ALCOHOL/HIV-INDUCED NEURODEFICIT AND CIRCUMVENTION BY A NEUROPROTECTIVE AGENT

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ABSTRACT

Alcohol Use Disorder (AUD) remains a major problem in the United States, with usage varying between acute (binge) and chronic (heavy usage) staging. Alcohol Use Disorders affect 14.5 million people, with 9 million men and 5.5 million women. In the case of alcohol abuse, the effect of alcohol has been very well studied on the fetus but understanding of the chronic effect of alcohol abuse on neurotoxicity in the adult population, especially with comorbid conditions, such as Wernicke's and Korsakoff's Encephalopathy, remains more limited. Alcohol use and infection with the Human Immunodeficiency Virus-1 (HIV-1) in the United States is relatively common, with 30 to 60 % of these individuals having AUDs. These patients are exposed to different viral proteins that are known to be neurotoxic in the central nervous system.

The combined effects of alcohol induced neurotoxicity with HIV-induced neurotoxicity are the interest of the current project. We have utilized the HIV-1 transactivator of transcription (Tat) protein as a model for HIV infection in an animal model. This is a widely accepted model that has been used to study aspects of HIV infection. Through use of this animal model, the work demonstrates that chronic

exposure to alcohol and Tat creates a deficit in neurocognitive function with concomitant changes in receptors, cytokines and other inflammatory substances and molecules. This is important to keep in mind about changes in receptors and cytokines because the levels and changes can lead to the perpetuation of that neurodeficit.

In this model, animals were treated with alcohol for 12 weeks. Tat is introduced by use of a transgenic animal line that has been treated the same way as control animals. The animals were subjected to a behavioral battery that tested different types of memory, anxiety, and motor function. We have found that treated animals exhibited additive, meaning the effects of the substances are combined, synergistic, meaning the substances work in concert to cause drug effect, or antagonistic, meaning the substances work against each other to cause no drug effects, which ultimately created an increased neurodeficit.

Importantly, this work has also identified sex differences that are evident with treatment by HIV-1 Tat and alcohol, and these may be clinically relevant for treatment of patients with HIV-1 infection combined with alcohol abuse. Understanding sex difference or how drugs and other xenobiotics affect the individual is important because of development of different treatment strategies. This work evaluated the use of peroxisome proliferator activated receptor (PPAR) agonist on its ability to help alleviate the neurodeficit caused by Tat. The animals completed the described behavior battery above allowed us to identify the drug effects and potential changes in sex difference.

In Chapter 1, we introduce the pharmacology and toxicology of alcohol, exposure plans and types of toxicity studies, HIV-1 Tat and HIV Associated Neurocognitive Disorders, and PPAR γ agonist pharmacology and toxicology. We also provide a detailed effort to link all of these topics together to further the study described below.

In Chapter 2, we propose and carry out a two-pronged study to demonstrate prolonged exposure to alcohol in a controlled setting. We also examine synaptic protein analysis to ascertain the neurodeficit incurred from prolonged exposure to alcohol.

In Chapter 3, we propose and carry out a three-pronged study to investigate prolonged exposure to alcohol, as well as to HIV-1 Tat. We also investigate how the resulting neurodeficit changes cytokine and receptors, and at how drug effect influences these changes.

In Chapter 4, we propose and carry out a three-pronged study to demonstrate the neuroprotective properties of PPAR γ agonist, Rosiglitazone. We investigate the efficacy of this treatment using an animal study, and further investigate the effects on receptors and cytokines. We also evaluate the type of drug effect rosiglitazone exerts in the system.

In Chapter 5, we conclude by giving an overview of our results. We also provide some commentary on the use of personalized, precision medicine approach to help with treatment of HIV-1 and alcohol abuse in populations. Further studies

using animal models and three different clinical trial options for expand the goals of this work that are ultimately proposed.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies have examined a thesis titled “Alcohol/HIV-Induced Neurodeficit and Circumvention by Neuroprotective Agent,” presented by Daniel C. Schwartz, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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CHAPTER 1 - INTRODUCTION

1.1 Alcohol

Alcohol production and use in human populations dates to prehistoric times and continues up through today. Alcohol remains the most researched, consumed, and abused substance around the world. It also remains the most widely consumed psychoactive substance in the United States. The effect of alcohol consumption has been broadly discussed in society, and there are a host of negative aspects to alcohol consumption, abuse potential, and alcohol use disorders that are recognized from different social, medical, and economic perspectives.

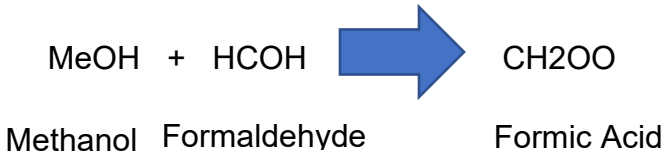
Alcohol production has remained relatively unaltered from prehistoric to current times (Winger 2004). While there are different types of alcohols available for consumption, the most common and generally least toxic is ethanol (Winger 2004; Yip 2011; Bruckner 2008). Ethanol can be toxic at high levels but does not produce toxic metabolites unless metabolic process is compromised. The most common formula to produce alcohol is using starches and sugars together with yeast, which allows for fermentation (Levine 1999; Winger 2004; Yip 2011). To harvest the alcohol from the mash, the mash must be distilled.

Once allowed to ferment the mash is allowed to be distilled off, and water is left in the pot and the alcohol vapor is collected (Levine 1999; Winger 2004; Yip 2011). Higher proof spirits are achieved with consecutive distillations. The beginning steps of alcohol production are used to make beer and wine beverage.

Most alcoholic beverages contain what are called congeners. Congeners can be best explained as by-products of the distillation process (Winger 2004; Lachenmeier, Haupt, and Schulz 2008; Bruckner 2008; Rodda et al. 2013; Strubelt et al. 1999). Congeners can be substances such as aldehydes, ketones, or other volatile alcohols. They can also be artifacts of aging or other processes e.g., Caribbean rum casks used to age scotch. Congeners account for different smells, tastes, and colors of alcohol-containing beverages (Winger 2004; Lachenmeier, Haupt, and Schulz 2008; Rodda, et al. 2013; Strubelt, et al. 1999); they are also responsible for some of the specific undesired effects of alcohol ingestion, such as headache and dizziness. One other aspect to alcohol production, the ability to determine to proof or drink strength of the alcoholic beverage.

Drink strength is determined by the percentage of alcohol in the beverage (Lachenmeier, Haupt, and Schulz 2008). Typically, alcoholic beverages are proofed. Proofing alcoholic beverages was typically used on the high seas and was measured by how long the alcohol being proofed would burn compared to burning black powder. Common alcoholic beverages consumed include beer, which has an average alcohol percentage of 5% or 10 proof; and wine, which has an average alcohol percentage of 12% or 24 proof, but can contain up to 23% alcohol percentage or 46 proof (Logan, Case, and Distefano 1999). Distilled spirits are much higher in alcohol concentration, at percentages starting at 25% or 50 proof and up. Higher proof drinks can be obtained by more distillations, which will bring about higher purity (Winger 2004). Other alcohols can be ingested to get additional psychoactive effect but have toxic implications.

Other alcohols, such as methanol and isopropanol form toxic metabolites once metabolized by the body (Winger 2004; Bruckner 2008). Methanol is a common alcohol that can be found in lots of different solutions, can be found in copier toner, but is best known as wood alcohol. Methanol metabolism is shown below:



This compound, methanol, has been ingested by people historically dating back to as recently as prohibition. Alcoholics who have been starved for alcohol will ingest anything that contains alcohol to stop from starting the detox process, with symptoms that can include but not limited to hallucinations, delusions, and seizure (Winger 2004).

Alcohols that have been known to be ingested are isopropanol and ethylene glycol, which are toxic to humans (Winger 2004; Yip 2011). Isopropanol is a commonly used antiseptic substance used in medicine, example cleaning surfaces and skin. When ingested, the final metabolite is acetone. The effect to the individual can be headache and possible acidosis (Levine 1999). The other alcohol that has been known to be ingested by alcohol seeking individuals is ethylene glycol. Ethylene glycol is used in cars as antifreeze. Ethylene glycol is not as volatile (bp. 197°C) as isopropanol (bp. 82.5°C). The final metabolic product of ethylene glycol is conversion to an insoluble diol to dicarboxylic acid (Levine 1999). The dicarboxylic acid reacts with calcium and produces insoluble calcium oxalate crystals, which are visible in the urine. The calcium that is removed to

make calcium oxalate crystals precipitates in the kidneys and the brain. These crystals are viewable in the brain and can be visualized in the tissue sections during post-mortem examination of the brain by using a plane polarizing light microscope (Levine 1999).

The consistent, widespread, and enduring use of alcohol in human populations has led to frequent studies about the effects of alcohol on human populations as it relates to medical issues, societal issues, and even economics (NIAAA 2022b). With alcohol use as common as it has been in the US according to NIAAA, 85.6% people have reported having an alcoholic beverage some time in their life (NIAAA 2022a). There is controversy, medicinal effects of alcohol, has occasionally developed as to the “benefits” of use of alcohol including the potential health effects of congeners of distillation, or even the concentrated effects of substances from fermentation of grapes or other fruit (Rodda, et al. 2013; Logan, Case, and Distefano 1999). Nevertheless, most of the literature shows that alcohol use has had a negative impact on individuals and should be limited (Yang et al. 2018; Hasin 2003; Walter et al. 2017). This is especially true when other diseases and conditions are present. However, US society is still affected by overall high levels of alcohol consumption with a high economic burden in the 249 billion dollars in the year 2010 (NIAAA 2022a). Our study allows for the studying of how alcohol and co-morbidities may interact in a system and allow for an understudied topic to be studied more (NIAAA 2022b; Yang, et al. 2018; Walter, et al. 2017).

1.1.1 Alcohol Absorption, Distribution, Metabolism, and Elimination (ADME)

1.1.1.1 Absorption

While most humans consume alcohol orally, in truth alcohol administration can take multiple routes, and absorption can occur in many forms. Routes of administration include inhalational, dermal, intravenous, and oral. These different routes allow for the absorption of alcohol, and can be very effective for alcohol studies and research activities (Levine 1999; Yip 2011). These routes do not mimic traditional human exposure to alcohol and are ultimately not ideal for human exposure studies. To better mimic human conditions of exposure to alcohol in animal studies, the best method for alcohol exposure is to feed the animal alcohol orally (Jeanblanc et al. 2019; Lovinger and Crabbe 2005; McBride and Li 1998; Spanagel 2000; Wallace 1990).

Once the alcohol has been ingested, it is moved through the gastrointestinal system, stopping in the stomach first, specifically the pyloric region and entry into the small intestine (Levine 1999; Yip 2011). The small intestine is the principal organ of absorption for alcohol due to the large surface area and high exposure to vasculature via diffusion (Levine 1999; Yip 2011). Diffusion is moving a substance from areas of high concentration to areas of low concentration. Because of the increased surface area of the small intestine, the diffusion process allows 75% of the dose to absorbed and sent systematically. (Levine 1999; Yip 2011).

1.1.1.2 Distribution and Blood Alcohol Concentration

Alcohol distribution follows a systematic process. The dose starts in the small intestine and diffuses across the mucosal membrane. The dose then enters the systemic circulation by the hepatic portal vein (Levine 1999; Yip 2011). The dose is then moved to the heart on the right side where unoxygenated blood is brought to be re-oxygenated and distributed to the body. The blood is then transferred to the lungs for gas exchange. This is extremely important for the volatility property of alcohol (Levine 1999; Winger 2004; Yip 2011; Cowan et al. 2016). The volatility property that is exhibited is because of alcohol vapor in the air inside the lungs. The lung capacity for air is 2100 L to 1 L of blood, which allows for alcohol to be detected by breathalyzer (Levine 1999). After the lungs, the oxygenated blood is distributed out to the rest of the body for oxygenation and the alcohol is sent with the blood to other organs and tissue.

After this distribution occurs, alcohol has a high affinity for tissue and fluids that have high water content. Tissue that contains high levels of water will have a high alcohol content as determined by Widmark (Levine 1999; Winger 2004; Yip 2011). For the males and females, these individuals will have differing body water content (Levine 1999). This means that males and females of similar weights drinking the same drinks will have a different blood alcohol concentration (BAC) (Cowan, et al. 2016; Bruckner 2008; Alfonso-Loeches, Pascual, and Guerri 2013; Baraona et al. 2001; Dilley et al. 2018; Frezza et al. 1990; Mitchell Jr., Teigen, and Ramchandani 2014). On average, males have a 68% body water vs. female's body water of 55% (Levine 1999; Bruckner 2008). Similarly, two men of different

weights one heavy, more distribution space, and the other lighter, less distribution space, will have differing BAC (Levine 1999).

While the individual is in the absorptive phase, BAC level can differ between arterial and venous blood. In early-stages absorption, the arterial blood circulation will have a higher BAC level (Levine 1999; Baraona, et al. 2001; Dilley, et al. 2018; Frezza, et al. 1990; Mitchell Jr., Teigen, and Ramchandani 2014). Once absorption is completed, there is no difference between BAC levels in the arterial vs. venous circulations (Cowan, et al. 2016). Serum and plasma show difference also with water content. Serum contains more water than plasma and is preferred sample for alcohol determination. Serum is preferred due to serum having a content of 12 to 20% red blood cell (Levine 1999; Bishop 2005). Serum will have a higher BAC than plasma for this reason. Typically, the average adult will eliminate 16 mg% per hour alcohol (Bruckner 2008). So, if a person consumes alcohol to a blood alcohol level of 120 mg%, they would require at least 8 hours to bring down the blood alcohol level to negligible levels (Bruckner 2008; Baraona, et al. 2001; Dilley, et al. 2018; Mitchell Jr., Teigen, and Ramchandani 2014).

1.1.1.3 Metabolism

Alcohol metabolism follows a complicated system, with a goal of detoxifying and making the molecule more hydrophilic. It is complicated because ethanol elimination can occur in multiple ways. Alcohol can be metabolized by several different enzymatic pathways. The main pathway is found in the microsomal fraction, or the microsomal ethanol oxidation system (MEOS) handles further metabolism (Levine 1999; Winger 2004; Yip 2011; Fenna et al. 1971; Wilson and

Matschinsky 2020). The MEOS is part of the cytochrome P450 isoenzyme system (CYP). CYP 2E1 is considered the principal enzyme used to handle the metabolic needs of this system for alcohols, halogenated alcohols, and aromatic alcohols (Levine 1999; Winger 2004; Yip 2011; Bruckner 2008). This system converts alcohol to an aldehyde, acetaldehyde, and from there converted further to a ketone and excreted. Other enzymatic pathways used for the elimination of alcohol are the alcohol dehydrogenase, catalase, and aldehyde dehydrogenase (ADH) systems and show variations based upon sex, racial lines, and genetic lines (Levine 1999; Winger 2004; Yip 2011; Bruckner 2008; Fenna, et al. 1971; Wilson and Matschinsky 2020). These systems are all inducible systems. Therefore, people suffering from alcohol use disorders are said to have higher than normal metabolic rates than people who do not suffer from alcohol use disorders (Levine 1999; Winger 2004; Yip 2011; Fenna, et al. 1971; Wilson and Matschinsky 2020).

1.1.1.4 Elimination

After the alcohol has undergone metabolism, the compound should be more hydrophilic. Once alcohol has been metabolized enzymatic pathways, it will be broken down to acetaldehyde and further broken down to carbon dioxide and water (Levine 1999). The principal way that alcohol is eliminated is by urine but can be eliminated by feces and vomiting. Typically, elimination of alcohol is subject to zero-order kinetics, meaning that for 1 mole of alcohol to 1 mole of enzyme used to eliminate the molecule (Levine 1999). This is observed at high BAC levels. At low BAC levels, first order kinetics is observed which follow Michaelis-Menten kinetics (Levine 1999).

1.1.1.5 Factors affecting ADME

There are numerous factors that can contribute to alteration in the ADME of alcohol.

1. Disease state of the individual. If the liver is compromised, the alcohol metabolism and elimination will be hindered as build-up of toxic substances will take place (Levine 1999; Yip 2011).
2. Gastric Motility. Increased gastric motility will cause increased absorption; while decreases in gastric motility will cause decreased absorption. Conditions that shift more blood to the GI tract will cause increased absorption of alcohol (Levine 1999; Yip 2011). Drugs that the individual may take can cause changes in the gastric motility that can mimic disease states (Levine 1999).
3. Genetics. Genetic factors can interfere with or increase ADME of alcohol. The metabolizer status of an individual can alter the rate at which metabolism occurs. Some people may be slow, fast, rapid, or ultra-rapid metabolizers (Levine 1999; Yip 2011). This has to do with the race and sex of the individual because of single nucleotide polymorphisms, which are substitutions at specific locations in the genome of individuals. (Birley et al. 2009; Fenna, et al. 1971; Wilson and Matschinsky 2020).
4. Administration. Administration of certain compounds such as fructose, glycine, alanine, and drugs may alter the elimination of the alcohol. Drugs such as ranitidine and cimetidine can influence BAC levels (Levine 1999).

These can also inhibit the action of gastric ADH, which is important in the elimination of ETOH.

5. Carbonated Beverage. Use of carbonated beverages can enhance absorption, distribution, metabolism, and elimination of alcohol (Levine 1999).
6. Drink Strength. Drink strength has also been known to contribute to absorption. The higher the concentration of the drink the faster the absorption; while more dilute drinks will be less absorbed or may absorb more slowly (Levine 1999). This same logic would continue to be true but at higher concentrations of alcohol gastric mucosa becomes irritated and gastric emptying occurs as well as excess mucous production.
7. Food Intake. Food can cause changes in absorption of alcohol while drinking. Food plays two major roles when it comes to absorption. The effects on full stomach and drinking versus (vs.) empty stomach and drinking can influence BAC levels. Empty stomachs cause peak intoxication quicker than what would normally be achieved on a full stomach (Levine 1999). Alcohol competes with food for absorption in the small intestine. With presence of the competition of food, alcohol is more likely to be eliminated by the body (Levine 1999).

1.1.2 Tolerance

Differences in alcohol levels in individuals are demonstrated in the effects on the individual during consumption. This is called tolerance. Tolerance is defined as the effect that results from chronic usage of a drug where a larger dose

is needed to achieve desire effect (Elvig et al. 2021; Levine 1999). The metabolic status of each individual drinker can be different. In general, people suffering from alcohol use disorders can metabolize alcohol quicker than the occasional drinker. This is because the metabolic systems are more active; while the learned behaviors are treated differently and accounted for differently by the individual which is acquired tolerance (Levine 1999). Acquired tolerance is a process where, through learning and experience, the individual can compensate for the depressing effects of a given substance (Levine 1999). These people are able conceal the effect of overt intoxication, unlike the occasional drinker (Levine 1999). Acute tolerance also known as the Mellanby effect is a phenomena where the alcohol concentration is greater when the BAC is ascending (increasing) than descending (decreasing) (Holland and Ferner 2017; Levine 1999). With the Mellanby effect, impairment remains whether BAC is increased or decreased.

