavior observed in OF, but not SP or EPM. The correlations were observed between spontaneous inhibitory postsynaptic current frequency and distance in OF, as well as spontaneous excitatory

postsynaptic current amplitude and velocity in OF. This finding is the first step towards the creation of a statistical model for the prediction of neural signaling in the PFC.

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Chapter 1

Introduction

Anxiety Disorders

Anxiety disorders are a category of mental disorders characterized by persistent feelings of fear and anxiety (Lenze and Wetherell 2011). According to the 5th Edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5), patients are diagnosed with an anxiety disorder if they express excessive anxiety or fear for a period of 6 months or more, relating to different activities such as work or school. Based on recent national surveys, the yearly prevalence of anxiety disorders in U.S. adults is estimated at 19% (Kessler et al. 2005). From this data, there is a higher prevalence of anxiety disorders for women (23.4%) than men (14.3%). Additionally, an estimated 31.9% of 13-18 years olds has, or is currently suffering from, an anxiety disorder, with 8.3% suffering from severe impairment (Merikangas et al. 2010). Hence, the prevalence of anxiety disorders is higher is children and teens than adults, implying a generational shift to a more anxious population. The prevalence of having any anxiety disorder was similar across age groups, but was higher for girls (38.0%) than for boys (26.1%), exemplifying the persistent sex differences in relation to mental disorders across the lifetime (Bandelow and Michaelis 2015).

Based on the European Study of the Epidemiology of Mental Disorders, only 20.6% of patients suffering from anxiety disorder seek treatment from accredited health care facilities. Typical treatments for anxiety disorders include a combination of psychotherapy and

pharmacotherapy, both chosen based on the patient's history, the severity of illness, previous treatment attempts, and with other psychiatric or health disorders (Cuijpers et al. 2013; Wedekind, Engel, and Bandelow 2006). The general treatment recommendations regarding first-line medications are Selective Serotonin Reuptake Inhibitors (SSRIs) and Selective Serotonin Norepinephrine Reuptake Inhibitors (SSNRIs) (Cassano, Baldini Rossi, and Pini 2002). Though the exact mechanism of action of SSRIs is unknown, they are thought to increase the extracellular levels of 5-hydroxytryptamine (5-HT) by selective blocking of the 5-HT transporter. The resulting increased concentration of 5-HT can lead to improve mood and reduced levels of depression and anxiety. SSNRIs supposedly work in a similar manner except that they not only increase the levels of 5-HT but also norepinephrine in the synapse, again by blockage of the neurotransmitters' respective transporters (Lambert and Bourin 2002; Sangkuhl, Klein, and Altman 2009).

The psychotherapy side of treatment includes two categories—behavioral and cognitive. Behavioral therapy for the treatment of anxiety disorders involves a therapist who will help the patient combat undesirable thoughts associated with anxiety and will often involve learning relaxation and deep breathing exercises (Otte 2011). As for cognitive therapies, a therapist helps the patient adapt their thought patterns into thoughts that are healthier.

The relapse rate of patients suffering from anxiety disorders is ambiguous and relies on a few studies. According to the Harvard/Brown Anxiety Research Project (HARP), the relapse rate over a 12-year follow-up period was 56% for panic disorder patients, 58% for patients suffering from panic disorder with agoraphobia, 38% for social phobia patients, and 45% generalized anxiety disorder patients (Bruce et al. 2005). Another study by Calkins et al. (2009) looked at the relapse rate of anxiety disorders during a 3-year follow-up of women. From the 643 women studied, 65% reported a recurrence of anxiety disorder as defined by meeting criteria for anxiety

disorders following a disease-free state over a significant period of time (Calkins et al. 2009). Hence, anxiety disorders have high rates of relapse, resulting in a sustained burden on the individuals' quality of life. According to Hoffman and colleagues (2008), anxiety disorders are significantly correlated with substantial human and economic costs. Patients suffering from anxiety disorders reported difficulties both on a physiological and emotional level which resulted in great social impairments (Hoffman, Dukes, and Wittchen 2008). Additionally, anxiety disorders are significant cost-drivers with respect to use of family physicians, medical specialists, and work cut-back days. As seen here, anxiety in itself is a truly debilitating disease, but it also has a high comorbidity with alcohol use disorders which tend to worsen its effects on human and economic burden (Smith and Randall 2012; Kushner, Abrams, and Borchardt 2000). Indeed, anxiety and alcohol use disorder (AUD) are two types of disorders that feed off each other as described by the mutual maintenance model, thus explaining the high rate of progression of the diseases if left untreated (Stewart and Conrod 2008).

As defined by the National Institute of Health, AUD is a chronic relapsing brain disease characterized by compulsive use or loss of control over alcohol consumption, resulting in negative emotional state when inaccessible (National Institute on Alcohol Abuse and Alcoholism, 2018). To be diagnosed with AUD, patients must meet specific criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013). Based on the DSM-5, one must meet at least two of the 11 criteria during a given 12 month period to receive a diagnosis of AUD. Depending on the number of criteria a patient fulfills, the severity of AUD would range from mild, moderate, to severe. According to the 2015 National Survey on Drug Use and Health, approximately 15.1 million of American adults ages 18 and older, or 6.1% of this age group, suffered from AUD (Center for Behavioral Health Statistics and Quality, 2016). As for

American adolescents, about 623,000 of the population ages 12-17, or 2.5% of the population in this age group, suffered from AUD (Center for Behavioral Health Statistics and Quality, 2016). An estimated 88,000 people die from alcohol-related causes annually. In addition to the loss or deterioration of lives, the misuse of alcohol accounted for approximately $249 billion of cost for the U.S. government (Sacks et al. 2015). Alcohol misuse and deaths related to it were not problems solely seen in the United States. 3.3 million deaths or 5.9% of the global deaths were due to alcohol consumption as reported in 2012 (Global Status Report on Alcohol and Health, 2014). In 2014, the World Health Organization reported that alcohol contributed to more than 200 diseases and injury-related health conditions, in particular, alcohol addiction and cirrhosis of the liver, and is considered a significant risk factor for multiple types of cancers (Global status report on alcohol and health, 2014). With such prevalence of AUD, adequate treatments that prove successful are a necessity. Multiple types of procedures are currently available to AUD patients which are divided into three categories: behavioral therapies, medications, and mutual-support groups, similar to those used for anxiety disorders (Seneviratne and Johnson 2015). Unfortunately, in the United States, only 6.7% of adults and 5.2% of adolescents who had AUD in the past year received adequate, government-approved treatments for their condition (Center for Behavioral Health Statistics and Quality, 2016).