1.1.3 Alcohol Effects on Central Nervous System (CNS)

Predominantly, the effect of alcohol is on the brain and the CNS which causes depression (Levine 1999). The depression that is experienced from

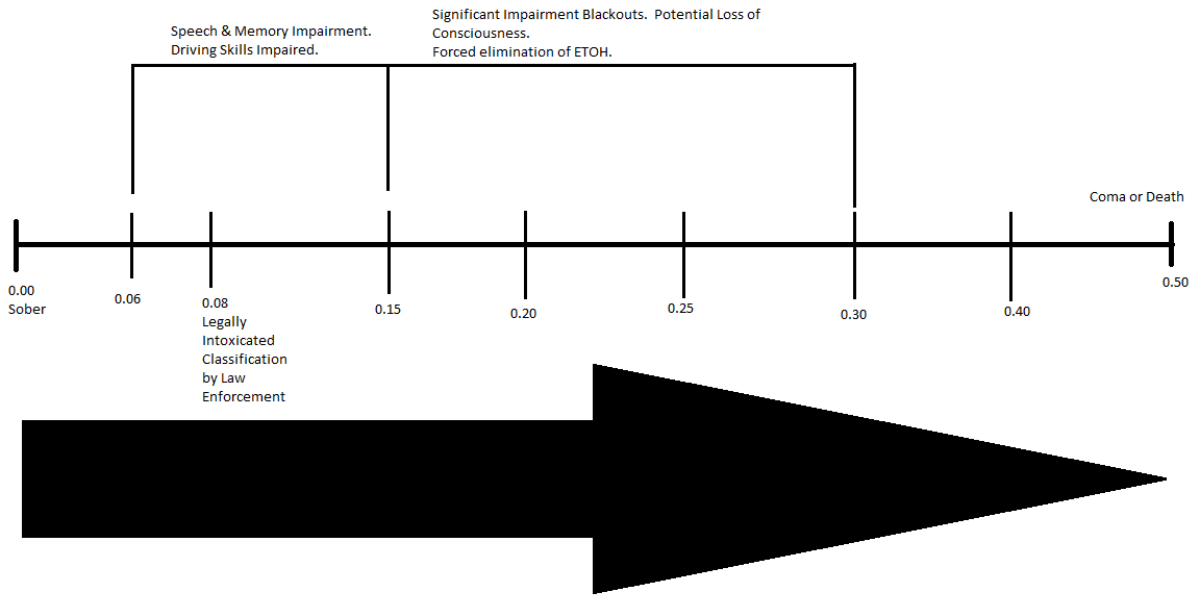


Figure 1.1 – ETOH continuum. The figure demonstrates that as the blood alcohol percentage increases there are certain effects that occur. Example: Speech and memory impairment occur between BAC levels 0.06% to 0.15% BAC. At 0.08% BAC most states consider a person legally intoxicated and should operate motor vehicles or heavy machinery.

alcohol should viewed on a continuum, as shown above in figure 1.1. At low BAC, impairment is minimal and may range from talkativeness to increased sociability. As the alcohol concentration increases, simpler functions are depressed including decision making and changes in decision making. At the point of max intoxication, the respiratory centers in the brain can become completely depressed (Levine 1999; Winger 2004; Yip 2011), which leads to coma and death. For this work, the BAC concentrations are low. The animals experience only the depression of complex functions.

1.1.4 Alcohol Neurochemistry

Alcohol neurochemistry is very complex. Alcohol can have action on various receptors inside the brain, including gamma amino butyric acid (GABA),

glutamate, opioid, serotonin, adenosine, and dopamine receptors (Levine 1999; Vrij-Standhardt 1991; Winger 2004; Yip 2011). All cause various effect on the user. Alcohol binds to the GABA receptor, which acts as the major inhibitory neurotransmitter in the brain (Levine 1999; Vrij-Standhardt 1991; Winger 2004; Yip 2011). Alcohol has been noted to have similar activity at receptors for commonality of benzodiazepines and barbiturates (Levine 1999; Winger 2004; Yip 2011). Alcohol also binds the N-methyl-D-aspartate (NMDA) receptors. These receptors bind glutamate which acts as the major excitatory neurotransmitter (Levine 1999; Winger 2004; Yip 2011). Once bound to NMDA receptor, it exerts the action of blocking the glutamate channels, which produce euphoria, unlike phencyclidine (PCP) and ketamine which causes surgical anesthesia (Joffe et al. 2018; Levine 1999; Winger 2004; Yip 2011; Vrij-Standhardt 1991). Prolonged exposure and binding of the NMDA receptor can cause alcohol tolerance formation, addiction, alcohol-related dementia (ARD), and can lead to eventual withdrawal syndromes with cessation of administration of the drug (Cui et al. 2015; Elvig, et al. 2021; Kamal et al. 2020; Levine 1999; Oslin et al. 1998; Winger 2004; Yip 2011).

1.2 Animal Models and Exposure Protocols

Animal models have long been a tradition in biomedical research and are referred to as descriptive animal studies. Descriptive animal studies are used and function to show that exposure of the animal to the substance in question in high doses is a necessary and applicable method to discovering doses that may be harmful to humans and is necessary toxicological testing (Spanagel 2000). Numerous models that have been used to study numerous different topics. There

have been models created just to study addiction and long-term and short-term toxicologic consequences of different substances thought to be toxic or known to be toxic (Jeanblanc, et al. 2019; Lovinger and Crabbe 2005; McBride and Li 1998; Spanagel 2000; Wallace 1990). Models and exposure protocol systems are setup to ascertain certain consequences that can be drawn from research and applied to other systems and possibly other substances, such as looking at the anti-depressive effects of dimethyltryptamine (DMT) (Cameron et al. 2019). The goal of most animal studies is to be able to translate those findings to human systems (Spanagel 2000; Wallace 1990; Lovinger and Crabbe 2005; Ferdowsian and Beck 2011; Ghasemi and Dehpour 2009).

Choosing the right strain of animal is important when testing alcohol consumption. Selecting strains that are typically used in alcohol research, C57BL6/J, assure the researcher that alcohol will be routinely consumed, and the study will give an accurate depiction of experimental conditions and mimicking conditions that will translate to humans (Acheson et al. 2013; Jeanblanc, et al. 2019; McBride and Li 1998; Spanagel 2000; Wallace 1990). The diet with these types of animals is also an important factor to consider. When performing studies that work to understand facts about alcohol, high sugar diets should not be used (Spanagel 2000; McBride and Li 1998; Wallace 1990). The use of a high sugar diet will decrease alcohol consumption, and the effect that the researcher desires will not be achieved (Spanagel 2000; Spanagel 2017; Coleman et al. 2008). Circadian rhythm is another important consideration. Alcohol consumption should take place at the same time each day as to not allow variation which may alter

consumption of the substance (Spanagel 2000). Animal activity takes place mostly at night and lends to showing a controlled environment and eliminating factors of variation.

1.2.1 Types of Exposure Studies

Many different types of studies can be conducted to ascertain clinical and toxic effects. These studies can be grouped into various classifications. These classifications offer a tiered approach to studying the therapeutic effect or toxic effect from the substance. The classes are as follows:

1. Acute Toxicity Studies: These are typically used to provide information on known toxicity of the substance and can be used to determine clinical manifestations that may occur from consumption of toxicant (Eaton 2008; Boekelheide and Campion 2009). This study design can allow the researcher to ascertain the best species to perform the research with. This type of study allow the researcher to notice any reversibility from use of the toxicant that is noted when the toxicant is no longer being administered (Eaton 2008; Boekelheide and Campion 2009). This study will allow for exploration of possible research design and allow for dose selection of the upcoming study. These studies normally last only few days to a week before termination of the experiment.
2. Subacute Toxicity Studies: These types of studies are performed on the toxicant to ascertain information about repeated dosing and further dose selection for sub-chronic toxicity testing (Eaton 2008; Boekelheide and Campion 2009). These tests are typically carried out by dosing an

animal through the animal's food and then performing chemistry assays (Eaton 2008; Boekelheide and Campion 2009). These studies last typically up to 14 days before termination of experiment.

3. Sub-chronic Toxicity Studies: These tests are known to last different time periods of exposure. The most common is 90 days of exposure to the toxicant. The goals of this type of experiment are two-fold. The first goal is to establish a No Observable Adverse Effect Level (NOAEL) (Eaton 2008; Boekelheide and Campion 2009). The second goal is to further show and describe organ systems or organs that may be affected by repeated exposure to the toxicant (Eaton 2008; Boekelheide and Campion 2009). These studies generate data to establish where toxicity will occur and show underlying issues that may have occurred.
4. Chronic Toxicity Studies: These studies are performed similarly to how a sub-chronic study would be performed, but the period of exposure is longer than the sub-chronic exposure (Eaton 2008). Once the design is known for this type of study, the dose can be carried forward from the sub-chronic study. These studies typically last 90 days or more.

When these studies are carried out, the animals should be weighed weekly, and the data recorded and stored for later usage. These studies can help elicit information that will continue to benefit researchers long after the study has been concluded (Eaton 2008).

1.2.2 Animal Exposure Protocol and Models for Alcoholism

Models of alcoholism used to study alcoholism are numerous and can be complex. Researchers have setup studies to mimic different phases of the disease itself. Being able to draw conclusions from the studies is dependent on understanding the model, what is being tested, and how comparable any model is to the other (Jeanblanc, et al. 2019; McBride and Li 1998; Spanagel 2000; Wallace 1990; Lovinger and Crabbe 2005). There are a few things the researcher must remember when designing the model system and choosing the right animal. The researcher must know of animal behavior that coincide with natural behaviors. The animal in the wild would eat rotten food allowing for the animal to consume food stuffs that have fermented and produced alcohol (Spanagel 2000; Wallace 1990). This allows for checking the ability of the animal to become intoxicated by alcohol. This fact makes animals the ideal model system to mimic e.g., alcohol reinforcement (McBride and Li 1998; Spanagel 2000; Wallace 1990). The researcher can also study certain behaviors that are noted from alcohol intoxication and extend the study into how the intoxication affects brain physiology. Any model that is to be used to model alcohol or drug seeking behavior should have predictive value to human situations of addiction or toxicant exposure (Spanagel 2000; Hitzemann 2000).

1.2.2.1Two-Bottle Choice and Preference Study

A commonly used model to observe alcohol consumption is called two-bottle choice or preference studies. In this model the cage is fitted with a bottle of water and a bottle of alcohol. The concentration of the alcohol is typically increased in tiered approach or can be ascertained by performing a literature

search. Typically, when selecting alcohol solutions, low concentrations of alcohol, (6% by weight/by volume,) has a sweet taste which means the animal will drink more (Spanagel 2000; Wallace 1990). Selection of a high dose (50% or more by weight/by volume,) will have a bitter taste and have aversive taste to the animal (McBride and Li 1998; Spanagel 2000; Wallace 1990). The animal will consume less of this alcohol solution. Variation of the alcohol solutions should be kept to a minimum and can be used as a challenge, which can be used to increase the dose gradually, to get the animals to a more stable alcohol dose. Once the dose of the alcohol is known, the animal is allowed to drink from both bottles at will (McBride and Li 1998; Spanagel 2000). The weight of both bottles is recorded prior to administration to the animal and after administration.

There have been some noted problems with the preference study or two bottle choice. The first problem that has been elucidated is that this model does not consider that addictive behavior does exist in the animals (Spanagel 2000). This model at its root is a systematic controlled exposure to alcohol over a predetermined period.

Typically, most alcohol models must demonstrate the ability to trigger compulsive and uncontrolled behavior (Spanagel 2000). The reinstatement model allows the researcher to address the phenomenon of relapse and cravings. This model type can be used to test other substances, such as opioids. These two events, compulsive and uncontrolled behavior, do not allow for measurement of preference that is used in the two-bottle choice or preference study. At the start of the reinstatement model, the animal is trained to use a lever, a form of operant

conditioning, to get the alcohol dose. This is a delivery device that is used to deliver substances and can be used to simulate compulsive and uncontrolled behavior. After learning how the apparatus works, the animal is allowed to complete the task (McBride and Li 1998; Spanagel 2000). The task has been learned but the alcohol will be withheld from the animal, and the alcohol is replaced with water to force the extinction process (Spanagel 2000). The extinction step is important in this type of study because this allows learned behaviors to be unlearned and give accurate measurement of, in this case, compulsive and uncontrolled behavior (Milad and Quirk 2012). After the animal stops pressing on the switch, the extinction has been learned and achieved. Next, the researcher will introduce the alcohol to the apparatus. The reintroduction of the substance allows the researcher to assess the ability to reinstate the drug seeking, compulsive, and uncontrolled behavior, which in this case would be pressing the lever (Spanagel 2000).

There are three other ways to cause reinstatement in animal models and those are listed below:

1. Injection of a small amount of drug.
2. Induction of stress.
3. Using conditioned stimuli that was used previously during the training sessions (Spanagel 2000).

Reinstatement models are typically used to test opioids and psychoactive drugs. This model has trouble to transferring conditions to the use of alcohol. This paradigm or approach has been used to study the effects of putative anti-craving

and anti-relapse drugs, e.g., acamprosate, disulfiram, and naltrexone (McBride and Li 1998; Spanagel 2000).

Limitations to the reinstatement model do exist and they are as follows:

1. There is controversy as to whether the animals are truly dependent on ETOH. This would be the sense that the administration of the drug is uncontrolled, or cravings have been achieved (Spanagel 2000).
2. Extinction of the lever or task plays a minor role in alcohol cravings. When former alcohol seeking individuals are looking to remain abstinent, these individuals will try to avoid all cues that may induce consumption of alcohol (Spanagel 2000). These patients may experience cravings and possible relapse from consumption due to cues being present. Cues that might cause relapse would be the sight of a bar or the smell of alcohol (Spanagel 2000).
3. One of the big issues with the reinstatement model is that it does not reflect being abstinent from alcohol cravings but predicts and shows the individual relapsing instead. This situation is better mimicked using alcohol deprivation studies. This effect is represented in another model called long-term alcohol self-administration with repeated alcohol deprivations (Spanagel 2000).

1.2.2.2 Long Term Alcohol Self-Administration with Repeated Alcohol Deprivation Phases

This paradigm is used best to measure or model compulsive, uncontrollable drug or alcohol seeking, and/or drug or alcohol taking behavior that is seen in drug and alcohol addicts (Spanagel 2000; Wallace 1990).

To start, the animals are housed with free access to food and water. Alcohol solutions of 5, 10 and 20 percent are placed in the cages (McBride and Li 1998; Spanagel 2000). The animals are allowed free access to the alcohol solutions. This free access lasts for at least two months or as determined by the researcher. After this time, the alcohol is removed from the animals. This is referred to as the deprivation phase, and deprivation phases last as determined by the researcher (Spanagel 2000; Egli 2005). The administration of alcohol each time is followed by the deprivation phase. This allows for the measure of increased compulsive and drug seeking behavior (Spanagel 2000; Egli 2005). This type of behavior has been noted in other species, not just rodent. These changes are characterized by changes seen in the animal's alcohol intake. Animals have been noted during this model to consume alcohol during inappropriate time, i.e., during the light phase (Spanagel 2000). Animals are typically inactive during this time while rodents are typically more active at night.

This model can be used under operant conditions. The alcohol intake increases with performance of a given task i.e., pressing a lever. Use of operant conditions allows the researcher to observe motivation for drug and alcohol seeking behavior, which has been classified as craving (Spanagel 2000; Egli 2005). Increased motivation doesn't meet criteria for addictive behavior, but also loss of control must be present during this time. To test this loss of control the

researcher will most likely add either quinine or a solution with a high sugar content to either alcohol or the water (Spanagel 2000). The researchers have found that animals will still consume the alcohol but at higher levels followed by a single deprivation phase. This shows that drinking habits and behaviors at a certain point, will become inflexible and uncontrollable (Spanagel 2000). The fact these conclusions have been made over multiple models by multiple researchers demonstrates that chronicity, a state of long lasting duration, implies compulsive, uncontrolled drug-seeking and drug-taking behavior and is way outside the norms of normal eating and drinking habits (Spanagel 2000; Lovinger and Crabbe 2005; Egli 2005; Tabakoff and Hoffman 2000).

Alcohol deprivation studies show a non-nutritional component of alcohol taking but overriding the ability to seek alcohol for pharmacological properties provide the motivation for drug and alcohol seeking behaviors (Spanagel 2000; Egli 2005). During the deprivation phases, the animals show high levels of noted anxiety-like behavior (Lovinger and Crabbe 2005; Spanagel 2000). This is like what is present in humas who are undergoing alcohol or drug withdrawal processes. This type of model has been accepted as current model of addiction and fulfills criteria laid out in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) (Hasin et al. 2013; Tabakoff and Hoffman 2000). This model has also been validated using both acamprosate and naltrexone (Spanagel 2000; Egli 2005).

The deprivation model has been validated by using naltrexone, which is a partial agonist antagonist of opioid receptor. Administration of naltrexone has been

shown to decrease alcohol craving and decrease consumption of alcohol in animals as well as humans (Spanagel 2000; Cui, et al. 2015; Ron and Berger 2018; Winger 2004; Yip 2011). There has been discussion over the usage and frequency of administration of naltrexone. Some researchers have discussed the intermittent usage and chronic usage of naltrexone as therapy (Spanagel 2000; Egli 2005). The researchers found the most beneficial use of naltrexone was intermittent, which caused decrease in consumption and cravings (Spanagel 2000; Egli 2005). The administration of the substance that is desired to be discontinued must meet the following two criteria:

1. The agent that is being discontinued must be continuously administered, and
2. Animals receive repeated intermittent injections of naltrexone during the study (Spanagel 2000; Egli 2005).

When naltrexone is administered chronically, the animals show increased preference for alcohol consumption and craving. Pharmacologically, total blockade of the opiate receptors forces more receptors to be made, which cause increased susceptibility to alcohol cravings and consumption (Spanagel 2000; Howland 2011; Winger 2004; Yip 2011). The intermittent or moderate dosing of naltrexone pharmacologically does not cause changes in opiate receptors. The opiate receptor number stays constant, which will decrease cravings and drug seeking behavior (Spanagel 2000; Winger 2004; Yip 2011; Egli 2005).

The deprivation model has also been validated using acamprosate. Acamprosate is used primarily in developed and European countries. The effect

of acamprosate has been well studied in various deprivation models. Acamprosate exerts action by modulation of the NMDA receptor, which uses glutamate. Alcohol at low levels inhibits the actions of the NMDA receptor (Winger 2004; Yip 2011; Spanagel 2000). To administer acamprosate to animals inside study conditions, the animals will receive various doses of acamprosate and saline. The animals also receive various levels of alcohol during the administration of acamprosate. The resulting exposure to acamprosate causes a marked decrease in consumption and preference of alcohol in a dose-dependent manner (Spanagel 2000). With increased levels of acamprosate, the drinking behavior that is learned either fall below the baseline levels or is terminated.

The use of both naltrexone and acamprosate together or separately show decreases in alcohol and/or drug seeking behavior (Spanagel 2000; Egli 2005; Tabakoff and Hoffman 2000). The use of these two agents serves two purposes. First, demonstration of an acceptable treatment strategy. Lastly, the use gives possible model system for future discovery for agents that may aid in drug and alcohol addiction (Egli 2005; Hitzemann 2000; Tabakoff and Hoffman 2000).