Prolonged excessive alcohol consumption as seen in AUD triggers a change in the brain's reward and stress systems (Ma and Zhu 2014). There are neuroadaptive changes associated with not only alcohol consumption, but also dependence and withdrawal. Those changes are thought to contribute to the transition from moderate and controlled use of alcohol to excessive, symptomatic alcohol use (Becker 2012). The neuroadaptive changes within the brain reward system is thought to be the cause of the multiple symptoms observed in individuals suffering from alcoholism. When

alcohol withdrawal is attempted by an individual, characteristics symptoms ensued which can sometimes be life threatening and induced by the stress systems. Many withdrawal symptoms associated with this negative emotional state persist for an extended period and constitute a dominant motivational force promoting the perpetuation of alcohol use/abuse, as well as enhancing vulnerability to relapse (Becker 2014). Because alcohol relapse is associated with external stimuli and comorbidities that relate to alcohol abuse, there is a need for better treatment and improved prognosis.

Precision Medicine

Based on the lack of long-term positive outcomes of current treatments for AUD and anxiety disorders, precision medicine is a promising therapeutic approach to address these disorders effectively. The term "precision medicine" has become very popular in recent years, fueled by scientific and political outlooks (Roden and Tyndale 2013). Precision medicine has superseded the term "personalized medicine" since doctors already treat each patient on a personalized level, a switch in terminology that implies novelty and the incorporation of a wide array of individual data, including clinical, lifestyle, genetic, and biomarker information (König et al. 2017). The current understanding that clinicians, scientists, and the general population have of "precision medicine" is that it consists of highly complex machinery dedicated to a greater understanding of the molecular and physiological basis of diseases (Xue et al. 2016). An improved understanding of precision medicine could be the key to greater diagnostic and prognostic efficiencies, in addition to more powerful pharmacological tools.

The National Institute of Mental Health (NIMH) has launched the Precision Medicine Initiative for the treatment of mental health disorders (Ghitza 2015). The NIMH Research Domain Criteria is defined as a research method for new approaches spanning different levels of physiology from genomics to behaviors to explore dimensions of functions spanning the whole range of human behaviors in the normal and diseased state (National Institute of Health, 2018). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) has also accepted the precision medicine model whose goal is for health professionals to identify the most effective alcoholism treatment on a per person basis. The NIAAA and other organizations conduct and promote research to identify genetic markers capable of predicting how alcoholism patients may respond to certain treatments. Such endeavor is the first step in optimizing the way we treat and care for patients suffering from addiction (NIAAA, 2018). Previous studies have shown there are strong genetic influences on prefrontal cortical functions in the adolescents, especially those that later develop mental disorders such as schizophrenia (Harris et al. 2009; Anokhin et al. 2010). Hence, the prefrontal cortex, a crucial region in goal-oriented and higher order behaviors, is a great candidate for the prediction of mental disorders.

Role of Prefrontal Cortex

The existing literature assessing the underlying neurocircuitry of anxiety disorders implicate the amygdala and the prefrontal cortex (PFC) as having a major role in his pathology. The following studies were all performed via functional magnetic resonance imaging in humans which looks at blood-oxygenated level dependent changes during anxiety tasks. In a mixed cohort of patients with anxiety disorder and social phobia, patients intolerant to uncertainty showed

heightened amygdala activity during a decision-making task (Krain et al. 2008). Additionally, studies have shown that patients suffering from anxiety disorders demonstrate frightened facial expressions while anticipating aversive photographs (McClure and Pine 2007; Nitschke, Oathes, and Hilt, 2010). Though there are only few studies studying the circuitry of anxiety disorder, they tend to point towards the activation and/or dysregulation of the amygdala and the PFC.

The circuitry of AUD, and addiction in general, has been proposed to be divided in a network of four, interconnected, circuits: reward related circuits located in the nucleus accumbens and the ventral pallidum, motivation/drive related circuits located in the orbitofrontal cortex and the subcallosal cortex, memory/learning related circuits located in the amygdala and hippocampus, and finally control related circuits located in the prefrontal cortex and anterior cingulate gyrus (Gilpin and Koob 2009). Those circuits all receive dopaminergic innervation, but are also connected to one another through glutamatergic projections. In general, addictive behaviors are thought to follow a cyclical model involving three phases according to the model of plasticity in addiction characterized by Dr. George Koob, the current director of the NIAAA (Robinson and Kolb 2004). The first phase is the binge/intoxication phase which involves the dorsal striatum, the ventral tegmental area and the cerebellum. The second phase, withdrawal/negative affect, involves the basolateral amygdala and the central amygdala. The third phase, preoccupation/anticipation, involves the hippocampus and the prefrontal cortex (Herman and Roberto, 2015). It is still unclear how all those circuits come to play together into one general pattern of addiction. However, neuroimaging studies have shown the clear dysfunction of the prefrontal cortex in addictive behaviors (Goldstein and Volkow, 2011).

The PFC is a multimodal association cortical area mediating various sensory modalities (Siddiqui et al. 2008). Its primary task is integrating and interpreting incoming cortical information

to develop responses reflecting present and future circumstances. The functions of the PFC include goal-oriented behaviors, attention, short-term memory, planning, stimulus detection, and other executive functions (Siddiqui et al. 2008). The PFC sends projections throughout the brain, including regions such as the bed nucleus of the stria terminalis (Crowley et al. 2016), and other amygdala regions which are vital for the expression of fear (Marek et al. 2018) and anxiety (Parfitt et al. 2017; Sang et al. 2018). The PFC is the center of many neurobiological studies, as its dysfunction is involved in attention deficit/hyperactivity disorder, bipolar disorder, anxiety, and AUD (Gamo and Arnsten 2011). There is growing evidence that these diseases result from altered glutamatergic neurotransmission induced by projections of the PFC motivational and higher order cognitive processes that initiate and control goal-oriented behaviors (Jentsch and Taylor 1999; Kalivas 2010). Additionally, the results of several studies suggest that the plasticity of associative learning overlap with the development of addictive behaviors (Berke and Hyman 2000; Gass and Chandler 2013). An important feature of the PFC is its functional and structural adaptability, a major component of cortical cognitive processing strongly influenced by past experiences. Hence, the reward information such as that provided by the VTA dopaminergic system has widespread and lasting influence on the activity of the PFC. Although much research has been done in an attempt to understand the circuitry of the PFC and the critical signaling changes following perturbations (Crowley et al. 2016; Pleil et al. 2015b), the basal variability in PFC signaling is unknown, as well as its potential to predict deficits in patients with anxiety disorders or AUD.

Animal Models of the Disorders

Animal models of psychiatric disorders have offered critical advancements to the understanding of diseases (Nestler and Hyman 2010). The use of animal models is particularly useful for developing structural and predictive models within neuropsychiatric research, as it has become necessary to identify biomarkers for the prediction of behaviors related to a disorder and specialized treatments (Gomez-Mancilla et al. 2005). In this study, behavioral tests for the investigation of changes in anxiety-related behaviors and anhedonia were used and are described below.

The Open Field (OF) was designed in 1934 to study behavior in rodents and became one of the most utilized behavioral experiments in neuropsychology (Lezak, Missig, and Carlezon 2017). The OF offers an easy and rather quick evaluation of defined behaviors which do not necessitate training of the subject. The open maze consists of an area surrounded by walls of sufficient height to prevent the subject from escaping. The maze's shape is either square or round with a sufficiently large area to generate a sense of openness in the center of the maze. Most variables studied in the OF are locomotor activities which are used to study anxiety-related behaviors in the animals.

The Elevated Plus Maze (EPM) has been described by Pellow and colleagues as a method to evaluate the anxiety responses of rodents (Pellow et al. 1985). As its name indicates, the maze is elevated and plus shaped with four arms, two closed and two opened (Handley and Mithani 1984). The rodents are placed at the intersection of the four arms of the EPM and their behavior is usually recorded for 5 minutes. The common way of evaluating anxiety in rodents with the EPM is by studying the time spent in open arm and closed arm which is linked with the level of anxiety

of the animal. The behavior in EPM reflects the conflict between the rodent's preference for protected areas and its innate motivation to explore new environments (Prut and Belzung 2003).

In addition to behavioral models investigating anxiety-related behaviors, a test for anhedonia was also included in this study. The sucrose preference test (SP) is used to examine the loss of pleasure seeking or reward seeking (Eagle, Mazei-Robison, and Robison 2016). Rodents naturally consume sugary foods and selectively drink sweet drink rather than water when given the option. However, rodents that showed significant levels of stress or depression showed little to know preference for sweet drink, hence displaying anhedonia. Thus, the SP is a great way to measure anhedonia in stress/depressed rodents by giving a choice test of two bottles measuring the preferred nutrient intake, usually between water and 1-2% sucrose in water.

Electrophysiology

In vitro patch clamp recording is an electrophysiological technique developed in the 1970s as a way to study ion channels in live tissue (Verkhratsky and Parpura 2014). Whole cell patch clamp recordings can be used to study the electrical behavior of neurons which is usually carried out *in vitro* on brain slices. Patch clamping on brain slices is a significant advantage as it provides a physiologically relevant environment and the brain circuits are relatively well preserved compared to cell culture preparations, a method in which neurons are grown in vitro outside of their natural environment and under controlled conditions (Liem et al. 1995). This also allows visual identification of brain regions within slices, thereby affording high regional specificity. Additionally, patch clamping allows for the use of micropipette filled with internal solutions allowing for the direct study of communication between neurons via the assessment of

electrochemical properties of the cell membrane and interactions due to neurotransmitters (Di Berardino and Mckinnell 2004).

Synaptic transmission is generally induced by chemical transmission which can be due to ionotropic receptors or to metabotropic receptors (G protein coupled receptors) (Suszkiw 2011). Ionotropic transmission involves excitatory, glutamatergic receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartatereceptor (NMDAR) as well as inhibitory receptors γ-aminobutyric acid (GABA)A. The opening of ion channels resulting from the activation of those receptors lead to the flow of cation/anion in and out of the cells. The flow of cations and anions across the cell membrane can be measured in terms of spontaneous excitatory post synaptic currents (sEPSC) from glutamate transmission and spontaneous inhibitory post synaptic currents (sIPSC) from GABA transmission (Smith et al, 2000). Glutamate transmission leads to an increase of resting membrane potential which drives the firing of action potentials. On the other hand, GABA transmission decreases the resting membrane potential thus raising the difficulty of producing action potentials (Harvey et al. 2000). The sEPSC and sIPSC can offer information about the environment of a neuron based on the frequency and amplitude of currents observed (Purves, Augustine, and Fitzpatrick 2012). The amplitude of spontaneous post synaptic currents can be related to the number of glutamatergic or GABAergic receptors at the synapse. As for the frequency of spontaneous post synaptic currents, those are related to how many vesicles are released per action potential, including the number of neurotransmitters packed in each vesicle, as well as frequency of vesicle release (Kandel and Schwartz, 2014).

The primary cells of interest in the PFC in the current study are pyramidal neurons which are glutamatergic and represent 80% of its neuronal population (Malkin et al. 2015). The pyramidal neurons are divided in layers (I-VI) and establish glutamatergic connections with subcortical zones. In general, cortical pyramidal neurons can be divided into two classes: regular spiking neurons, which present a rather weak/slow adaptation with repetitive firing, and the intrinsic burst neurons, which present more complex firing patterns (Yang, Seamans, and Gorelova 2018). Whole cell patch clamping of PFC pyramidal cells will allow for a fast, precise measurement of electrical communication within and between neurons, allowing for a greater understand of the electrophysiology of the PFC in anxiety-related behaviors.

Creation of a Model to Predict Behavior Using Neurophysiology

As demonstrated, there is a gap in the knowledge concerning proper treatments for anxiety disorders and AUD. Not only do we lack a complete understanding of the physiological basis of anxiety-related disorders and AUD, but we also lack the patient-by-patient characterization of mental illnesses. Hence, there is a need for precision medicine in psychiatric disorders. The new field of "precision psychiatry" coined by Fernandes and colleagues (2017) point to the lack of advanced diagnostic and therapeutic technologies that other medical specialties have benefitted from (Fernandes et al. 2017). With the growing acceptance of psychiatric disorders as having an actual physiological and not just psychological basis, precision psychiatry can only be accomplished with the development of new diagnostic and prognostic tools, personalized and guided treatments for patients, as well as precise and novel pharmacological treatments.