1.2.2.3 The “Point of No Return” model

This model recognizes that alcohol consumption occurs in stages. The first stage is known as acquisition. During this stage, the individual will experiment with various alcohols and doses. This may last an undetermined period, which leads into the transition phase. This phase is characterized as the period between normal consumption and the increased or excessive consumption phase (Spanagel 2000; Spanagel 2017; Egli 2005; Hitzemann 2000; Tabakoff and

Hoffman 2000). The concept is that there are two distinct phases of alcohol consumption that the individual undergoes with a transition period present (Spanagel 2000; Spanagel 2017).

The model system starts out with the animals having free access to food, water, and alcohol. The consumption of alcohol is noted to increase, and it has been noted that every other day an alcohol abstinence period does exist for the individual. The thought is that the odor of alcohol increases or influences the drinking behavior (Spanagel 2000; Spanagel 2017; Hitzemann 2000; Tabakoff and Hoffman 2000). During the acquisition phase, the animal is assessing the alcohol psychotropic effects and intake and consumption is adjusted as needed by the animal (Spanagel 2000; Spanagel 2017). This is noted to be the development of a pattern of exposure. This phase is noted to be stable for months at a time. The exposure of alcohol consumption is controlled by internal and external factors. These factors are known as social rank and order, behavior, and social isolation (Spanagel 2000; Spanagel 2017; Hitzemann 2000; Tabakoff and Hoffman 2000). These factors influence the frequency and consumption of alcohol for the animal.

With free access to alcohol during study, drinking behaviors will change. The change in consumption is noted to occur over a period of several months.

There is an important factor when performing these types of tests and that is the ability to demonstrate uncontrolled drinking behavior. This model achieves this by three important factors.

1. Use of taste adulteration: Non-addicted animals will drink significantly less than addicted animals in the presence of quinine.

2. Short-Isolation: Non-addicted animals will increase alcohol intake while isolated. The addicted animals will not consume more alcohol because of the isolation (Spanagel 2000; Spanagel 2017; Hitzemann 2000; Tabakoff and Hoffman 2000).
3. Social rank: Non-addicted animals will increase alcohol consumption with increase in social rank; while subordinate animals drink twice as much. Addicted animals drank the same amount as the control animals. Social rank does not play a role for the addicted animal in increasing the consumption (Spanagel 2000; Spanagel 2017; Hitzemann 2000; Tabakoff and Hoffman 2000).

With the use of this model, the researchers were able to determine that there was a point of no return when it comes to addiction from the following conclusions that can draw from this study (Spanagel 2000; Spanagel 2017; Koskela et al. 2021; Egli 2005; Hitzemann 2000; Tabakoff and Hoffman 2000).

1. Addicted animals do not have influence from external and internal factors.
2. Transition from controlled alcohol consumption to uncontrolled drinking is an irreversible event.

This model has also been validated pharmacologically. The validation primarily relies on the use of the mesolimbic dopaminergic systems (Spanagel 2000; Spanagel 2017; Hitzemann 2000). These systems are found to have the ability to help with reinforcement and adaptation to exposure to toxicants. Exploitation of the dopaminergic system, especially of the D2 receptor, can assist

with cravings and help fight relapses back into alcohol or drug usage (Spanagel 2000; Spanagel 2017; Hitzemann 2000; Koskela, et al. 2021). Unfortunately, the use of dopaminergic agents, such as lisuride and flupenthixol, can increase alcohol and drug intake in both addicted and non-addicted animals (Spanagel 2000; Spanagel 2017; Egli 2005; Spanagel and Hölter 2000). This model can be used to predict how an individual responds to pharmacologic therapy for addiction.

1.3 Immunology and Toxicology of the Central Nervous System

1.3.1 Important Cells of the Central Nervous System (CNS)

The CNS is comprised of many cells. There are neurons, glial cells, and dendritic cells just to name a few. We will focus on the class of cells called glial cells. This class contains Schwan cells, astrocytes, oligodendrocytes, and microglia (Rao 2011; Moser 2008; Colton 2009). We will focus on the astrocytes. The astrocytes are important cells for nervous system health.

Astrocytes have numerous functions, and those are enumerated below. These cells play important roles when it comes to homeostasis of the extraneural environment, providing energy substrates for processing, and limiting the repercussions of oxidative stress (Rao 2011). The energy substrates that are provided by astrocytes include lactate, citrate, alanine, glutathione, and α-ketoglutarate which are all used by neurons. Astrocytes serve as gate keepers for heavy metals that are needed by the brain to function because of the production of metallothioneins (Rao 2011). Astrocytes use glutamate as energy for cellular production, which is an excitatory neurotransmitter (Rao 2011; Moser 2008). Astrocytes are known to produce molecules, such as superoxide dismutase and

glutathione peroxidase, and proteins that are used to deal with the production of free radicals (Rao 2011). The astrocyte is used to control tight junctions of the blood brain barrier and the blood cerebrospinal fluid barrier (Strazza et al. 2011; Moser 2008). Astrocytes are dependent on the release of trophic factors that are involved in synaptic plasticity. Astrocytes modulate the growth of neurites, which are branches of neuronal cell bodies which will become axons and dendrites (Moser 2008; Rao 2011). Astrocytes are also used to promote angiogenesis, detect neuronal injury, perform immune modulation, and help regulate neurotransmitter production (Moser 2008; Rao 2011). Astrocytes also play an important role and makeup the blood brain barrier and the blood cerebrospinal fluid barrier by projecting end feet onto the backside of the barrier (Moser 2008; Rao 2011).

1.3.2 Blood Brain Barrier and Transport of Toxicants

The nervous system enjoys an exclusive and protected status that most other organs do not possess. The nervous system is made up of the brain and spinal cord which are covered in meningeal surfaces, while the peripheral nerves are covered in perineural cells (Moser 2008; Rao 2011; Alvarez et al. 2011; Chow and Gu 2015). The brain is protected by barriers that exist to keep undesirable substances and compounds. The blood brain barrier functions as below:

1. Barrier function. This is an anatomic barrier, which keeps the compartments separate making the brain and the rest of the nervous system a closed system (Moser 2008; Rao 2011; Chow and Gu 2015).

2. Enzymatic barrier. The fact that the substances must use transportation methods, such as active transport or other chemical processes to gain entry to the brain is another barrier that has to be circumvented by substances to exert the effect on the individual (Moser 2008; Rao 2011; Smith 2003).
3. This barrier acts as a control point of access to the brain. There are also some pathways that are available to the individual that are necessary for blood brain barrier integrity. One system is the Hedgehog pathway. This pathway acts as a repair mechanism or use as an anti-inflammatory system (Alvarez, et al. 2011).

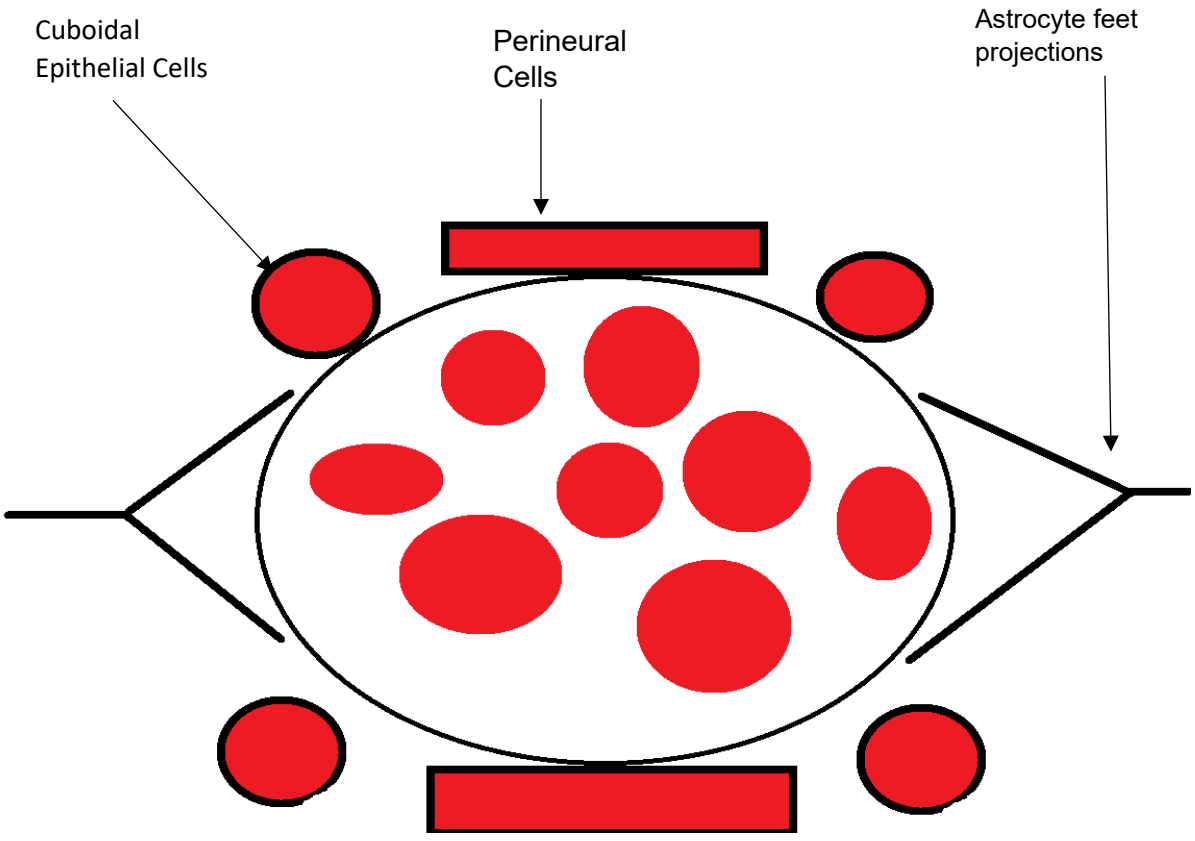


Figure 1.2 – Blood Brain Barrier Schematic. Cross section of blood vessel. Astrocyte foot projections are present. Blood cells are seen in the lumen. Cuboidal epithelial cells and perineural cells present.

The blood brain barrier is made up of specialized cells that are tightly placed together. The tight junctions that are present in the blood brain barrier are 50 to 100 times smaller than peripheral blood capillaries. These cells exist in the microvasculature of the brain and spinal cord (Moser 2008; Rao 2011).

These endothelial cells use multiple transport methods of bringing substances across the barrier and into the brain. The cells use different methods to get substances across the barrier. These methods are active transport, endocytosis, or diffusion (Moser 2008; Rao 2011; Smith 2003; Terasaki and Ohtsuki 2005). Active transport takes energy to move substances across the barrier. The endothelial cells contain transporters, such as the multi-drug resistant protein and p-glycoprotein, that move substances across the cells out of the brain (Moser 2008; Rao 2011; Terasaki and Ohtsuki 2005; Zheng and Ghersi-Egea 2020). These transporters participate in active transport. The ability of the toxicant to cross the barrier depends on the lipophilicity of that toxicant (Moser 2008; Rao 2011; Smith 2003). The more lipophilic the molecule, the better the molecule crosses the vasculature into the brain.

Another transport mechanism is transportation of substances by the use the glial cells or tissue macrophages, which how the brain becomes a persistent reservoir for viral particles (Ko et al. 2019; Machado Andrade and Stevenson 2019). These macrophages will harbor viral particles inside of intracellular vesicles and release once inside the neural environment (Koppensteiner et al. 2012). The substance can diffuse into the glial cell, be transported actively into the glial cell and moved through the blood brain barrier or can be endocytosed by the glial cell

and moved across the barrier that way (Moser 2008; Rao 2011). Otherwise, the substance must cross into the brain from the blood stream using diffusion. Once the integrity of the blood brain barrier has been compromised, substances can pass at will and the individual will suffer undesired effects of intoxication by substances ingested or by infectious processes (Zheng and Ghersi-Egea 2020).

There are exceptions to this systems, which include use of transport by the spinal and autonomic ganglia and comprise the second transport barrier of a substance to enter the brain (Moser 2008; Rao 2011). This barrier is known as the blood cerebrospinal fluid barrier or, blood-CSF barrier. This barrier does not contain specialized endothelial tight junctions, and instead makes use of blood tissue barrier but use astrocytic feet processes to make up this barrier (Moser 2008; Rao 2011; Strazza, et al. 2011). This barrier exists to account for fluctuation of levels of hormones in the blood.

1.3.3 Immune Regulation of Nervous System

When the nervous system is exposed to toxicants, there are varying degrees of response to toxicant exposure. There are several key steps that occur with the exposure to alcohol specifically per Kaminski 2008.

1. Increased depletion of CD4 and CD8 positive cells.
2. Suppression of innate immune system with inhibition of toll like receptors, especially toll like receptor 3. This has been noted cause a pleiotropic effect.
3. High dose binges have been shown to decrease or suppress proinflammatory cytokines and chemokines.

These are key events that have been noted to occur with exposure to alcohol, but there is a lack of information about chronic exposure to alcohol as it pertains to the immune system and response in the brain.

When activated by certain toxicants, including alcohol, cytokines can show elevation or increased production. Alcohol exposure has been shown to trigger interleukin 1 (IL-1) type cytokine, either the α or β subtypes, and these can induce the production of IL-8 and tumor necrosis factor α (TNFα) (Bruckner 2008; Chen, Zhang, and Huang 2016; Kaminski 2008; Ramesh, MacLean, and Philipp 2013; Achur, Freeman, and Vrana 2010; Crews et al. 2006; Harper 2007; Ibáñez et al. 2019; Kamal, et al. 2020; Kelley and Dantzer 2011; Neupane et al. 2016; Qin et al. 2008; Vrij-Standhardt 1991; Yip 2011). In rodents, the primary isoform of IL-1 is the α subtype. These molecules are proinflammatory and can cause other responses in the nervous system as well as other areas of the body. The induction of interferon γ (IFNγ) and TNFα by alcohol causes the activation of macrophages, other cells used for antigen presenting, and can act as a proinflammatory cytokine (Bruckner 2008). The production of IL-6 is also stimulated by the presence of ETOH (Míguez et al. 2012; Gruol et al. 2021). Increased alcohol exposure triggers increased cytokine production due to increase in expression of NF-κB expression in the cells (Kozlov et al. 2020). This is seen as a product of alcohol addiction and other substances (Crews, Zou, and Qin 2011). Also, the use neurotrophins in the brain can help alleviate concerns of toxicant induced neurotoxicity and cell death.

1.3.4 Neurotrophins

The neurotrophins have been shown to protect the brain through a variety of pathways that encourage and promote brain cell survival and protect from neuroinflammation. Brain Derived Neurotrophin Factor (BDNF) plays a role of increasing neuroplasticity by using PI3k/Akt pathways (Kozlov, et al. 2020). These neurotrophins also use other pathways to increase neural environment survival. The use of calcium modulin kinase II (CamkII) phosphorylates the CREB factor which leads to eventual synaptic plasticity increases. The phosphorylation event allows for the regulation Bcl-2 genes, which are antiapoptotic, to be expressed in the nervous system (Kozlov, et al. 2020). This event allows for BDNF to regulate survival of different parts of the nervous system.

With exposure to alcohol intoxication and dependence, there is alteration in the amount of BDNF that is present in the brain. Studies have been completed to show the changes in the levels of BDNF and plasma. The researchers found that there was a substantial decrease in the amount of BDNF and found drew the conclusion that decreased levels of BDNF contributed to decrease in neuronal survival and addictive tendencies (Joe et al. 2007; Zanardini et al. 2011). This has suggested that possibly with other intoxications the addicts have more ability to become addicted. This may suggest that other systems, such as PI3k/Akt pathways, and this would be altered which would alter survival of neurons (Joe, et al. 2007; Kozlov, et al. 2020; Zanardini, et al. 2011).

The changes in cytokines and other factors can lead to a perpetuation by the co-infection of the human immunodeficiency virus (HIV), which leads to the acquired immune deficiency syndrome (AIDS) (Monnig 2017).

1.4 HIV-1 Pathophysiology and the Component HIV-1 Tat

1.4.1 HIV-1 Disease Processes and Replication Processes

HIV-1 is contracted by different risky behaviors, such as unprotected sex and sharing of needles, but may also be transmitted through blood transfusions, and between mother and child at birth. The virus infection can be potentiated and made worse by the use of substances, such as alcohol (Hahn and Samet 2010). It aides in causing more promiscuous behavior, may lead to delays in testing, and generally is associated with poor decision making (Hidalgo, Atluri, and Nair 2015; Pandrea et al. 2010). HIV-1 belongs to the family retroviridae, which are also known as retroviruses (Forbes 2007). These viruses are unique because they contain reverse transcriptase. The uniqueness of these viruses falls to the reverse transcriptase by looking at the amino acid sequence. The reverse transcriptase is made up of two pieces p66, 560 amino acids, and p51, 440 amino acids (Sarafianos et al. 2009). Once sequences are known for this viral family, virus can be further subdivided into many different groups (Sacktor 2018). These viruses are unique because of the ability of this family to start with ribonucleic acid (RNA) and then transcribe to deoxyribonucleic acid (DNA) and back to RNA to finish viral packaging to release from the host cell (Forbes 2007). The viruses contain different factors that allow for optimal viral replication. HIV-1 follows a highly ordered replication process (Sarafianos, et al. 2009).

The infection of this virus follows a sequence as shown below. The initial symptoms start as flu like symptoms, which is characterized as the acute phase. One of the first places the virus will enter is the brain. In HIV infected patients, the

supporting cells, such as the microglia and the astrocytes, are infected, while the neurons remain uninfected (Henderson et al. 2020; Herskovitz and Gendelman 2019; Kramer-Hämmerle et al. 2005; Ton and Xiong 2013; Yadav and Collman 2009). The cellular contents remain infectious and cause an increased inflammatory state. Once the initial phase occurs in the brain and the rest of the body, the disease will progress to the latent phase that can last years (González-Scarano and Martín-García 2005). This phase lasts years due to combined antiretroviral therapy (cART) and highly active antiretroviral therapy (HAART). During this period, viral replication is producing millions of viral particles. This process knocks down the CD4+ counts or the T helper cells that are found to help arm and activate the adaptive immune response (Vidya Vijayan et al. 2017). HIV looks specifically for the CD4+ cells that express the CCR5 coreceptor (Vidya Vijayan, et al. 2017). These cell receptor types are primarily found in the mucous membranes and not in the blood stream. This depletion of these cells is caused by the CD8+ cells or cytotoxic T cells that are looking for cells to induce apoptosis (Vidya Vijayan, et al. 2017; González-Scarano and Martín-García 2005; Kreuzer and Rockstroh 1997). Eventually, the CD4+ cell levels rebound as the infected cells are cleared, which is the hallmark of the latent phase of the disease (González-Scarano and Martín-García 2005; Kreuzer and Rockstroh 1997; Ko, et al. 2019; Killian and Levy 2011). After the latent period the patient transitions to AIDS. This characterized as the CD4+ cell counts to fall below 200 cells/ml of blood. At this time there are more viral copies circulating and being made than their cells to fight the infection (González-Scarano and Martín-García 2005). The

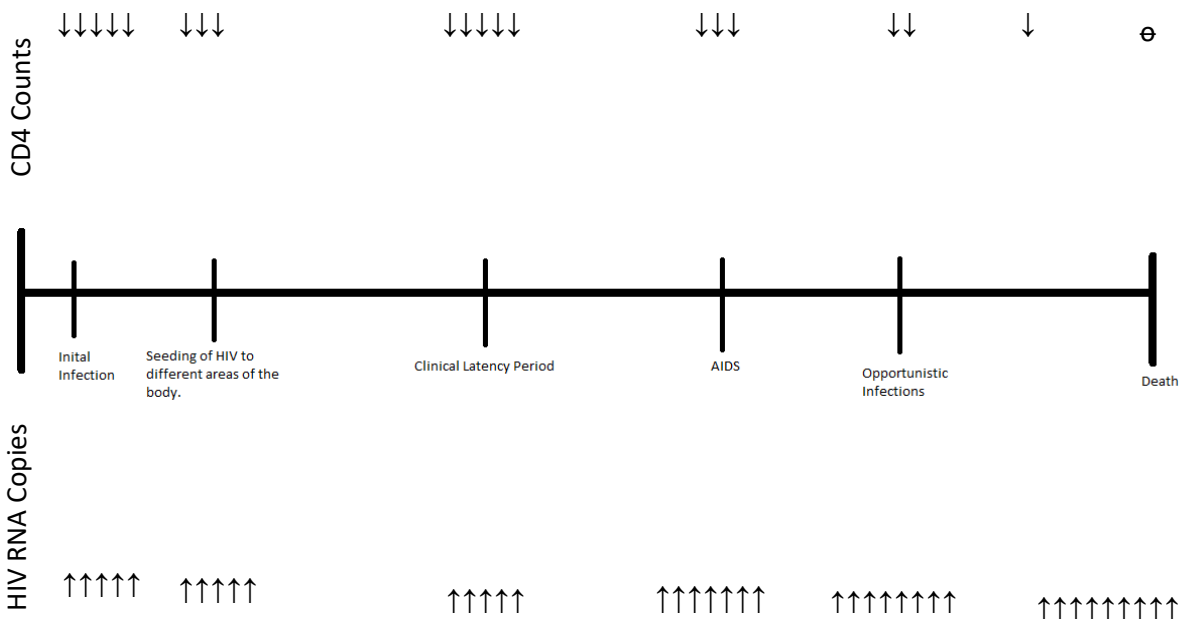


Figure 1.3 – HIV Disease Continuum. As disease progresses CD4 decreases, and viral copies increase.

infected patient is typically overtaken by opportunistic infections and different cancers, which at this point the patient will succumb to these infections and expires (Fauci 2003; Killian and Levy 2011). The virus infection can be potentiated and made worse by the use of substances, such as alcohol. Alcohol contributes to more promiscuous behavior, delays in testing, and poor decision making. The viral replication process follows an ordered process.

As shown below, the HIV-1 viral life cycle starts with attachment via the use of glycoprotein 120 (gp120) and fuses with the host cell causing viral material to enter the host cell. The viral replication machinery is now moved from the outer parts of the cell to the interior or the nucleus of the cell for integration of viral genetic material (Killian and Levy 2011; Fauci 2003). The replication process is managed

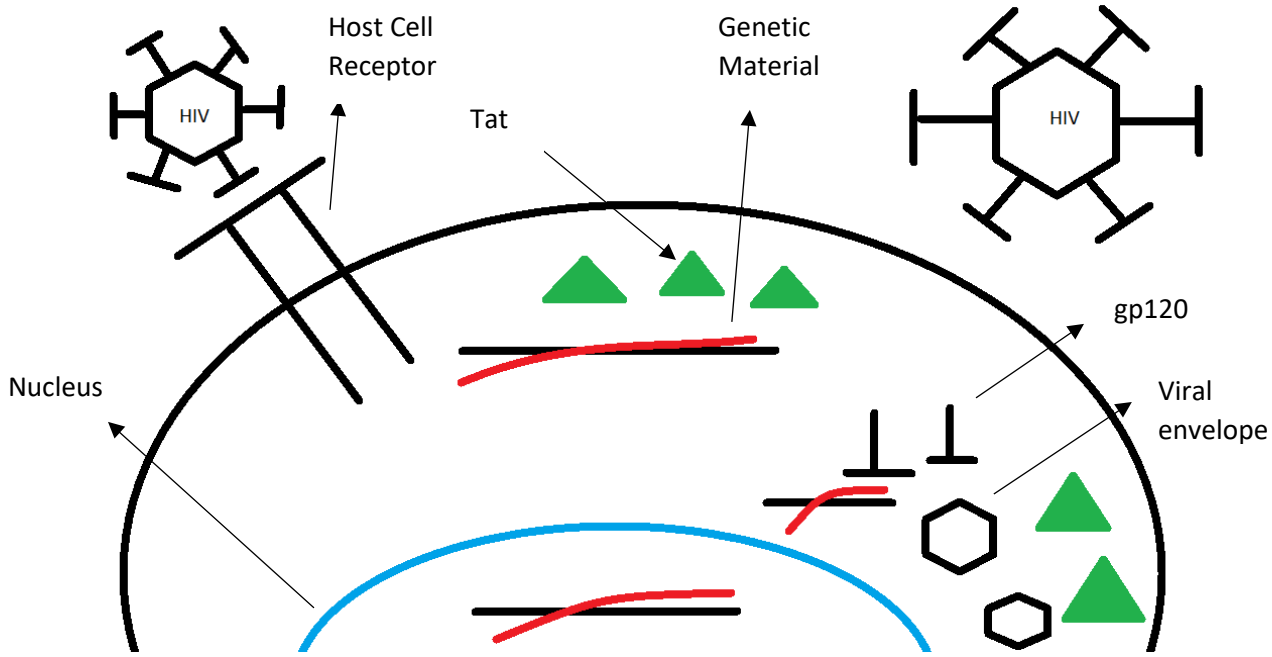


Figure 1.4 – HIV-1 Replication Process. HIV injects into cell and releases viral components. Replicates and makes viral particles in the host cell. Last Step is release of completed viral particle.

by a protein called transactivator of transcription (Tat). The viral genetic material is integrated via viral integrase into the host cell and then copies of that material are made using the formation of complimentary DNA (cDNA) (Fauci 2003; Killian and Levy 2011). Viral particles are made and manufactured using host cell machinery and repackaged for further spread and infection. Once the particles force the cell to capacity, the cell is lysed, and viral particles are released, and the process continues throughout the patient (Fauci 2003; Killian and Levy 2011). This process can be accelerated by the presence of the HIV-1 Tat protein.

1.4.2 HIV-1 Tat Purpose and Research Use

The trans-activator of transcription (Tat) protein subunit inside the HIV virus and is comprised of between 86 and 101 amino acids. This protein length is entirely dependent on the subtype of Tat. The Tat protein has been shown to have

been identified as a major player in viral replication process (Fauci 2003; González-Scarano and Martín-García 2005; Killian and Levy 2011; Bagashev and Sawaya 2013). This protein increases the replicatory properties of the HIV-1 virus. With low presence of this protein, the transcript process is low, meaning low levels of viral particles are present in the host cell (Bagashev and Sawaya 2013; Jadhav and Nema 2021; Rice 2017). Once the protein is present in high amount, the viral particles increase a substantial amount by transcription of the HIV dsDNA (Bagashev and Sawaya 2013). This is made possible by phosphorylation and binding of certain factors that ultimately causes increase in all the needed HIV genes for replicatory processes (Bagashev and Sawaya 2013). This is how the virus defeats the host immune response.

The function of Tat is to increase viral replicatory processes and overcome the host immune system. To activate, as shown below, Tat must bind to an RNA stem loop which causes a trans-activating response (TAR) on the 5' end of the HIV transcripts (Bagashev and Sawaya 2013; Saylor et al. 2016). The alteration of these transcripts allows for the recruitment of positive elongation complex which is dependent on CDK9 and cyclin T1. The requirement for CDK9 is for phosphorylation of two subunits termed NELF and DSIF and the phosphorylation events of the carboxyl terminal domain (CTD) of the RNAP II allow for elongation and causes increase in viral RNA transcription (Bagashev and Sawaya 2013; Saylor, et al. 2016; Haughey et al. 2001). Once the cofactors that comprise the complex have bound to Tat, the replication process proceeds and binds to the RNA polymerase II. The attachment of the Tat protein to the RNA polymerase II during

elongation step on the full-length TAR transcripts suggest that it takes two Tat molecules to activate this process (Bagashev and Sawaya 2013; Saylor, et al. 2016). This suggests that this is the minimum requirement to assist and ramp up viral replicatory process for efficient viral replication. Once this complex is assembled, it is termed the super elongation complex (SEC). This process is regulated by the binding of NF-κB (Bagashev and Sawaya 2013; Saylor, et al. 2016). Once viral replication is complete, Tat can also be found outside of this complex.

The Tat protein can be found in the blood stream of patients infected with the HIV virus and can be viewed as a toxin deliverable to uninfected host cells (Bagashev and Sawaya 2013; Jadhav and Nema 2021; Meyaard et al. 1992; Nath et al. 1999; Rice 2017; Hahn et al. 2015). Once released into the cellular environment by lysis of infected cells, Tat acts by causing cellular dysfunction, toxicity, and eventually cell death. Once the toxin has been allowed to absorb into these cells, the immune response causes apoptosis of uninfected host cells (Fauci 2003; Killian and Levy 2011). Tat protein is implicated in the mediation of apoptosis, the increase of blood brain barrier permeability, and the enhancement of the secretion of proinflammatory cytokines (Fauci 2003; Killian and Levy 2011; Strazza, et al. 2011; Hahn, et al. 2015; Nath, et al. 1999). Tat, itself, is known to play a part in the process that is known as HIV Associated Neurocognitive Disorders also known as (HAND) (Bagashev and Sawaya 2013; Fauci 2003; Hahn, et al. 2015; Killian and Levy 2011; Marino et al. 2020).

1.4.3 HIV Associated Neurocognitive Disorders

HIV Associated Neurocognitive Disorders (HAND) are a spectrum of diseases that have been recognized since 2007 development of diagnostic nomenclature (Saloner and Cysique 2017). The spectrum of disease includes several different severities of neurocognitive disorders. These diseases include asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD) (Crews et al. 2009; Eggers et al. 2017; Elbirt et al. 2015; Saylor, et al. 2016; Ghafouri et al. 2006; Omeragic et al. 2020; Alfahad and Nath 2013; Ances and Ellis 2007; Bougea et al. 2019; Gannon, Khan, and Kolson 2011; Spudich 2013). The most common form that is experienced by patients is HAD, which is found to be fatal in most cases (Saylor, et al. 2016; Elbirt, et al. 2015; Saloner and Cysique 2017; Wallace 2022). With the addition of combined antiretroviral therapy (cART), deaths have decreased over time (Crews, et al. 2009; Eggers, et al. 2017; Elbirt, et al. 2015; Omeragic, et al. 2020; Saylor, et al. 2016; Ghafouri, et al. 2006). Prior to the implementation of cART, there were a high number of deaths from patients that had AIDS. With further understanding, pairing different HIV therapies together has led to a 50% decrease in deaths of patients from AIDS (Crews, et al. 2009; Eggers, et al. 2017; Elbirt, et al. 2015; Saylor, et al. 2016; Ghafouri, et al. 2006; Omeragic, et al. 2020; Alfahad and Nath 2013; Ances and Ellis 2007; Bougea, et al. 2019; Gannon, Khan, and Kolson 2011; Spudich 2013).

HAND progression now varies because of the presence and usage of cART and can remain stable over long periods of time. The use of cART has allowed patients to suppress viral production and remain aviraemic. Lower CD4+ counts

means that the degree of immunosuppression in these patients is high, which makes this quality a risk factor for transitioning through the different phenotypes from ANI to MND and finally progression to HAD (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Omeragic, et al. 2020; Saylor, et al. 2016). HAND typically presents with loss of executive functions and memory impairment. Prominent loss and disruption of attention also occurs. The ability to multitask, impulse control, and judgement decreases (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Omeragic, et al. 2020; Saylor, et al. 2016; Alfahad and Nath 2013; Ances and Ellis 2007; Bougea, et al. 2019; Gannon, Khan, and Kolson 2011; Spudich 2013). The ability to recover memory is attenuated for these patients. Motor coordination skills such as gait, motor coordination, and bradykinesia are present and are known symptoms of HAND. There have also been reported changes in spatial learning and memory which are considered the most common symptoms of HAND (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Omeragic, et al. 2020; Saylor, et al. 2016). Overall, the initiation of cART has resulted in fewer HIV and AIDS complications but cognitive impairment still remains (Valcour et al. 2011).

The risk of developing HAND is thought to be linked to a variety of environmental and comorbidities. The status of the individual's cardiovascular health has found to be important, with studies also specifically looking at obesity, diabetes, lipid disturbances, and tobacco usage as factors that will affect cognitive performance (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Omeragic, et al. 2020; Saylor, et al. 2016; de Almeida et al. 2013). The age of the

patient has been shown to be a factor with respect to formation of dementia and ultimately HAND (Guo and Buch 2019). Some patients have reported and have undergone increased cognitive problems from the presence of HAND (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Omeragic, et al. 2020; Saylor, et al. 2016; Silverstein, Kumar, and Kumar 2014). Other factors that should be considered when a patient has chance of diagnoses with HAND are substance-abuse status, nutritional status, presence of traumatic brain injury, obstructive sleep disturbances, psychiatric diseases such as anxiety disorders, major depressive disorder, and bipolar disorders (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Omeragic, et al. 2020; Saylor, et al. 2016). Other factors that should be considered with patients who are taking cART would be age, CD4+ count levels, substance abuse status, HCV, cerebrovascular disease status, sleep disturbances, and psychiatric disease status (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Guo and Buch 2019; Hidalgo, Atluri, and Nair 2015; Mattson, Haughey, and Nath 2005; Omeragic, et al. 2020; Saylor, et al. 2016; Silverstein, Kumar, and Kumar 2014; Alfahad and Nath 2013; Ances and Ellis 2007; Bougea, et al. 2019; Gannon, Khan, and Kolson 2011; Spudich 2013).

There are biomarkers that influence factors that have been determined to affect the severity of HAND. Biomarkers that have been determined to have influence on cognitive status have been grouped as to function to aid in diagnosis of HAND. The groups are cell stress, neuronal injury/protection, oxidative stress, energy metabolism, immune activation, and the status of glutamate regulation ability (Crews, et al. 2009; Eggers, et al. 2017; Elbirt, et al. 2015; Kaul, Garden,

and Lipton 2001; Marino, et al. 2020; Mattson, Haughey, and Nath 2005; Pocernich et al. 2005; Porter and Sutliff 2012; Saro et al. 2021; Saylor, et al. 2016; Alakkas et al. 2019; Alfahad and Nath 2013; Ances and Ellis 2007; Bougea, et al. 2019; Gannon, Khan, and Kolson 2011; Spudich 2013). The cell stress, neuronal injury/protection, and oxidative stress biomarkers focuses primarily on cerebrospinal fluid (CSF) content and other substance found in the brain such as sphingomyelin levels, β-amyloid levels, iNOS levels, and other carbonyl levels (Alakkas, et al. 2019; Crews, et al. 2009; Eggers, et al. 2017; Elbirt, et al. 2015; Kaul, Garden, and Lipton 2001; Mattson, Haughey, and Nath 2005; Saylor, et al. 2016; Alfahad and Nath 2013; Ances and Ellis 2007; Bougea, et al. 2019; Gannon, Khan, and Kolson 2011; Spudich 2013). The energy metabolism group primarily looks at the levels of brain choline levels. The immune activation group focuses on cytokines such as IL-1α/β, IL-12, MCP-1, TNFα and various cluster differentiation markers as well as CSF content and chemistry (Crews, et al. 2009; Eggers, et al. 2017; Elbirt, et al. 2015; Kaul, Garden, and Lipton 2001; Marino, et al. 2020; Mattson, Haughey, and Nath 2005; Pocernich, et al. 2005; Porter and Sutliff 2012; Saro, et al. 2021; Saylor, et al. 2016; Alakkas, et al. 2019). The ability to regulate glutamate is an important consideration when evaluating patients for cognitive function and staging of HAND progression (Haughey, et al. 2001). These markers will help stage patients according to status, but this highlights the need for better biomarkers to highlight different stages of the disease to allow staging.

There have been numerous animal models developed to study HAND. The models have been made in many different species with the most popular being in

non-human primates and rodents (Gorantla, Poluektova, and Gendelman 2012; Jaeger and Nath 2012; Kim et al. 2003; Langford et al. 2018; Nookala et al. 2018; Sil et al. 2021). The non-human primate models are especially important and very useful because the use of SIV-infected macaques have been found to mimic HIV alterations that occur in humans (Saylor, et al. 2016). The use of macaques for the purpose of researching HAND has allowed the collection of samples, such as CSF, plasma, and other CNS samples at various time points throughout the study (Saylor, et al. 2016; Gorantla, Poluektova, and Gendelman 2012; Jaeger and Nath 2012; Kim, et al. 2003; Langford, et al. 2018; Nookala, et al. 2018). The use of these animals has also allowed the researchers to study HAND from the asymptomatic stages to the most terminal stages of the disease, and the use of these models has allowed researchers to give cART and study the disease in the proper context.

There have also been rodent models developed. These models make usage of transgenic animals with genetic additions that are typically under glial fibrillary acidic promoter (GFAP) control (Gorantla, Poluektova, and Gendelman 2012; Jaeger and Nath 2012; Kim, et al. 2003; Langford, et al. 2018; Nookala, et al. 2018). The animals normally have to be administered an agent to turn on the gene that has been bred into these animals, which is typically doxycycline. These animals have been known to show synaptic pathology, spatial learning and memory deficits, and anxiety-like behaviors (Saylor, et al. 2016; Gorantla, Poluektova, and Gendelman 2012; Jaeger and Nath 2012; Kim, et al. 2003; Langford, et al. 2018; Nookala, et al. 2018). Some limitations of the use of rodent

models are that they cannot mimic complex stages of the disease such as invasion of the brain by HIV-infected and uninfected monocytes and macrophages, or by free unbound virus, which is a hallmark of HAND (Gorantla, Poluektova, and Gendelman 2012; Jaeger and Nath 2012; Kim, et al. 2003; Langford, et al. 2018; Nookala, et al. 2018; Saylor, et al. 2016).

There has been substantial work completed on the evolution and disposition of HAND. There are many risk factors and biomarkers that have been used to diagnose and stage HAND, itself. There have been models created that have allowed further research into the disease process and have allowed ways to mimic the disease progression of HAND that have helped study the disease. Therapies are available to help ameliorate the considerations that have been noted above. There are researchers that are working to repurpose drugs and find new therapies. One therapy that has been posed is the use of peroxisome proliferator activated receptor gamma drugs (PPARγ), namely rosiglitazone (Rosi) (Huang et al. 2014; Wen Huang et al. 2009; Huang et al. 2015; Omeragic et al. 2017; Omeragic et al. 2019; Potula et al. 2008).