Due to the nature of psychiatric symptoms, the modeling of neuropsychiatric disorders from humans to animals is challenging (Nestler and Hyman, 2010). However, the benefits of their models are systemic, accurate ways of diagnosing a disease based on observable behaviors. A few studies have shown the possibility of using behavioral predictors of neural states in AUD models in relation to mental disorders such as schizophrenia (Khokhar and Todd 2018; Brumback et al. 2016) . However, the study of anxiety-related behavioral predictors of neural states in AUD using a multivariate analysis method would be a novel way to model AUD in relation to mental disorders. Hence, the creation of a statistical and behavioral model capable of predicting high- and low-alcohol binge drinkers based on basal anxiety and neurophysiology could be a revolution for the treatment of AUD and anxiety related disorders. In this study, we hypothesize that anxiety phenotype in mice could predict the neuronal physiology at the level of the PFC as observed in patch clamp physiology. We used behavioral experiments that include EPM, OF, and SP tests on C57BL/6J male and female mice to score them for levels of anxiety-like and anhedonia behaviors. Following behavioral assessments, whole-cell patch clamp electrophysiology was performed to assess synaptic drive in the prefrontal cortex (a measurement of overall glutamate and GABA balance). Then, linear regression analysis was conducted to identify correlations between behavior and brain physiology, and importantly, identify the key behaviors which best predict anxiety-like behavior. The overall goal of this study was to predict electrophysiological patterns based on behavior, a crucial step towards precision treatment of stress and addiction.

Chapter 2

Methods

The goal of this project was to 1) investigate anxiety-related behaviors, 2) study the physiology of PFC pyramidal neurons via the use of patch clamp electrophysiology, and 3) perform linear regression analysis to identify behavioral predictors of neuronal physiology. Male and female C57BL/6J mice (strain 000664, The Jackson Laboratory) were maintained on a 12:12 hours light cycle in a temperature-controlled and humidity-controlled vivarium. Both male and female mice were used to account for sex-linked differences. The total sample size was 14, all of which underwent behavioral testing and 9 were used for electrophysiology. Once mice reached adulthood, they underwent behavioral testing for anxiety-related behaviors and anhedonia through EPM, OF, and SP which are thought to represent human forms of anxiety and depression. Electrophysiological experiments were then conducted 24 hours following the last behavioral test. More specifically, whole cell patch clamp electrophysiology was used to measure the excitatory and inhibitory currents of layer 2/3 pyramidal neurons in the PFC. Once data collection was completed, linear regression was performed with the purpose of defining subgroups within the data allowing for the identification of specific behaviors that would predict electrophysiological states of PFC neurons.

Investigation of anxiety-related behavior

Mice underwent the following behavioral tests to measure anxiety and anhedonia levels. The order of the tests was randomized to account for possible order effects. All mice for this experiment were housed under reverse light cycle, and went through behavioral tests during the

dark/active phase of the cycle. All tests were performed in the morning: EPM and OF tests occurred between 10am and 12pm while SP tests were started at 8am and ended 12 hours later at 8pm. Tests for each mouse occurred 24 hours from each other and the last one 24 hours before electrophysiology. All behaviors, except SP, were video recorded and underwent automated analysis (Ethovision XT, Noldus Information Technology). In the EPM test, mice were placed in a plastic standard EPM arena which consists of 2 clear open arms (30 x 5 cm; 90 lux) and 2 black closed arms (30 x 5 x 15 cm; 20 lux) extending from a 5 x 5 cm central area and elevated 47 cm from the ground (Holmes and Rodgers 1998). Each mouse was placed in the arena for a total of 5 minutes, and its movements were recorded using a CCD camera. Automated video analysis was performed (Ethovision, Noldus) to score the following variables in EPM: time spent in open arm, time spent in closed arm, frequency of entry into open arm, frequency of entry into closed arm, total distance moved, and time spent in center zone. The center zone was defined as the open area at the center of the EPM which mice must cross to enter a different arm. In the OF test, mice were placed in a square white plexiglass arena measuring 50 cm per side (Seibenhener and Wooten 2015). Each mouse was placed in the arena for a total of 5 minutes, and its movements were recorded using a CCD camera. Automated video analysis was used to score the following variables in OF: total distance moved, average velocity, time spent in center zone, frequency of entry into center zone. Here, the center zone was defined as the zone in the center of the field that is considered dangerous for the mouse. In the SP test, mice were allowed to drink from testing pipettes, large glass pipettes that mice can easily drink from filled up to 25mL, for 12 hours with a choice of water and 2% sucrose. The position of water and sucrose was randomized. The total sucrose and water consumption were analyzed as a measure of anhedonia.

Electrophysiology of pyramidal neurons in the PFC

Twenty-four hours following the last behavioral test, mice were decapitated while under anesthesia (5% isoflurane) to isolate their brain for electrophysiological analysis. Each brain was then prepared as described in previous work (Crowley et al., 2016b; Lowery-Gionta et al., 2015). Brains were rapidly removed, and coronal slices were cut at 300 μM on a vibrating microtome (Compresstome, Precisionary Instruments) while in ice-cold sucrose solution (mmol/L: 194 sucrose; 20 NaCl, 4.4 KCl, 2 CaCl2, 1 MgCl2, 1.2 NaH2PO4, 10 glucose, 26 NaHCO3). Slices were promptly placed in artificial cerebrospinal fluid (ACSF, in mmol/L: 124 NaCl, 4.4 KCl, 2 CaCl, 1.2 MgSO4, 1 NaPO4, ten glucose, 26 NaHCO3) maintained at a constant temperature of 30° C and allowed to recover for an hour before visualization. Recording electrodes (3-6 MΩ when filled) were pulled on a Narishige PC-100 pipette puller (Amityville, NY) using thin-walled borosilicate glass capillaries. Infrared video microscopy (Olympus, Center Valley, PA, USA) was used to visualize neurons. Signals were acquired using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), digitized at 10 kHz and analyzed using Clampfit 10.2 software (Molecular Devices, Sunnyvale, CA, USA). The input resistance and access resistance were continuously monitored throughout all experiments and those in which access resistance fluctuated more than 20% were excluded from all data analyses. A cesium methanesulfonate-based intracellular solution (in mM: 135 cesium methanesulfonate, 10 KCl, 1 MgCl2, 0.2 EGTA, 2 QX-314, 4 MgATP, 0.3 GTP, 20 phosphocreatine, pH 7.3, 285-290 mOsmol) with cells held at -55 mV allowed access to spontaneous glutamate transmission. The same neurons were then used to record spontaneous GABA transmission with cells held at +10mV.

Linear Regression Analysis

Regression analysis is a powerful statistical method to study the relationship between multiple variables (Alexopoulos 2010). In the context of behavioral analysis, regression analysis is used to look for predictors of electrophysiological characteristics of behavior in the PFC. All analysis was performed using SAS software and GraphPad Prism. Correlations were determined to be statistically significant when the p-value was found to be smaller than 0.05, hence providing strong evidence that the observed results were not due to random chance. The SAS software was used to perform regression analysis with multiple independent variables. The procedure used was "proc glm" and a "manova" statement was used to encompass all independent variables of interest. The output for each independent variable was two tables. The first table was an ANOVA table listing the sum of squares and mean squares. A second output table offered the parameter estimates, standard errors, t-value, and associated p-value. Then, there was a final output table for the multivariate tests of the model which offered the correlations and statistical significance values for each combination of variables observed in this experiment. The results of interest from this final output were then graphed using GraphPad Prism.