1.5 Pharmacology and Toxicology of PPARγ and Rosiglitazone

1.5.1 Pharmacology of PPARγ

PPARγ receptors belong to a superfamily that are primarily associated with steroid and thyroid receptors (Katzung 2015). These receptors are found primarily in muscle, adipose tissue, brain, liver, large intestine, and macrophages (Katzung 2015; Warden et al. 2016). These receptors have been found to be involved in a variety of functions including glucose metabolism and availability, blood vessel health in the body and the brain, adipocyte differentiation, insulin signal transduction, release of various factors that affect lipid metabolism, and infectious disease processes (Barnstable, Zhang, and Tombran-Tink 2022; Castro, Gonçalves-de-Albuquerque, and Silva 2022; Giralt, Domingo, and Villarroya 2009;

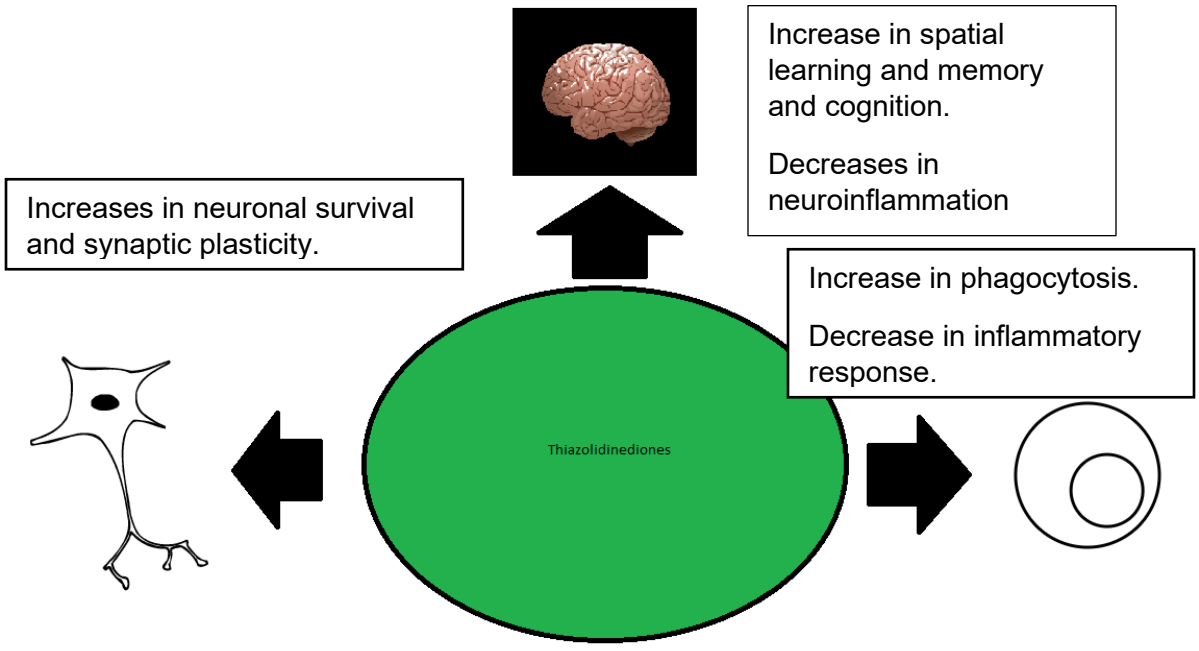


Figure 1.6 – The pharmacological benefits of Thiazolidinediones (TZDs). These drugs show several different benefits. Increasing brain health and decreasing inflammatory responses.

Huang, et al. 2015; Katzung 2015; Koethe 2017; Layrolle, Payoux, and Chavanas

2021; Morsy et al. 2022; Porter and Sutliff 2012; Vázquez-Carrera and Wahli 2022; Huang, et al. 2014; Marciano et al. 2015; Olefsky and Saltiel 2000; Semple, Chatterjee, and O'Rahilly 2006; Wang, Dougherty, and Danner 2016). The activity of these receptors has also been shown to be anti-inflammatory with decreasing levels of C-reactive protein and various interleukins, namely IL-6 (Huang, et al. 2015; Katzung 2015; Park et al. 2022; Vázquez-Carrera and Wahli 2022; Zamanian et al. 2022). These receptors have been reported to be implicated and described as possible therapy of different neurological disorders, namely Alzheimer's Disease, Parkinson's disease, and different forms of dementia (Aghaei et al. 2019; Aleshin et al. 2013; Barnstable, Zhang, and Tombran-Tink 2022; Behl et al. 2021; Cai et al. 2018; Cai et al. 2015; Jahrling et al. 2014; Liu, Chen, and Chang 2022; Michailidis et al. 2022; Ogura and Yamaguchi 2022; Shrestha et al. 2022; Sola et al. 2022; Sundararajan et al. 2006; Tufano and Pinna 2020; Zamanian, et al. 2022).

The effects of this receptor and drug usage have had the ability to increase neuronal survival, memory cognition, decrease levels of β amyloid levels, and decrease inflammatory processes just a name a few things (Katzung 2015; Huang, et al. 2015; Justin et al. 2020; Labandeira et al. 2022; Michailidis, et al. 2022; Ogura and Yamaguchi 2022; Omeragic, et al. 2017; Ramesh, MacLean, and Philipp 2013; Villapol 2018; Warden, et al. 2016; Zamanian, et al. 2022; Bosse 2011; Wang, Dougherty, and Danner 2016). There are multiple drugs that have been available for usage that react with these receptors, most are members of the glitazone family. These drugs are pioglitazone, rosiglitazone, and troglitazone.

Rosiglitazone and pioglitazone are still both available for prescription usage. Troglitazone was removed from the market due to fatal side effects resulting in liver failure (Bosse 2011; Katzung 2015).

The current work focuses on rosiglitazone.

1.5.2 Pharmacology of Rosiglitazone

Rosiglitazone is a drug that belongs specifically to the thiazolidenones (TZD) family of medications that work on the PPARγ superfamily of receptors (Katzung 2015; Bosse 2011; Cai, et al. 2018; Sundararajan, et al. 2006; Warden, et al. 2016; Yu et al.). This drug continues to be primarily protein bound and metabolized with minimal metabolite formation. The isoenzymes that are used to perform metabolic procedures are CYP2C8 being major isoenzyme used while CYP2C9 is the minor user for metabolic procedures, and the drugs work by causing decrease in glucose levels by using the GLUT 1 and GLUT 4 receptors (Bosse 2011; Katzung 2015; Marciano, et al. 2015; Olefsky and Saltiel 2000; Semple, Chatterjee, and O'Rahilly 2006; Wang, Dougherty, and Danner 2016). Rosiglitazone can either be used as a monotherapy, but also can be used as a combination therapy with metformin, which is a biguanide. The combination has been shown to not cause hypoglycemia in patients. The use of rosiglitazone specifically has been shown to cause increase in lipid levels (Cholesterol, HDL, and LDL levels) but does not really affect the levels of triglyceride (Bosse 2011; Katzung 2015).

1.5.3 Toxicology of Rosiglitazone

There are several damaging side effects associated with the usage of rosiglitazone for glycemic control or decreasing inflammation (Bosse 2011; Katzung 2015). Rosiglitazone has been shown to cause an increase in angina pectoris, impaired blood flow to the heart, and the incidence of myocardial infarction but was noted to have similar side effects of other hypoglycemic agents (Berthet et al. 2011). Due to these considerations, the usage of this drug has been limited in Europe and the United States (Berthet, et al. 2011).

Fluid retention has occurred in some patients that are also using insulin therapy for glycemic control. For patients who are experiencing heart failure or have the risk for heart failure, this class of drug should be avoided. For women, the loss of bone density and bone fracturing have been noted (Bosse 2011; Katzung 2015; Marciano, et al. 2015). The fracturing is thought to be from the lack of osteoblast presence in the bone (Bosse 2011; Katzung 2015). Anemia has been reported to occur, because of increased levels of plasma volume in the blood not due to decreases in the red cell mass (Bosse 2011; Katzung 2015). Weight gain has been reported for patients that use sulfonylurea or insulin in combination with PPARγ drugs that are available for usage. The weight gain is thought to be from excess fluid, but not from an increase in fat mass (Bosse 2011; Katzung 2015).

1.6 Linkage of Alcohol, HIV, and PPARγ Agonist

There are three major points to remember when considering how the different topics brought together in this work may interact during research activities as well as application in clinical usage. The use of alcohol and PPARγ agonist has been documented to be used together to ameliorate the concerns of growing

neurodeficit (Barnstable, Zhang, and Tombran-Tink 2022; Cai, et al. 2018; Fotio et al. 2021; Sola, et al. 2022; Sun et al. 2012; Wallace 2022; Brust 2010). The use chronic alcohol while being co-infected with HIV is a long-standing issue in the field of HIV research and clinical work. We will consider these agents, and how to create a system to study the interactions between alcohol, HIV, and the PPARγ agonist. The work and conclusions ascertained from this study will help to move usage of PPARγ agonist drugs to potential clinical practice and usage. The usage of this drug will help alcohol dependent individuals and HIV infected individuals with developed neurodeficit.

1. Alcohol and HIV are known to be used together by patients, which exacerbates the effects of both (Atluri 2016; Chilunda et al. 2019; Hidalgo, Atluri, and Nair 2015; Pandrea, et al. 2010; Silverstein and Kumar 2014).
2. The use and presence of HIV and alcohol allows cellular environments to change and receptor constitution to change on a continuous basis (Crews et al. 2017; Erickson et al. 2019; Flora et al. 2005; Gruol, et al. 2021; Hauser and Knapp 2014; Pandrea, et al. 2010; Silverstein and Kumar 2014; Liu et al. 2009; Matavele Chissumba et al. 2015). The use and presence of both HIV and alcohol can cause changes in synaptic plasticity and neurochemical makeup (Atluri et al. 2015; Avdoshina, Bachis, and Mocchetti 2013). These effects may to cause neurodeficit either together or separately with prolonged exposure.

3. The use of exposure protocols and animal models. There are multiple animal models that have been developed to mimic a disease or show a certain disease characteristic for the study of diseases (HIV) or substances (alcohol and PPARγ agonist) (Acheson, et al. 2013; Fitting et al. 2013; Gorantla, Poluektova, and Gendelman 2012; Jaeger and Nath 2012; Jeanblanc, et al. 2019; Kim, et al. 2003; Langford, et al. 2018; Lovinger and Crabbe 2005; McBride and Li 1998; Nookala, et al. 2018; Park, et al. 2022; Sil, et al. 2021; Spanagel 2000; Spanagel 2017; Wallace 1990; Piątkowska-Chmiel et al. 2022). There have multiple models to ascertain how HIV causes brain pathology and causes neurodeficit in animals that have been allowed to transfer those affects to humans.
4. The use and repurposing of PPARγ agonists as neuroprotective agents. The PPARγ receptors reside in the brain. These agents specifically rosiglitazone have been shown to decrease or circumvent the neurodeficit that is experienced by patients that suffer from Alzheimer's disease, Parkinson's disease, and different forms of dementia (Cai, et al. 2015; Justin, et al. 2020; Sagheddu et al. 2021; Shrestha, et al. 2022; Sola, et al. 2022; Tufano and Pinna 2020; Kummer and Heneka 2008; Zolezzi et al. 2014). HIV associated neurocognitive disorders have been noted to have characteristics that resemble pathology that is seen in Alzheimer's disease, with the dementia and resulting neurodeficit with

memory and alterations in motor coordination (Eggers, et al. 2017; Elbirt, et al. 2015; Ghafouri, et al. 2006; Marino, et al. 2020; Saylor, et al. 2016).

With all of this in mind, several questions must be addressed:

1. How long must exposure to alcohol persist for it to cause a significant change in spatial learning and memory and motor coordination changes in an animal system?
2. How long must exposure to alcohol persist for significant synaptic plasticity changes to occur?
3. Do HIV-1 Tat and alcohol work cause alterations in spatial learning and memory, motor coordination, receptor constitution changes, and change in cytokine levels?
4. Does the addition of Rosiglitazone to HIV-1 Tat exposed animals cause circumvention of neurodeficit with respect to spatial learning and memory, alterations in motor coordination, receptor constitution changes, and change in cytokine levels?

To help answer these questions, the researcher has made several hypotheses:

1. We hypothesize that with prolonged duration of exposure to alcohol, there will be changes in motor coordination and spatial learning and memory as well as changes in synaptic plasticity.
2. We hypothesize that with prolonged exposure HIV-1 Tat and alcohol will cause alterations and significant changes in spatial learning and memory, motor coordination, receptor constitution, and cytokines.

3. We hypothesize that the addition of rosiglitazone to HIV-1 Tat exposed animals will circumvent the neurodeficit and associated changes in spatial learning and memory, motor coordination, receptor constitution, and cytokines.

CHAPTER 2

LONG TERM ALCOHOL USE CAUSES NEURODEFICIT: AN EXPOSURE PLAN AND CONSEQUENCES

2.1 Introduction

Alcohol Use Disorders (AUD)s remain a problem worldwide. In the US, a significant number of people have and will be affected by AUDs in their lifetime (Grant et al. 1994; Patrick and Schulenberg 2013). This has led to a plethora of research to understand AUDS and mitigate their effect.

Researchers have evaluated these conditions on many different levels. AUDs have been studied and many different models have been used to study them on a short- and long-term basis. Most models that are used to study AUDs focus on a particular aspect of alcohol use disorders, which makes conclusions easier to draw from the research that is completed (Jeanblanc, et al. 2019; Lovinger and Crabbe 2005; McBride and Li 1998; Spanagel 2000; Spanagel 2017; Wallace 1990). Researchers use *in vivo* models to mimic portions of this disease. These models make sense because of the simple fact that animals in nature will consume rotten foods that have been allowed to ferment (Spanagel 2000; Wallace 1990). After consuming these foods, animals have been noted to exhibit properties of alcohol intoxication, which allows all alcohol models to have the principle of self-administration. That animals complete these behaviors allows them to be used for alcohol reinforcement training and behavior (Spanagel 2000; Wallace 1990).

Administration methods vary widely across researchers and models. The most common method of administration is the process of “two bottle choice.” Two

bottle choice allows for the researcher to give alcohol to the animal while taking advantage of alcohol seeking behavior (Spanagel 2000). The method works by fitting the cage of the animal with a bottle of water and a bottle of alcohol. The animal is allowed to drink for a predetermined amount of time; afterward, the alcohol bottle is documented for the amount drank (Spanagel 2000). Researchers have also used gastric gavage (Jeanblanc, et al. 2019; Lovinger and Crabbe 2005; McBride and Li 1998; Spanagel 2000; Wallace 1990). This involves placing a feeding needle down the animal's throat and delivering the metered dose of alcohol to the animal. This can be done at set points of the day. Researchers have used vapor chambers and injections of alcohol, but these methods do not mimic how humans typically consume alcohol (Spanagel 2000; Wallace 1990).

As stated above, there have been many models used to study AUDs. Some of the models are used for long-term as well as short-term administration (Jeanblanc, et al. 2019; Spanagel 2000; Eaton 2008). When selecting the appropriate model for studying a certain aspect of alcoholism, the researcher should consider what is the end goal of the research (Spanagel 2000). Most models that have been used for alcohol research have the feature and the ability to trigger compulsive and uncontrollable drinking behaviors that are known to be associated with tendencies toward full-blown alcohol use disorders (Spanagel 2000).

Models like the reinstatement model, deal specifically with the relapse and craving features of AUDs where preference does not need to be measured. In studies where preference is important or needs to be measured, the researcher

should use the two-bottle choice model. This model can be used in a long-term and short-term setting; however, this model does not consider addictive and compulsive behavior (Spanagel 2000). However, some models are developed for long-term use only.

The long-term alcohol self-administration with repeated deprivation phases is such a paradigm. In this paradigm, the animal is monitored for alcohol-seeking and taking behavior that traditionally seen in people affected by alcohol use disorders (Lovinger and Crabbe 2005; Spanagel 2000; Wallace 1990). The animal is subjected to free access to the alcohol for a period of two months and then the solutions are removed from the cage for a pre-determined amount of time (Spanagel 2000). After the deprivation phase, the solutions are placed back in the cage and this process is repeated. This process in meant to induce compulsive behavior, which has been noted in different species, e.g., mice, rats, monkeys, and humans. This model also meets the criteria for alcohol use disorder, as to be a suitable model to study alcohol use disorder (Hasin, et al. 2013; Grant, et al. 1994). This method can be pharmacologically validated using drugs that are used to block cravings and relapses.

The last model is known as the “point of no return.” This model examines alcohol seeking behavior on a continuum. The model starts off by the animal having free access to alcohol and water. These animals may consume a significant amount of alcohol; alternatively, the animal may abstain from drinking alcohol (Spanagel 2000; Wallace 1990). After this period, animal alcohol seeking behavior is altered by smell and taste of the solutions. The animal will either drink

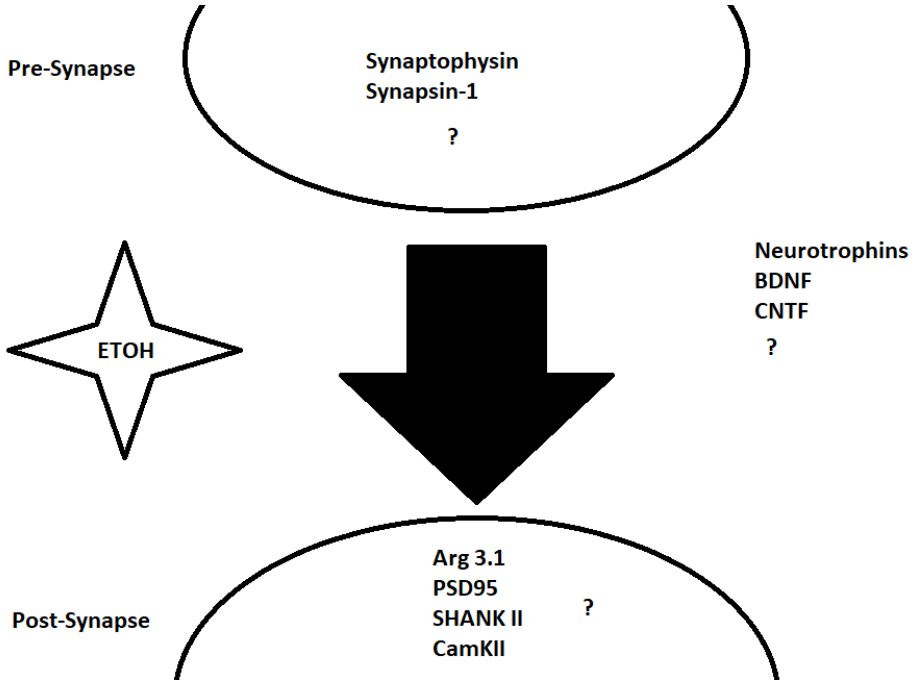


Figure 2.1 – Summary schematic for hypothesis. The figure above illustrates a synapse with protein markers being illustrated in pre-synaptic, synaptic cleft, and post-synaptic locations.

more or will drink less. This is the phase where the animal will learn to access the psychotropic effects of alcohol, which will also influence alcohol seeking behavior (Spanagel 2000; Wallace 1990). This model continues for several months at varying strengths of solutions. Without changing the animal surroundings, the animal increases their alcohol intake and volume. At this point and time, the animal is said to have crossed the point of no return (Spanagel 2000; Wallace 1990).

Behavioral testing can be completed as a supplement to these studies. Alone they measure important aspects of alcohol use disorder. When comparing the use of behavioral assays and these models, there was no significant difference when conducted in different labs, when looking at variation between labs (Jury et al. 2017). The use of behavioral assays allows for other measurements to be taken and the ability to apply different parameters to categorize varying pathologies and

neurobiological changes (Nookala, et al. 2018). Behavioral changes allow for other conclusions to be drawn and may offer more information for the researcher.