The independent variables studied for EPM was the total distance moved, the frequency of entry in each zone (center point, open arms, and closed arms) as well as the time spent in the zones. The variables studied for OF were the total distance moved, the velocity of the mouse, the time spent in the center zone, and the frequency of entry in the center zone. For the SP test, the only variable studied for the sucrose preference as a percentage. As for the electrophysiology, the frequency and amplitude of sEPSCs and sIPSCs were studied as well as the synaptic drive which is a relation of the frequency and amplitude of excitatory input to the frequency and amplitude of inhibitory input (**Figure 1**). The last variables studied using linear regression were the sex of the

mouse to account for sex-specific patterns of anxiety in behavior and electrophysiology as well as their age.

$$Synaptic\ Drive = \frac{Excitatory\ (frequency)(amplitude)}{Inhibitory(frequency)(amplitude)}$$

Figure 1. Equation for the synaptic drive.

Chapter 3

Results

Alexandre Bourcier performed all behavioral experiments and prepped brain slices for patch clamp electrophysiology. Electrophysiology was performed by Dr. Nicole Crowley and Alexandre Bourcier while the linear regression analysis was performed in consultation with Dr. Max Crowley from the Human Development and Family Studies Department. The p values presented in the following figures are to indicate evidence against the null hypothesis, and therefore statistical significance. As for the r values presented, they stand for the correlation coefficient measuring the strength and direction of a linear relationship between two variables.

The following are results of the linear regression analysis performed between behavioral and electrophysiological data. Strong negative correlations were observed between the sEPSC frequency and the synaptic drive, as well as the sIPSC frequency and the sEPSC frequency (Figure 2). The first correlation observed between sIPSC and sEPSC shows that the synaptic drive decreases as the sEPSC frequency increases which, based on the synaptic drive equation presented in Figure 1, could be due to sIPSC frequency increasing at a greater rate than sEPSC frequency. Additionally, the sEPSC and sIPSC amplitudes must be considered when mentioning the synaptic drive equation. On the other hand, the negative correlation between sEPSC frequency and synaptic drive is intriguing since it is counter-intuitive to the synaptic drive equation in which the synaptic drive is likely to increase with increasing sEPSC frequency. However, as explained above, such

result could be explained by changes in sEPSC and sIPSC amplitudes with the synaptic drive though no strong correlations between those variables were observed.

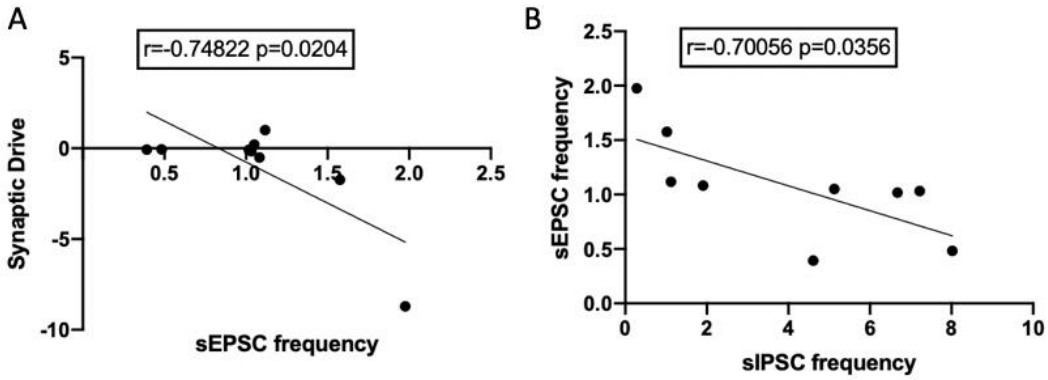

Figure 2. Correlations for electrophysiology variables. Graph A presents sEPSC frequency vs synaptic drive with a correlation coefficient of -0.74822 defining a strong negative correlation and a statistical significance of 0.0204. Graph B presents sIPSC frequency vs sEPSC frequency with a correlation coefficient of -0.70056 defining a strong negative correlation and a statistical significance of 0.0356.

The correlations between multiple EPM variables served as an internal control for measurement validity (Figure 3). As observed, the time spent in the closed arm was negatively correlated with the frequency of entry into the open arm and the time spent in the open arm was negatively correlated with the frequency of entry into the closed arm. Additionally, the frequency of entry into the open arm was positively correlated with the total distance moved and the time spent in the closed arm was negatively correlated with the time spent in the open arm. Similar behaviors positively correlated with each other while different behaviors (anxiolytic vs anxiogenic) were negatively correlated, thus confirming our behavioral analysis for EPM.

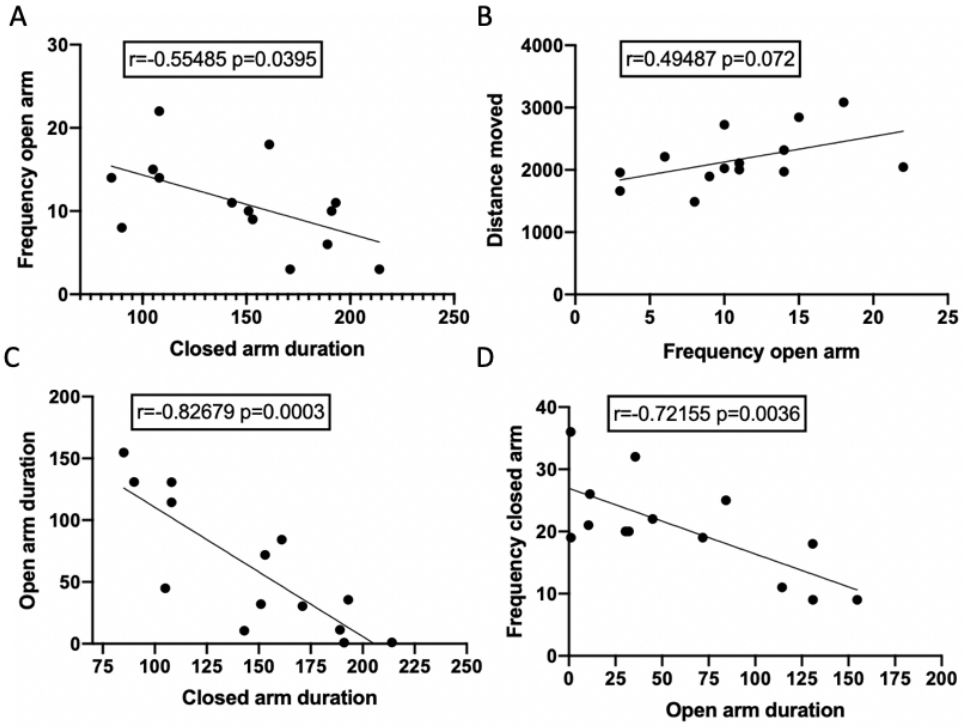

Figure 3. Correlations for behavioral variables. Graph A presents closed arm duration vs frequency of entry into open arm with a correlation coefficient of -0.55485 defining a negative correlation and a statistical significance of 0.0395. Graph B presents frequency of entry into open arm vs total distance moved with a correlation coefficient of 0.49487 defining a positive correlation and a statistical significance of 0.072. Graph C presents closed arm duration vs open arm duration with a correlation coefficient of -0.82679 defining a strong negative correlation and a very strong statistical significance of 0.0003. Graph D presents open arm duration vs frequency of entry into the closed arm with a correlation coefficient of 0.72155 defining a positive correlation and a statistical significance of 0.036.

The absence of a few correlations stood out. OF and EPM are two behavioral experiments for measuring anxiety behaviors which the literature has suggested can be used interchangeably or in tandem. However, there were no correlations observed between OF and EPM (**Figure 4**). As for the use of SP, a measure of anhedonia in mice, no statistically significant correlations between SP test and behavioral or electrophysiological data were observed. Finally, there were no

significant differences were observed between male and female mice although previous studies have demonstrated sexual differences in basal anxiety. Future work will investigate larger cohorts of mice to tease apart any sex differences in basal anxiety.

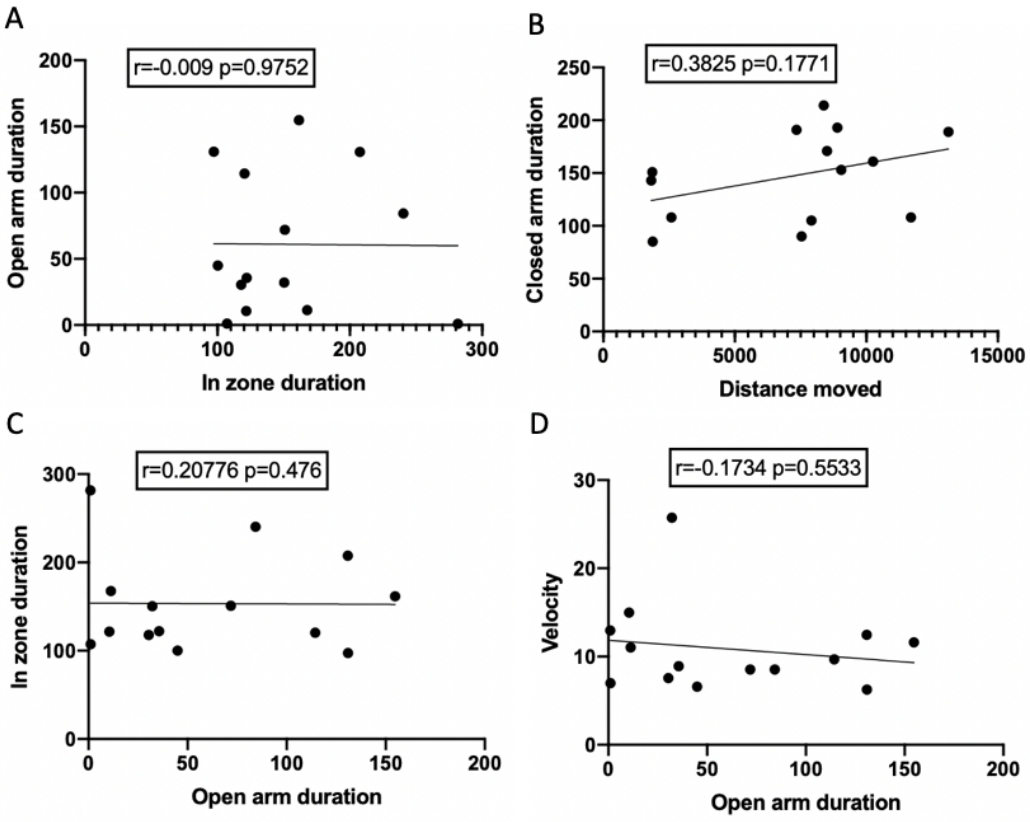

Figure 4. Correlations between OF and EPM variables. Graph A presents in center zone duration for OF vs open arm duration in EPM with a correlation coefficient of -0.009 and an insignificant p value of 0.9752. Graph B presents total distance moved for OF vs closed arm duration in EPM with a correlation coefficient of 0.3825 and an insignificant p value of 0.1771. Graph C presents open arm duration in EPM vs in zone duration for OF with a correlation coefficient of 0.20776 and an insignificant p value of 0.476. Graph D presents open arm duration in EPM vs velocity for OF with a correlation coefficient of -0.1734 and an insignificant p value of 0.5533.

The only significant correlations observed between behavioral and electrophysiological experiments were showed with OF test. Those results showed a positive correlation between the total distance covered by a mouse and the sIPSC frequency as well as a trend toward a negative correlation between the velocity of the mouse and the sEPSC amplitude. The positive correlation between the total distance covered by a mouse and the sIPSC frequency could potentially mean that a decrease in synaptic drive at the level of the prefrontal cortex would result in lower display of anxiety-like behaviors. Concerning the negative correlation between velocity and sEPSC amplitude, a decrease in synaptic drive at the level of the prefrontal cortex would also result in greater velocity of a mouse which shows lesser anxiety-like behaviors.