For this study, we wanted to answer the questions: 1. How long must exposure to alcohol persist for it to cause a significant change in spatial learning and memory and motor coordination changes in an animal system? 2. How long must exposure to alcohol persist for significant synaptic plasticity changes to occur? These questions allow us to make the following hypotheses: We hypothesize that with prolonged duration of exposure to alcohol, there will be changes in motor coordination and spatial learning and memory. We hypothesize that prolonged exposure to alcohol we cause changes in the regulation of synaptic plasticity as seen in figure 2.1.

2.2 Materials and Methods

2.2.1 Animals

Animals were obtained from the Jackson Laboratories and the colonies were maintained inside the Laboratory Animal Research Core (LARC) facility. We used 8-week-old C57/BL6J mice, both male and female mice, equaling n=60. The mice were divided among three time points, 4, 8, and 12 weeks of treatment. For the 4-week time point, the males (n=9) and females (n=10) were split into control and alcohol for their respective sexes. For the 8-week time point, the males (n=10) and females (n=10) being split between the control and alcohol groups for their respective sexes. For the 12-week time point, the males (n=8) and females (n=14) being split between the control and alcohol groups for their respective sexes. Food

and water were never restricted in this work. The mice were housed 3 to 5 animals to a cage inside the LARC facility. The animals were on a 6 am to 6 pm light and dark cycle. All behavioral testing was completed between the hours of 8 am to 5 pm till termination of the experiment. Animals were returned to the LARC at the end of the day after the experimentation was completed for the day. All procedures were approved and followed in accordance with the UMKC Institutional Animal Care and Use Committee.

2.2.2 Alcohol

Animals were given 20% alcohol by gastric gavage (diluted from Absolute Ethanol from Fisher, New Jersey). Animals were started on the alcohol at 8 weeks of age. The animals were weighed at the beginning of the experiment and weighted once a week till termination of the experiment for the animals assigned time point. Alcohol levels were determined based upon weight of the animals. The animals were challenged on the first three days with 1 g/kg by weight of 20% alcohol twice daily. After the 3rd day, the animals were given 2 g/kg by weight of alcohol twice daily until the experiment termination. The control mice were administered sterile water by gastric gavage at the start of the experiment and until termination. The sterile water was administered the same way the alcohol mice were challenged with the alcohol.

2.2.3 Rotor-Rod

The rotor-rod test is used to test the motor-coordination and locomotion. The more time spent on the rod means the animal has good motor-coordination and that locomotion is intact. At the start of the experiment, the animals were

brought from the LARC facility to the lab and the animals were left to sit undisturbed in the experiment room. After the hour, the mice were given their dose of alcohol (2 g/kg by weight) or water and then left undisturbed for 1 hour. The protocol is to give each animal 4 trials and speed the rod up 1 rpm by 15 seconds. During the course of the experiment, the time spent, and distance traveled on the rod are recorded each of the 4 trials.

2.2.4 Water Maze

Water maze test is a test of spatial learning and memory. The test takes place in a water tank. The water temperature in the water tank is regulated every day between 20 to 25 degrees Celsius. The escape island is located 1 cm below the water surface. The tank is laid out according to compass direction (Vorhees and Williams 2006). On the wall of the tank, there are cues on the walls of the water tank which allows the animal to navigate the water tank. The test takes place over the course of 6 days. Days 1 thru 5 are the test period with day six being the probe trial, which is a test of reference memory (Vorhees and Williams 2006). The animals are placed in the water tank for 4 random directions. For the test period, the escape platform is left in the water tank submerged for the 5 days in the southwest quadrant (Vorhees and Williams 2006). For the probe trial, the trial is 60 s per animal and the platform is removed from the southwest quadrant. The animals are tracked by the ANY-maze (version 4.99z) behavioral software. Spatial learning and memory deficit was analyzed by analyzing the escape latency (the time it takes to get to the escape platform) and the probe trial (time spent in the target quadrant) (Vorhees and Williams 2006).

2.2.5 Sample Collection

Mice were euthanized after the behavioral experiments were completed. The mice were anesthetized using a ketamine/xylazine solution. Once the mice were sufficiently under anesthesia, the mice's heart was perfused with 8 ml of isotonic PBS (Nookala, et al. 2018). The mice brain tissue was dissected after brain tissue extraction. The cerebellum was coronally bisected separating the medulla and the pons. The parietal cortex was coronally bisected separating the two hemispheres of the brain. The hippocampus was dissected out from the parietal cortex. All the brain tissue was collected, the brain tissue was snap frozen in liquid nitrogen (Nookala, et al. 2018). After the brain tissue was snap frozen in the liquid nitrogen, the brain tissue was moved to a freezer for storage.

2.2.6 Western Blot

The tissue collected from the mice was taken from the freezer and were placed on ice. The samples had a mixture of RIPA buffer and protease inhibitor added to them based upon the number of samples to be analyzed. The parietal cortex had 800 μl of the RIPA buffer mixture, the cerebellum had 800 μl of the RIPA buffer mixture, and the hippocampus had 400 μl of the RIPA buffer mixture to all the sections (Nookala, et al. 2018). The samples were homogenized and then spun at 4 degrees Celsius for 20 minutes at 14,000 rpms. Individual BCA assays were completed for each sample. The samples were then pooled to 1,000 ug of protein into their respective groups. A BCA assay was completed on the pooled samples. This allowed samples to have equal amounts of protein for the Westerns. The samples were made from pooled samples, and the samples contained 40 μg.

The samples were loaded and electrophoresed on a 10% polyacrylamide gel at 60 volts for 1 hour and then allowed to resolve at 100 volts for 2 hours. The gels were transferred to nitrocellulose membranes using the Trans-blot Turbo transfer system (Bio-Rad, Hercules, CA). The nitrocellulose membranes were probed with a variety of primary antibodies. Those antibodies were SHANK2, PSD95, synapsin-1, Arg 3.1, BDNF, synaptophysin, CamKII, and CNTF. GAPDH was used as the loading control to normalize all primary antibodies listed above. The blotting substrate that was used to visualize the membranes was the Clarity Western Substrate (Bio-Rad, Hercules, CA). The bands were quantified by using a gel imaging instrument (Fluorochem E 4.1.4) with the complimentary software Alpha View SA (version 3.4.0.0).

2.2.7 Statistics

Values are reported for all behavioral assays and Western blot as mean ± standard error of the mean (SEM). Statistical methods that were completed used IBM SPSS software (version 24) (IBM, Armonk, NY). Statistical methods that were used for the behavioral assays and the Western blots was two-way ANOVA with factors being sex and ETOH exposure. Only F values that showed significant interactions are represented in the text, while non-significant F values are not represented in the text. The Tukey post-hoc test was used for further testing with *a priori* significance level being set at $p < 0.05$. For the behavioral assays across the time points, three-way ANOVA was used with factors being sex, alcohol exposure, and time point. The Tukey post-hoc test was used for further testing

with *a priori* significance level being set at $p < 0.05$. On the figures, significant results are indicated by a *, and to indicate sex differences are depicted by a #.

2.3 Results

2.3.1 Effect of Alcohol on Locomotion and Motor Coordination

The effect of alcohol (ETOH) and sex was evaluated, and data analyzed using the rotor-rod assay over the course of the 3 time points.

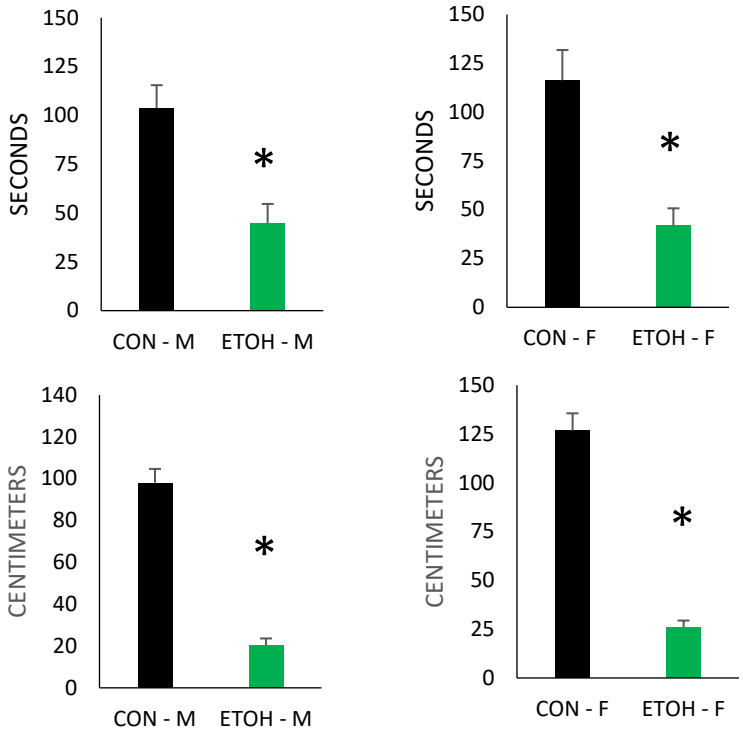


Figure 2.2 – 4 Week Roto-Rod. The test of motor coordination was performed. The sample groups were n=9 male and n=10 females. Significance was set at p=<0.05

In the 4-week rotor-rod assay, both male and female control groups showed consistent increase in time spent in the rotor-rod assay (male = 103.7s ± 11.79 and female = 116.25s ± 15.56). While in both alcohol groups, male and female, show decreased time traveled in the rotor-rod assay (male = 44.93s ± 9.75 and female = 41.85s ± 8.87). The control groups showed consistent increase in distance traveled in the assay (male = 98.06cm ± 6.62 and female = 126.63cm ±

8.94). While both alcohol groups, male and female, show decreased distance traveled in the assay (male = 20.37cm ± 3.25 and female = 26.07cm ± 3.36). These results were analyzed and show significant difference between the treatment groups in time spent on the rod ($df_{treatment}3, df_{error}60$ f = 14.034 p=1.97e-21) and distance traveled ($df_{treatment}3, df_{error}60$ f = 12.615 p = 1.14e-13). The alcohol groups spent significantly less time on the rod compared to control (p=<0.05) in the males; while, in the female groups they also spent significantly less time compared to control (p=<0.05). There was no significant sex difference observed in the time parameter for this test (p=0.845). The distance traveled of the ETOH groups showed that the animals traveled significantly less distance compared to control in males (p=<0.05) and female (p=<0.05). There was no significant sex difference observed in the distance parameter (p=1.00).

In the 8-week rotor-rod assay, both male and female control groups showed consistent increase in time spent in the rotor-rod assay (male = 82.94s ± 14.80 and female 114.21s ± 22.49). While in both alcohol groups, male and female,

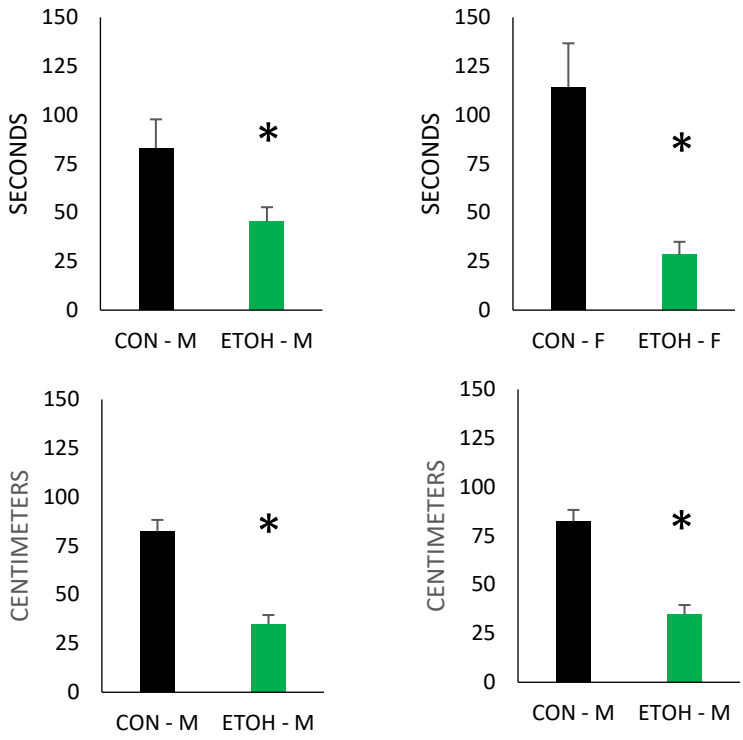


Figure 2.3 – 8 Week Roto-Rod. The motor coordination test was performed. The sample groups were n=10 male and n=10 female. Significance was set at p=<0.05

show decreased time spent in the rotor-rod assay (male = 45.32s ± 7.41 and female = 28.38s ± 6.63). The control groups showed consistent increase in the distance traveled in the rotor-rod assay (male = 82.34cm ± 5.95 and female = 114.91cm ± 12.38). While both alcohol groups, male and female, show decreased distance traveled in the assay (male = 34.93cm ± 4.63 and female = 13.40cm ± 1.59). These results were analyzed and showed significant difference between the treatment groups in time spent on the rod ($df_{treatment}3, df_{error}64$ f = 20.592 p = 1.87e-9) and distance traveled ($df_{treatment}3, df_{error}64$ f = 15.219 p = 1.39e-7). The alcohol groups spent significantly less time on the rod compared to control (p=<0.05) in the males; while, in the female groups they also spent significantly less time on the rod compared to control (p=<0.05). There was no significant sex

difference noted in the time parameter (p=0.496). The distance traveled by the alcohol groups was significantly less compared to control in the males (p=<0.05) and in the females (p=<0.05). There was no significant sex difference observed in this parameter (p=<0.05).

In the 12-week rotor-rod assay, both male and female control groups showed consistent increase in time spent in the rotor-rod assay (male = 90.39s ± 11.20 and female = 87.13s ± 7.46). While in both alcohol groups, male and female,

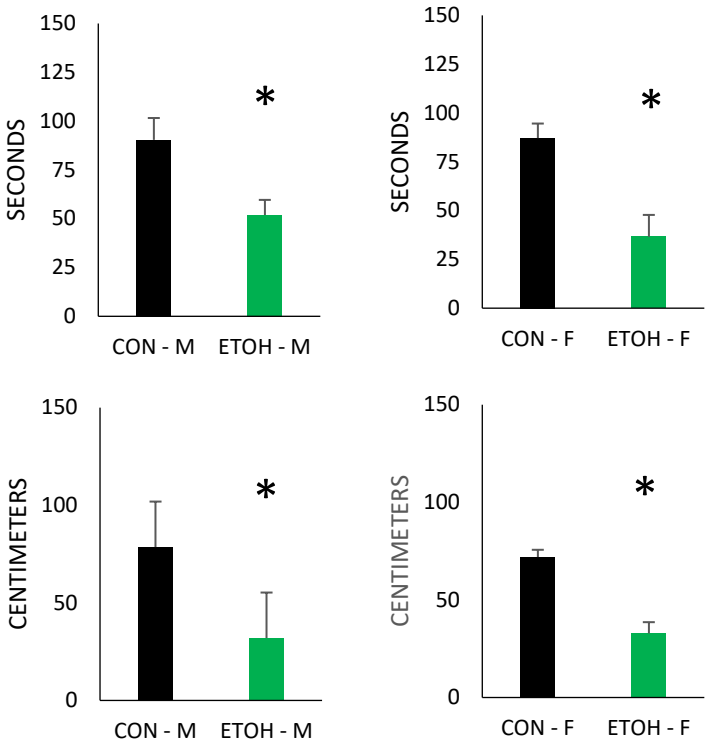


Figure 2.4 – 12 Week Roto-Rod. The motor coordination test was performed. The groups were p=8 male and n=14 female. Significance was set at p=<0.05.

show decreased time traveled in the rotor-rod assay (male = 51.89s ± 7.75 and female = 36.56s ± 11.22).

The control groups showed consistent increase in distance traveled in the assay (male = 78.57cm ± 6.01 and female = 71.96cm ± 3.71). While both alcohol

groups, male and female, showed decreased distance traveled in the assay (male = 31.97cm ± 2.79 and female = 32.81cm ± 5.78). These results were analyzed and show significant difference between the groups in time spent ($df_{treatment}3, df_{error}72$ f = 18.716 p = 4.46e-9) and distance traveled ($df_{treatment}3, df_{error}72$ f = 8.872 p = 4.40e-5). The alcohol groups spent significantly less time on the rod compared to control (p=<0.05) in the male; while the females also spent less time on the rod compared to control (p=<0.05). There was no significant sex difference observed in the time parameter of this assay (p=<0.05). The alcohol groups traveled significantly less distance compared to control in the assay in the males (p=<0.05); while in the females, they also showed decrease distance traveled in the assay (p=<0.05). There was no significant sex difference observed in the distance traveled parameter (p=0.994).

The individual time points showed that the alcohol groups showed significant decreases in both time and distance parameters. The data shows that alcohol causes locomotion and motor coordination impairment over the course of the three time points. When comparing treatment over the course of the time points comparing just treatment, there was a significant difference between the groups in the parameter of time ($df_{treatment}3, df_{error}232$ f=45.717 p=2.99e-23) and distance ($df_{treatment}3, df_{error}232$ f=33.903 p=6.14e-18). There was significant difference in the time parameter (control vs. ETOH male p=<0.05) and control vs ETOH female p=<0.05), and in the distance parameter (control vs. ETOH male p=<0.05 and vs control vs. ETOH female p=<0.05). There was no significant sex difference observed in either parameter (time p=0.317 and distance p=0.970).

There was no significant difference between the time points using either parameter (time p=0.412 distance p=0.754). When comparing the time point vs. the treatment there was no significant difference between the two parameters (time p=0.881 and distance p=0.870).