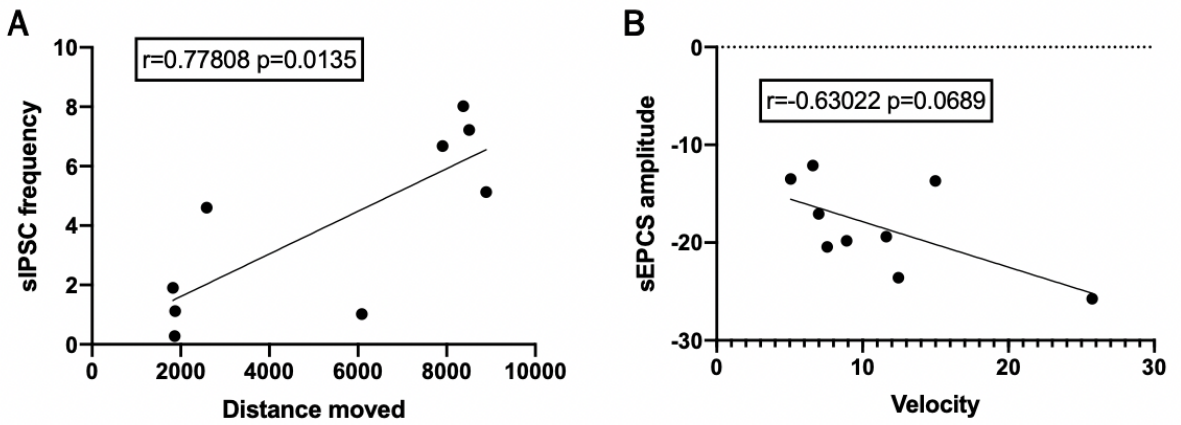

Figure 5. Correlations between electrophysiology and OF variables. Graph A presents distance moved in OF vs sIPSC frequency with a correlation coefficient of 0.77808 for a strong positive correlation and a significant value of 0.0135. Graph B presents velocity in OF vs sEPSC amplitude with a correlation coefficient of -0.63022 for a negative correlation and a p value trending to 5% significance at 0.0689.

Chapter 4

Discussion

The purpose of this experiment was to study the neurophysiological basis of anxiety-like behavior. This experiment was novel and serves as the stepping stone for what will be the development of a multivariate statistical and behavioral model capable of predicting the neuronal signaling underlying high and low anxiety behaviors, and in turn, high- and low- alcohol binge drinkers. It was hypothesized that anxiety phenotype in mice could predict the neuronal physiology at the level of the PFC as observed in patch clamp physiology. Our experiments involved using inbred strain of mice (C57BL/6J) which underwent a variety of behavioral assays related to anxiety and depression, and were then used for basal electrophysiology recordings.

The results of this experiment have pointed out a few variables from the behavioral experiments and electrophysiology that could be used for a predictive model. The strong negative correlation between the synaptic drive and the sEPSC frequency does not concord with the synaptic drive equation. However, this could be explained by the presence of one outlier. There are also a few data points which show either slightly positive or negative synaptic drive with the sEPSC frequency. In this case, the negative correlation between synaptic drive and sEPSC could be explained by greater sIPSC frequency to compensate. No correlations were found between synaptic drive and sIPSC frequency but increasing the sample pool could strengthen the data between synaptic drive and post synaptic current frequencies. The variability seen with the sEPSC, sIPSC, and overall synaptic drive is considered a strength of the experimental design: it suggests that inbred strains of mice, despite the assumption of relatively heterogenous behavioral performance, have a wide range of baseline neurological signaling.

A result of interest was the absence of correlations between EPM and OF. Despite the fact that much of the literature suggests they are interchangeable assays for the assessment of anxiety-like behaviors, this is not the first time EPM and OF are found not to correlate. In fact, there are studies showing contradictory results between EPM and OF such as the following. A study of mouse strains found that BALB/c were the least anxious and non-reactive strains based on EPM and OF tests, but a different study used OF to measure emotional behavior and locomotor activity in which they concluded that BALB/c mice were highly anxious and not very active. That second study also found that C57BL/6 mice had low levels of anxiety and showed high activity (Priebe et al. 2005; Brinks, 2008). Some studies attempted to measure how much of those discrepancies are due to the differences in analysis criteria between laboratories. The question is whether EPM and OF actually measure different underlying structures of mouse anxiety-related behavior. A study by Carola and colleagues (2001) assayed both BALB/c and C57BL/6 mice on both EPM and OF to investigate the functional significance of different parameters when analyzing behavioral data (Carola et al. 2004). They examined both simple and complex behavioral components of anxiety in mice and concluded that EPM and OF could be used interchangeably to study overall pattern of mouse anxiety. For this reason, it could be of great use to repeat these experiments with a different experimenter for the behavioral parts of the study as to validate the way that data was gathered. If the same weak correlation is found between OF and EPM, then there is room for further speculation as to whether those two behaviors can in fact be used interchangeably.

The correlations between electrophysiology and behavior were only present within OF, including the exciting correlations seen between sIPSC frequency and distance in OF as well as sEPSC amplitude and velocity in OF. The sIPSC frequency was shown to increase as the mouse moved greater distances in the OF. The increased distance in the OF is plausible as it would be

explained by increased frequency of inhibitory currents in the PFC, possibly allowing a mouse to be more active and feel less anxiety. There is also the possibility of the increased sIPSC frequency affecting a mouse's locomotion. Previous studies have shown patterns of increased locomotor activity in mice due to modulations of the PFC. In one study, disinhibition of the PFC via the use of GABA receptor agonists led to neurobehavioral changes similar to schizophrenia such as attentional deficits, enhanced prefrontal bursting and most relevant in our case, locomotor hyperactivity (Pezze et al. 2014). Therefore, our changes in GABA signaling and corresponding behavioral changes thematically (though not directly) replicate and complement the work by Pezze and colleagues. Another study attempted to induce symptoms of schizophrenia by reducing the synthesis of GABA which led to increased locomotor activity but no attentional deficits (Asinof and Paine 2013). Hence, an increase of inhibitory currents could be directly affecting locomotor activity instead of anxiety levels. Concerning the correlation between sEPSC amplitude and velocity in OF, the trend showed the sEPSC amplitude decreasing as the velocity of the mouse in the OF increased. This trend related to velocity of a mouse in OF could be interpreted in two ways. On one hand, a mouse could show high velocity due to heightened anxiety as a fight or flight response. On the other hand, there is the possibility of decreased velocity of the mouse due to a freezing response.