2.3.2 Effect of Alcohol on Spatial Memory and Learning Abilities

The effect of alcohol and sex was evaluated, and data analyzed using the Morris water maze assay over the course of the 3 time points. The water maze is

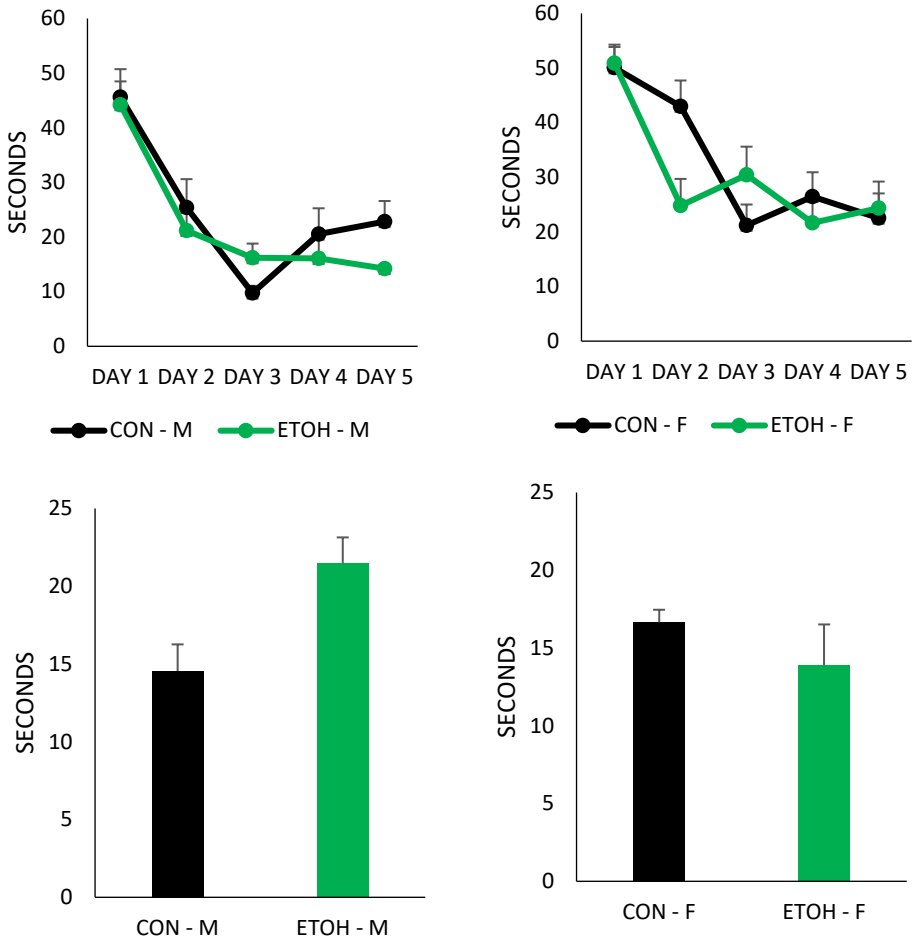


Figure 2.5 – 4 Week Water Maze. This is the test of spatial learning and memory. There was no significant change in the learning of the animals. The groups were n=9 male and n=10 females. The significance was set at p=<0.05.

used to show the ability of the animal to learn from spatial cues that are placed around arena.

In the 4-week time point, the control groups show an increase in time it took to find the platform on day 1 (male = 45.63s ± 5.10 and female = 50.03s ± 3.81) and decrease in time it took to find the platform on day 5 (male = 22.81s ± 3.78 and female = 22.50s ± 4.54). The alcohol groups show increase in time it took to find the platform on day 1 (male = 44.21s ± 4.28 and female = 50.86s ± 3.40) and decrease in time it took to find the platform on day 5 (male = 14.22s ± 3.77 and female = 24.34 ± 4.87). These results were analyzed and showed significant difference between treatment groups ($df_{treatment}3, df_{error}360$ f = 6.537 p = 2.58e-4) and difference between the days of which the assay was performed ($df_{day}4, df_{error}360$ f = 30.847 p = 4.29e-22). There was no significant difference in the time spent to find the platform for the alcohol groups compared to control (male p=0.814 and female p=0.832); while there was significant sex difference observed in the time parameter (p=<0.05). There is significant difference between the start of assay on day 1 and the end of the assay on day 5 (day 1 vs. day 5 p=<0.05).

The probe trial was completed for the 4-week time point. The probe trial is a test of reference memory. The control groups, both male and female, showed increased time spent in the target quadrant (male = 14.55s ± 1.71 and female = 16.65s ± 0.81). The alcohol groups showed mixed results with the males showing increased time in the target quadrant (21.48s ± 1.67) and female showing decrease in time spent in the target quadrant (13.90s ± 2.62). These results were analyzed and show significant difference between the treatment groups

($df_{treatment}3, df_{error}15$ f = 3.492 p=0.042). There was no significant difference between alcohol and control groups (male p=0.130 and female p=0.965) when pertaining to time spent in the target quadrant. However, there is significant sex difference between the sexes in this portion of the water maze assay (p=<0.05).

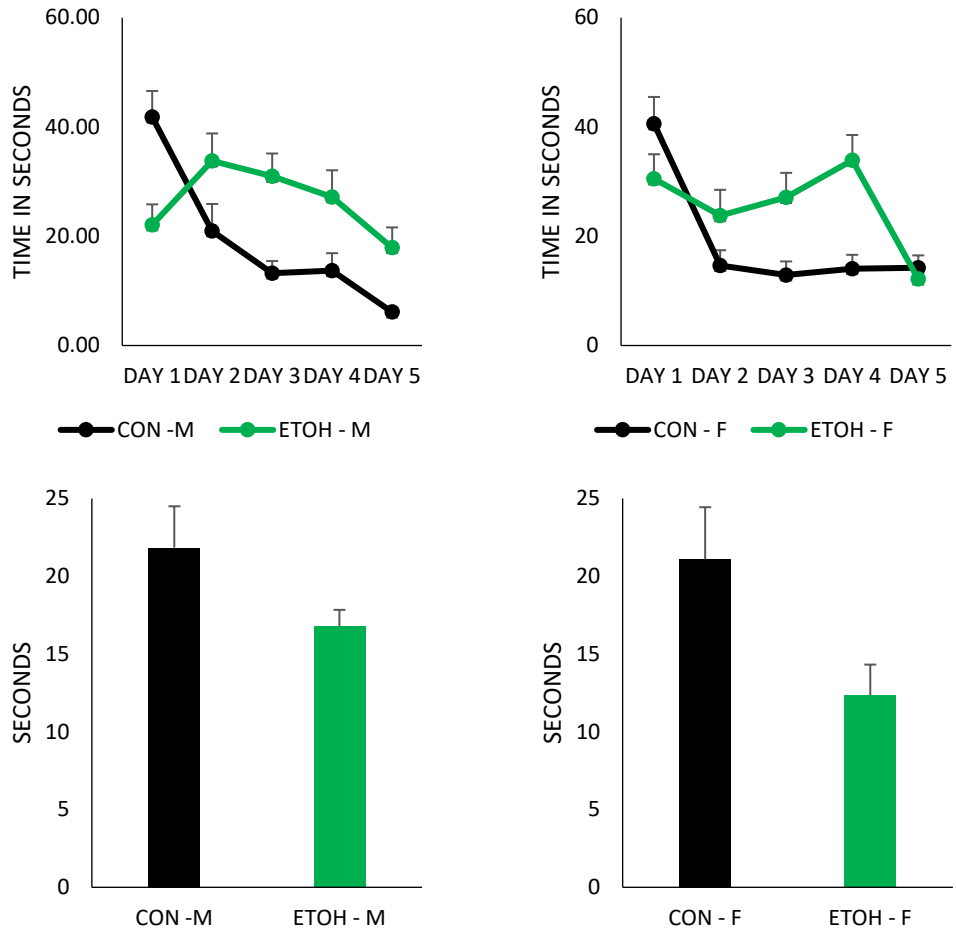


Figure 2.5 – 8 Week Water Maze. The test showed increased learning time in the males and decreased learning time in the females on day five. The groups were n = 10 males and n = 10 females. Significance was set at p = <0.05.

In the 8-week time point, the control groups show an increase in time it took to find the platform on day 1 (male = 41.81s ± 1.07 and female = 40.57s ± 1.10), and decrease in time it took to find the platform on day 5 (male = 6.12s ± 0.19 and female = 14.26s ± 0.50). The alcohol groups show increase in time it took to find the platform on day 1 (male = 22.08s ± 0.84 and female = 30.50s ± 1.10) and

decrease in time it took to find the platform on day 5 (male = 17.91s ± 0.83 and female = 12.16s ± 0.44). These results were analyzed and showed significant difference between treatment groups ($df_{treatment}3,df_{error}380$ f = 5.281 p=.001), and difference between start on day 1 and end of the assay on day 5 ($df_{day}4,df_{error}380$ f = 15.718 p = 6.50e-12). The alcohol groups show significant increase in time it takes to find the platform in males (p=<0.05) and in the females (p=0.044). There was no significant sex difference observed between the treatment groups in this parameter (p=0.982). There is significant difference between the start of assay on day 1 and the end of the assay on day 5 (day 1 vs. day 5 p=<0.05).

The probe trial was completed for the 8-week time point. The probe trial is a test of reference memory. The control groups, both male and female, showed increased time spent in the target quadrant (male = 21.84s ± 2.66 and female = 21.10s ± 3.34). The alcohol groups spent decreased time in the target quadrant (male = 16.78s ± 1.06 and female = 12.36s ± 1.36). These results were analyzed showed significant difference between groups ($df_{treatment},3,df_{error}16$ f = 3.315 p = .047). The alcohol groups compared to control did not show significant decreases in time spent in the target quadrant (male p=0.468 and female p=0.087). There was no significant sex difference observed in this portion of the water maze assay (p=0.577).

In the 12-week time point, the control groups show increase in time it took to find the platform on day 1 (male = 36.79s ± 5.16 and female = 44.50s ± 7.81). While on day 5 the control groups showed decrease in time it took to find the platform on day 5 (male = 11.14s ± 2.62 and female = 11.95s ± 3.90). The ETOH

groups showed increase in time it took to find the escape platform (male = 27.28s

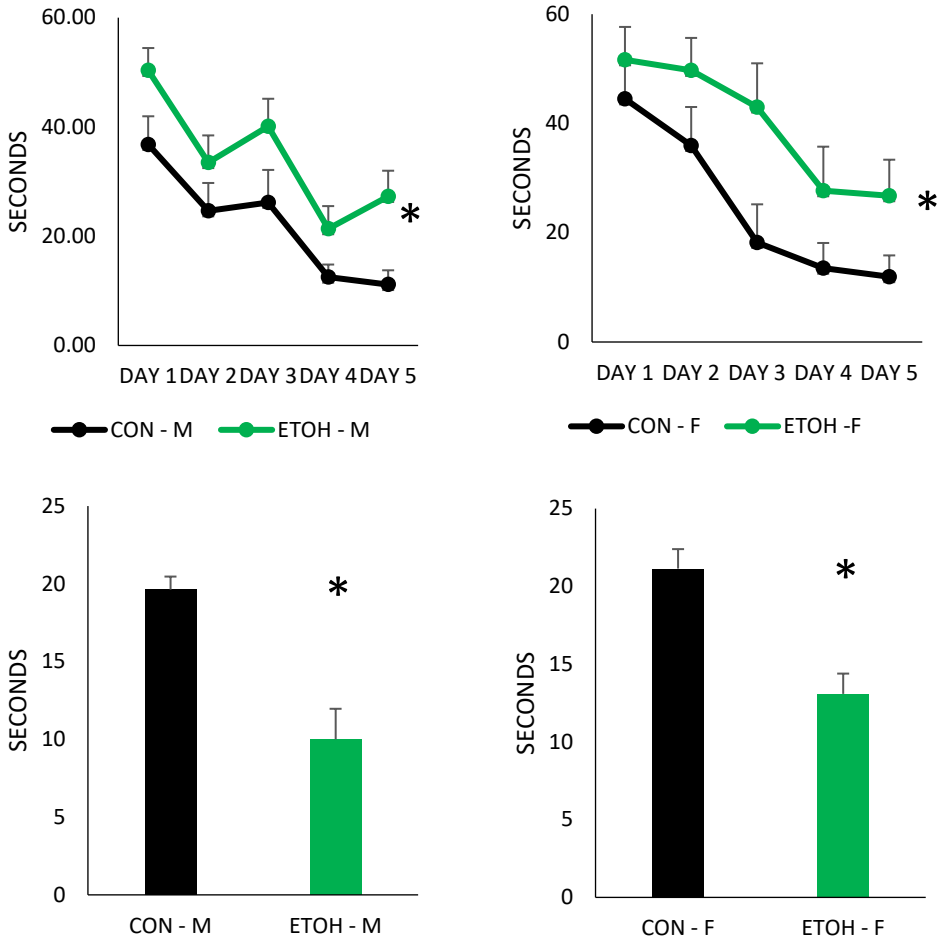


Figure 2.6 – 12 Week Water Maze. The alcohol groups showed significant decreases in the ability to find the escape platform and decreases in the time spent in the target region. Significance was set at $p<0.05$. * represents significant comparison.

± 4.71 and female = 26.77s ± 6.60). These results were analyzed between treatment groups ($df_{treatment}3$, $df_{error}420$ f=24.450 p=1.34e-14), and significant difference between start on day and end of the assay on day 5 ($df_{day}4$, $df_{error}420$ f=34.125 p=1.14e-24). The alcohol groups showed significant increase compared to respective controls (male p=<0.05 and female p=<0.05). There is no significant sex difference observed over the course for the treatment parameter (p=0.153).

There is significant difference between the start of the assay on day 1 and the end of the assay on day 5 (day 1 vs. day 5 p=<0.05).

The probe trial was completed for the 12-week time point. The probe trial is a test of reference memory. The control groups, both male and female, showed an increase in time spent in the target quadrant (male = 19.63s ± 0.84 and female = 21.14s ± 1.25). The alcohol groups, both male and female, showed decrease in time spent in the target quadrant (male = 10.00s ± 1.97 and female = 13.09s ± 1.31). These results were analyzed showing that there was significant difference between treatment groups ($df_{treatment}3$, $df_{error}18$ f=12.453 p=1.20e-4). The alcohol groups compared to control spent significantly less time in the target quadrant compared to control (male p=<0.05 and female p=<0.05). There is no significant sex difference in this portion of the water maze assay (p=0.495).

When comparing the time points, there were significant differences in treatment ($df_{treatment}3$, $df_{error}1160$ f=15.830 p=4.30e-10), time point ($df_{time\ point}2$,$df_{error}1160$ f=19.926 p=3.10e-9), day ($df_{day}4$,$df_{error}1160$ f=70.953 p=8.52e-54), treatment*time point ($df_{treatment*day}6$, $df_{error}1160$ f=9.076 p=1.02e-9), treatment*day ($df_{treatment*day}12$, $df_{error}1160$ f=2.527 p=0.003), time point*day ($df_{time\ point*day}8$, $df_{error}1160$ f=5.568 p=6.14e-7), and treatment*time point*day ($df_{treatment*time\ point*day}24$, $df_{error}1160$ f=2.464 p=1.13e-4). The treatment over the course of the time point showed that there is significant difference between the control vs. ETOH group (male p=<0.05 and female p=<0.05). There was significant sex difference demonstrated over the course of the three time points (p=<0.05). There was significant difference between the 4-week time point and the 12-week time point

(p=<0.05). Compared to day 1 to day 5 across the assay and time points, there was significant differences between day 1 vs. day 5 (p=<0.05).

Over the course of the time points, consistent learning and memory deficit because of the increased time spent finding the escape platform in the 12-week time point. The consistent increase in time to find the platform indicates that treatment period is adequate to induce neurodeficit for study. The probe trail shows consistent decrease in the time spent in the target quadrant. The decrease time spent in the target quadrant indicates that reference memory is compromised with prolonged treatment with alcohol.

2.3.3 Effect of alcohol on Protein Expression and Plasticity

Protein expression and plasticity changes were determined using the western blot technique after the animals were euthanized and the tissue was collected after completion of the behavioral testing for that time point. The brain to body weight ratios were calculated based upon necropsy weight and brain weight. We have checked three different regions, which would be parietal cortex, cerebellum, and hippocampus for each time point. The Morris water maze behavioral test concerns spatial learning and memory, which would concern the hippocampus and parietal cortex. The rotor-rod behavioral test concerns motor-coordination and locomotion, which would concern the cerebellum. The proteins that were probed to check for plasticity changes and protein expression were divided into four categories pre-synaptic protein (synaptophysin and synapsin), post-synaptic protein (SHANK 2, PSD95, and Arg 3.1), neurotrophins (BDNF and

CNTF), and protein involved in long-term potentiation (CAMKII) (Nookala, et al. 2018; Janz et al. 1999).

2.3.3.1 Effect of alcohol on Pre-Synaptic Proteins at 4-weeks

Synapsin-1 is a pre-synaptic protein that is present in the cytoplasmic surface of synaptic vesicles of nerve terminals and is involved in the formation and maintenance of synapses (Nookala, et al. 2018). Synapsin-1 showed a significant difference between groups ($df_{treatment}3$, $df_{error}15$ f=4.704 p=.021). In the 4-week time point, the parietal cortex saw a non-significant increase compared to control for the alcohol males (p=0.217) and the females showed a significant increase as

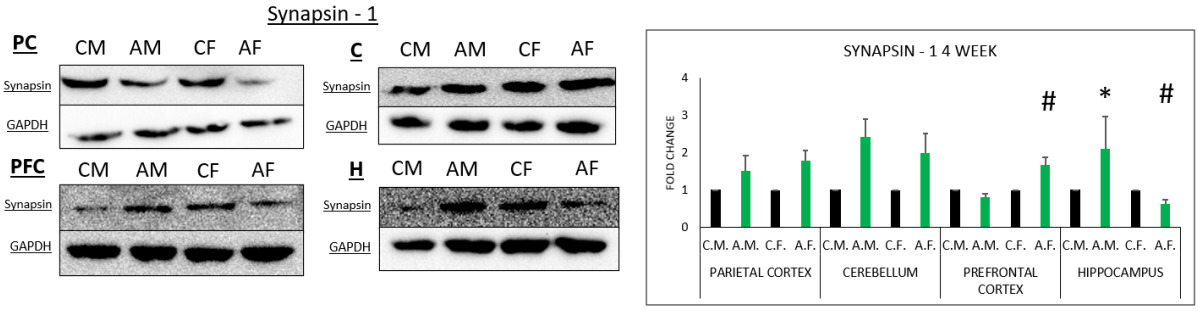


Figure 2.7 – Synapsin-1 4 week. The groups were n=9 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

compared to control (female = 0.041). The cerebellum showed a significant difference between the groups ($df_{treatment}3$, $df_{error}15$ f=6.875 p=0.004) in the 4-week time point. Compared to their respective controls, the males showed a significant increase (p=<0.05), but the females show a non-significant increase (p=0.065). The hippocampus showed a significant difference between groups ($df_{treatment}3$, $df_{error}33$ f=6.060 p=0.002) in the 4-week time point. Compared to their respective controls, the males showed significant increase (p=<0.05) while the females showed non-significant decrease (p=0.695). The protein showed significant sex difference in the hippocampus (p=<0.05), but non-significant sex differences were noted in the parietal cortex (p=0.801) and the cerebellum (p=0.739).

Synaptophysin plays a crucial role in the formation of synapses and vesicle endocytosis (Janz, et al. 1999; Nookala, et al. 2018). Synaptophysin in the parietal cortex showed significant difference between groups ($df_{treatment}3$, $df_{error}12$ f=5.928 p=0.010). Compared to their respective control, the males show non-significant decrease (p=0.767) while the females show significant increase (p=<0.05). In the cerebellum, there was no significant difference between groups in this region

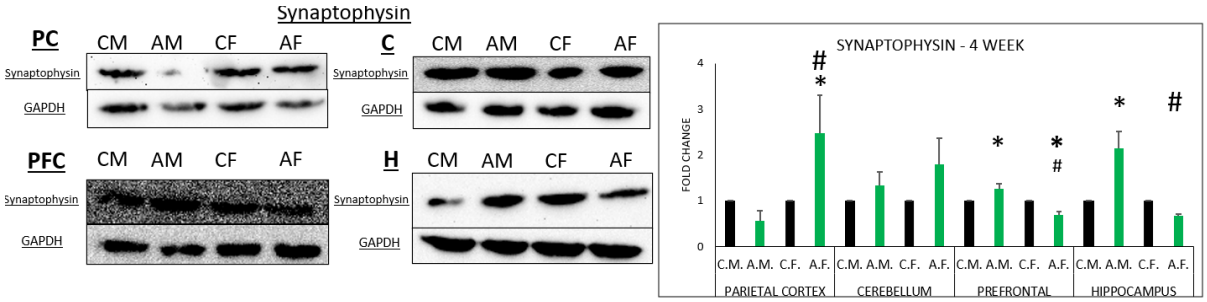


Figure 2.8 – Synaptophysin 4 week. The groups showed increases and decreases in an inconsistent manner. The groups were n = 9 male and n = 10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

(p=0.072). The hippocampus showed significant differences between the groups ($df_{treatment}3$, $df_{error}49$ f=15.985 p=1.49e-7) in the 4-week time points. The protein showed significant sex differences in the parietal cortex (p=<0.05) and hippocampus (p=<0.05), but no significant sex difference was noted in the cerebellum (p=0.578).