As presented in our introduction, there is a great need for better AUD treatments, specifically in the context of comorbidity with anxiety disorders. The current results are a great first step for the statistical model discussed prior, but it also brings up an issue. The strong correlations observed between locomotion and sIPSC could be due to prefrontal cortical circuit modulations of locomotion, and not anxiety. Hence, this brings up the difficulty that may arise when attempting to develop this model to mice who present abnormal levels of anxiety interpreted

as anxiety disorders, or even mice who have developed anxiety-like behaviors due to alcohol addiction. This has in fact been brought up by Strekalova and colleagues (2005) who demonstrated how stress-induced hyperlocomotion can be a confounding factor in animal models of anxiety and depression (Strekalova et al. 2005). This could be one of the potential limitations in future studies as well as this one, our ability to differentiate correlations arising purely due to differences in basal anxiety, or from other factors such as changes in locomotion inputs. Again, as mentioned in the introduction, the PFC is the seat to higher level behaviors which are difficult to measure or even clearly define at times. Interestingly, however, many research groups consider addiction to overlap with, or be related to, movement disorders (Deik, Saunders-Pullman, and Luciano 2012). Movement disorders can develop following chronic drug use and during withdrawal, bringing up the possibility that drugs of abuse directly modulate movement, such as psychostimulants (Asser and Taba 2015). Therefore, the overlap between electrophysiology and OF locomotion actually lead to important future questions between our statistical model and AUD: could basal locomotion predict mouse alcohol use?

Despite the limitations mentioned above, the electrophysiology and behavior used in this experiment as part of the model we hope to create is the first step towards better treatment for AUD and anxiety disorder patients. Developing an animal model allowing us to consistently predict neural physiology in the PFC based on behavior could be translated to humans. Ongoing work suggest that alcohol-used changes in electrophysiological properties of neurons are consistent between our mouse models of drinking and non-human primate models of drinking, suggesting that this work may have strong translational implications (Pleil et al. 2015a). In addition, the ability to predict brain physiology across a range of behaviors such basal states of anxiety, abnormal levels of anxiety, or anxiety resulting from alcohol addiction could allow

physicians to clearly define the physiological abnormalities of AUD or anxiety disorder patients. Hence, a physician could make treatment decisions based on those defined abnormalities on a per case basis, significantly improving patient outcomes.

This was, to our knowledge, the first study towards building a statistical model for the predictions of behaviors based on PFC electrophysiology. The data observed in this study is interesting but still preliminary as the sample pool was small with only 9 mice used for electrophysiological data and 15 for behavioral data. There were a few outliers which could have skewed the data slightly. It is crucial to increase the sample size to confirm the correlations observed and possibly see relationships that were not found in this preliminary study. Additionally, increasing the sample size would allow for the use of hierarchical latent profile analysis instead of linear regression which would allow for stronger prediction of anxiety-related behaviors. Future studies could focus on the basal anxiety levels of different outbred mouse strains since genetic variability is known to contribute to behavioral variability. As such, different predictors may arise based on the mouse strain used. Additionally, the use of a different strains may lead to similar results as this study and therefore strengthen the results observed. Once a statistical model is created on the basis of basal levels of anxiety, future studies will focus on the levels of anxiety-related behaviors of mice who have undergone binge drinking and how those behaviors can predict physiological changes in AUD models.

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ACADEMIC VITA

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Education

Schreyer Honors College, The Pennsylvania State University, University Park, PA 08/2015-05/2019
Bachelor of Science in Neurobiology, with Honors in Biobehavioral Health

Medical Experience & Community Service

Founder & President, *Remote Area Medical, Penn State University* 10/2017-05/2019

- Founded the first student chapter of RAM on the East Coast to organize trips to RAM clinics throughout the United States.
- Organized the first RAM Pennsylvania free clinic with the help of medical specialists and RAM officers in Susquehanna County in the summer of 2020.
- Worked with Pennsylvania state legislators to pass the Healthcare Act 2017 that would allow an easier access for healthcare providers from other states to volunteer at medical clinics in Pennsylvania.

Research Experience

Research Assistant, *Program for Global Surgery and Social Change, Harvard Medical School* 11/2018-Present

- Working under the mentorship of MD/MPH candidate and research fellow Rolvix Patterson
- Participating on pediatric research projects with surgeons at St-Boniface Hospital in Haiti
- Submitted research abstracts to the American College of Surgeons and Virginia Global Surgery conferences

Research Assistant, *The Crowley Laboratory for the Investigation of Addictive Behaviors, Penn State University* 01/2018-05/2019

- Research centered on the creation of a statistical model for the prediction of prefrontal cortex neurophysiology based on anxiety-like behaviors
- Overarching goal of getting a better understanding of addictive drinking and its relation with anxiety disorders
- Received funding from the Schreyer Honors College, the Eberly College of Science and the Biology Department

Summer Intern, *Schreyer MD/PhD program, Penn State College of Medicine* 05/2016-08/2017

- Participated in two consecutive summer programs in the Department of Neurosurgery
- Worked on multiple projects including the development of a new treatment for malignant peripheral nerve sheath tumors
- Shadowed MD/PhD physicians Dr.Ishmael and Dr.Holder as well as neurosurgeons Dr.Rizk and Dr.Zacharia

Peer-reviewed Publications

[1] Madhankumar, Mrowczynski, Slagle-Webb, Ravi, **Bourcier**, Payne, Harbaugh, Rizk, & Connor. (2017) Tumor targeted delivery of doxorubicin in Malignant Peripheral Nerve Sheath Tumors. *Plos One*.

[2] Mrowczynski**,** Payne, Slagle-Webb, **Bourcier**, Madhankumar, Mau, Aregawi, Harbaugh, Connor, & Rizk. (2017) MLN8237 Treatment in an Orthoxenograft Murine Model of MPNSTs. *Journal of Neurosurgery*.

[3] Mrowczynski, **Bourcier**, Liao, Langan, Specht, & Rizk. (2018) The Predictive Potential of Hyponatremia for Glioblastoma Multiform Patient Survival. *Journal of Neuro-oncology.*

[4] Mrowczynski**,** Payne, **Bourcier**, Mau, Slagle-Webb, Shenoy, Madhankumar, Harbaugh, Symons, Wolfe, Abramson, Connor, Rizk. (2018) Targeting IL-13Rα2 for Effective Treatment of Malignant Peripheral Nerve Sheath Tumors in Mouse Models. *Journal of Neurosurgery.*

[5] Mrowczynski, Zammar, **Bourcier**, Liao, Langan, & Rizk. (2018) Utility of Postoperative MRI after Glioblastoma Resection: Implications on Patient Survival. *World Neurosurgery.*

Publications in preparation, under review, or in revision

[1] Davanzo, Mrowczynski**, Bourcier**, & Rizk. (2019) Crista Galli vs Dermoid tumor: A Radiographical Analysis in Children.

[2] **Bourcier**, Mrowczynski, Dias, & Rizk. (2019) Pregnancy Associated Intracranial Hemorrhage due to Ruptured Aneurysms and Arteriovenous Malformations.

[3] Crowley, Magee, **Bourcier**, & Lowery-Gionta (2019). Animal models of alcohol use disorders: From casual drinking to dependence.