2.3.3.2 Effect of Alcohol on Post-Synaptic Proteins at 4-weeks

SHANK2 plays a crucial role in synaptogenesis by facilitating attachment of glutamate receptors to the NMDA receptor (Nookala, et al. 2018). The parietal cortex showed a non-significant difference between groups (p=0.055) in the 4-week time point. The cerebellum showed a significant difference between groups

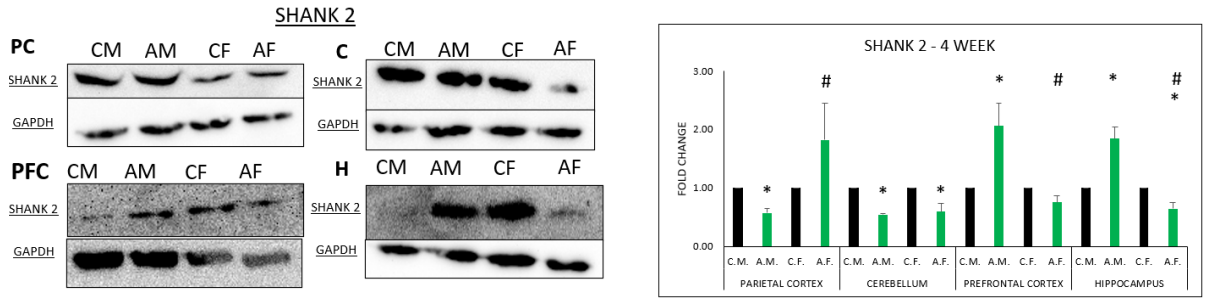


Figure 2.9 – Shank 2 4 week. The groups were composed of n=9 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

($df_{treatment}$3, df_{error}18 f=18.445 p=2.70e-5) in the 4-week time point. Compared to their respective controls, the males (p=<0.05) and females (p=<0.05) showed significant decreases in protein expression. The hippocampus showed a significant differences between the groups ($df_{treatment}$3, df_{error}28 f=31.283 p=1.27e-8) in the 4-week time point. Compared to their respective controls, the males showed a significant increase (p=<0.05); while the females showed a significant decrease (p=<0.05) in protein expression. The protein showed significant sex difference in the parietal cortex (p=<0.05) and hippocampus (p=<0.05) but showed non-significant sex differences in the cerebellum (p=0.941).

PSD95 plays a crucial role in the formation and maintenance of learning and memory formation (Nookala, et al. 2018). The parietal cortex showed significant difference between groups ($df_{treatment}$3,df_{error}38 f=28.524 p=1.63e-9) in

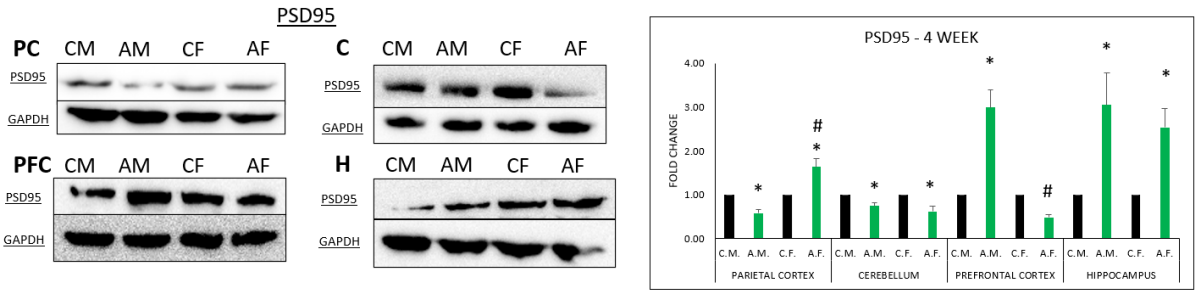


Figure 2.10 – PSD95 4 week. The groups were n= 9 male and n= 10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

the 4-week time point. Compared to their respective controls, the males showed a significant decreases in expression (p=<0.05); while the females showed significant increase in expression (p=<0.05). The cerebellum showed a significant differences between groups ($df_{treatment}$3,df_{error}36 f=11.854 p=2.00e-5). Compared to their respective controls, the males (p=<0.05) and females (p=<0.05) show a significant decreases in expression. The hippocampus showed a significant difference between groups ($df_{treatment}$3,df_{error}46 f=10.902 p=1.90e-5) in the 4-week time point. Compared to their respective controls, the males (p=<0.05) and females (p=<0.05) showed a significant increase in expression. The protein showed significant sex difference in the parietal cortex (p=<0.05), but the cerebellum (p=0.373) and hippocampus (p=0.749) showed a non-significant sex differences.

Arg 3.1 plays role in the spatial process, which is important in spatial memory (Nookala, et al. 2018). The parietal cortex showed a significant difference between groups ($df_{treatment}$3, df_{error}40 f=18.362 p=1.84e-7). Compared to their respective control, the males (p=<0.05) and females (p=<0.05) showed a significant decreases in expression. The cerebellum showed a significant

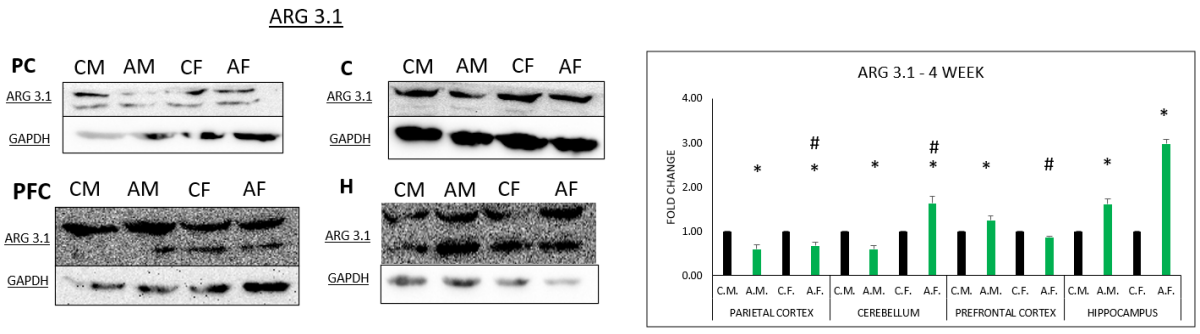


Figure 2.11 – ARG 3.1 4 week. The groups were n=9 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

difference between groups ($df_{treatment}3$, $df_{error}36$ f=24.443 p=1.63e-8). Compared to their respective control, the males showed significant decrease in expression (p=<0.05); while the females showed significant increase in expression (p=1.80e-5). The hippocampus showed significant differences between groups ($df_{treatment}3$, $df_{error}91$ f=12.624 p=6.14e-7). Compared to their respective controls, the males (p=0.003) and females (p=<0.05) both showed significant increase in expression. The protein showed significant sex difference in the cerebellum (p=<0.05); while the parietal cortex (p=0.679) and the hippocampus (p=0.260) showed non-significant sex difference.

2.3.3.3 Effect of Alcohol on Neurotrophins at 4 weeks

BDNF plays role in supporting the survival and encourage the growth of neurons and synapses (Nookala, et al. 2018). The parietal cortex showed a significant difference between the groups ($df_{treatment}3$, $df_{error}33$ f=51.383 p=6.23e-12) in the 4-week time point. Compared to their respective controls, the males showed a significant decrease (p=<0.05); while the females showed a significant

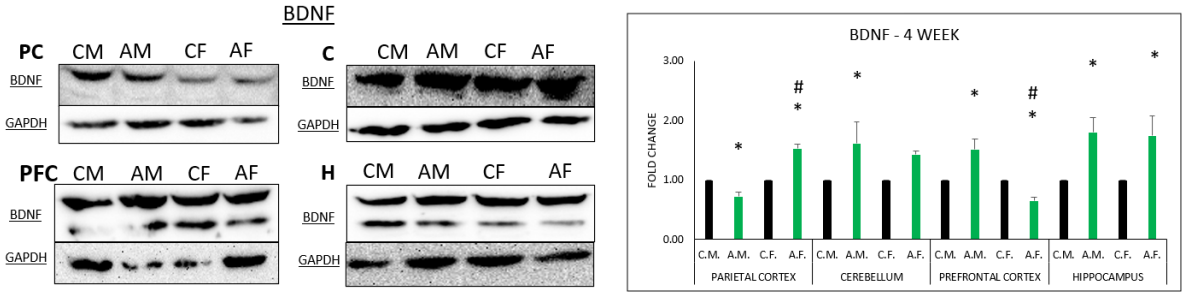


Figure 2.12 – BDNF 4 week. The groups were n=9 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

increase in expression (p=<0.05). The cerebellum showed a significant differences between the groups ($df_{treatment}3$, $df_{error}21$ f=5.583 p=0.007). Compared to their respective controls, the males showed a significant increase in expression

(p=<0.05); while the females showed a non-significant increase in expression (p=0.134). The hippocampus showed significant differences between groups ($df_{treatment}$3, df_{error}68 f=8.306 p=9.30e-5) in the 4-week time point. Compared to their respective controls, the males (p=<0.05) and females (p=<0.05) both showed significant increases in expression. The protein showed significant sex difference in the parietal cortex (p=3.59e-12), but the cerebellum (p=0.797) and the hippocampus (p=0.994) showed non-significant sex difference.

CNTF is involved various cells that are involved in sensory and motor neuron process (Nookala, et al. 2018). The parietal cortex showed a significant difference between groups ($df_{treatment}$3, df_{error}28 f=26.182 p=6.98e-5). Compared

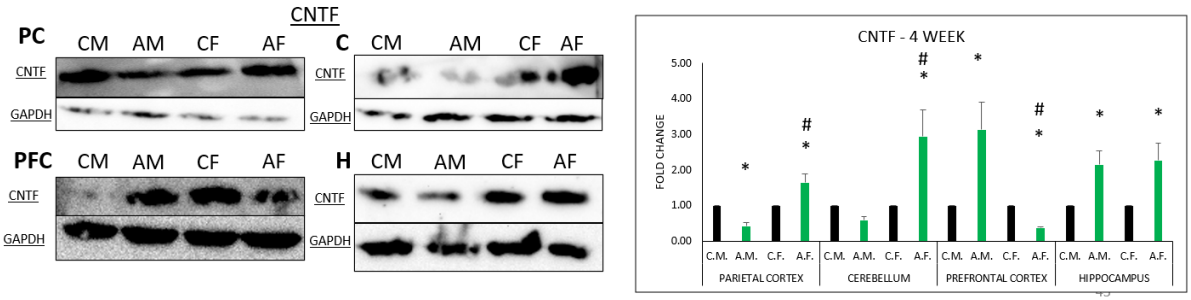


Figure 2.13 – CNTF 4 week. The groups were n=9 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

to their respective controls, the males showed a significant decrease in expression (p=<0.05); while the females showed a significant increase in expression (p=<0.05). The cerebellum showed a significant difference between groups ($df_{treatment}$3, df_{error}28 f=10.864 p=9.40e-5). Compared to their respective controls, the males showed a non-significant decrease in expression (p=<0.05); while the females showed a significant increase in expression (p=<0.05). The hippocampus showed a significant difference between groups ($df_{treatment}$3, df_{error}68 f=12.206 p=2.00e-6). Compared to their respective controls, the males (p=<0.05) and

females (p=8.80e-6) both showed significant increases in expression. Significant sex difference was noted in the parietal cortex (p=<0.05) and cerebellum (p=<0.05), but no significant sex differences were noted in the hippocampus (p=0.979).

2.3.3.4 Effect of Alcohol on CamKII levels at 4 weeks

Calcium modulin kinase II (CamkII) is involved the long-term potentiation of

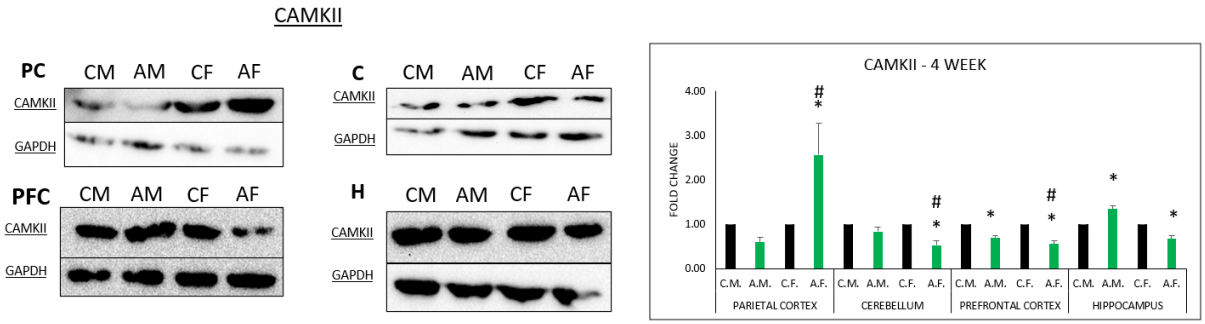


Figure 2.14 – LTP 4 week. The groups were n=9 male and n= 10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

memories in the brain (Nookala, et al. 2018; Lynch 2004; Bliss and Collingridge 1993). The parietal cortex showed differences between groups ($df_{treatment}3$, $df_{error}24$ f=7.881 p=0.001) in the 4-week time point. Compared to their respective controls, the males showed a non-significant decrease in expression (p=0.741); while the females showed significant increase in expression (p=<0.05). The cerebellum showed significant difference between groups ($df_{treatment}3$, $df_{error}24$ f=13.290 p=4.40e-5) in the 4-week time point. Compared to their respective controls, the males showed a non-significant decrease (p=0.391), but the females showed a significant decrease in expression (p=<0.05). The hippocampus showed significant difference between groups ($df_{treatment}3$, $df_{error}41$ f=42.586 p=3.11e-12) in the 4-week time point. Compared to their respective controls, the males showed

significant increase in expression (p=<0.05); while the females showed significant decrease in expression (p=<0.05). Significant sex difference was noted in the parietal cortex (p=<0.05), cerebellum (p=<0.05), and in the hippocampus (p=<0.05).

2.3.3.5 Summary of 4 Week Time Point

In the 4-week time point, the protein expression of all the region is mostly upregulated. The upregulation of key proteins on either side of the synapse demonstrates that toxic insults can have alteration on various proteins in the neural environment. Furthermore, the upregulation of these proteins suggests that they act like a protection mechanism for the neural environment.

2.3.4.1 Effect of Alcohol on Pre-Synaptic Proteins at 8 Weeks

Expression of synapsin-1 was checked in the four regions. The parietal

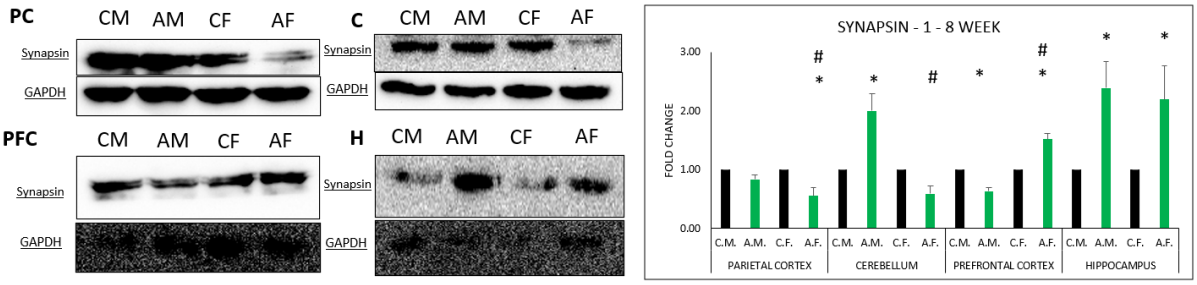


Figure 2.15 – Synapsin-1 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

cortex showed significant difference between the groups ($df_{treatment}3$, $df_{error}19$ f=10.770 p=4.06e-4) in the 8-week time point. Compared to their respective controls, the males showed a non-significant decrease in expression (p=0.283), but the females showed significant decrease in the expression (p=<0.05). The cerebellum was also checked for synapsin-1 levels. The cerebellum showed a

significant difference between the groups ($df_{treatment}3$, $df_{error}22$ f=16.153 p=1.80e-5) in the 8-week time point. Compared to their respective controls, the males showed a significant increase in expression (p=<0.05); while the females showed non-significant decrease in the expression (p=0.202). The hippocampus was checked as well for synapsin-1 levels. The hippocampus showed a significant difference between groups ($df_{treatment}3$, $df_{error}41$ f=8.221 p=2.43e-4) at 8 weeks of treatment. Compared to their respective controls, the males (p=<0.05) and females (p=<0.05) showed a significant increase in synapsin-1 expression. Synapsin-1 showed sex difference in the parietal cortex (p=<0.05) and cerebellum (p=<0.05), but no

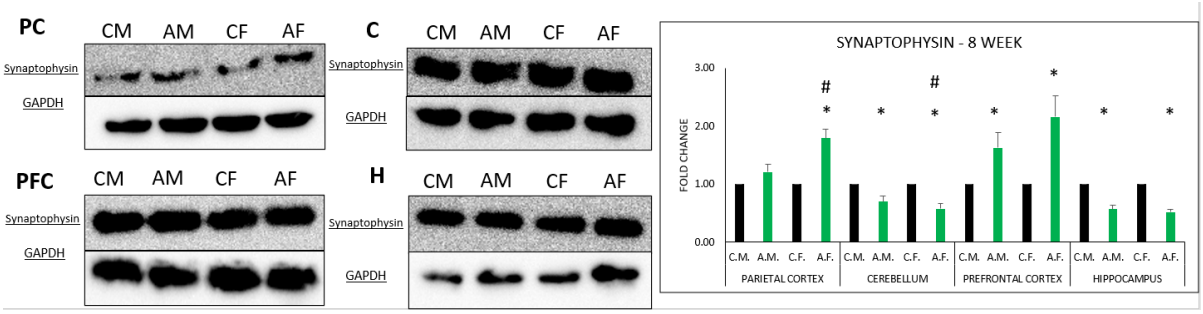


Figure 2.16 – Synaptophysin 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

significant sex changes were noted in the hippocampus (p=0.963).

Expression of synaptophysin was checked in the four regions. The parietal cortex showed significant difference between the groups ($df_{treatment}3$, $df_{error}29$ f=15.860 p=5.00e-6) after 8 weeks of treatment. Compared to their respective controls, the males showed a non-significant increase in expression (p=0.398); while the females showed significant increase for expression (p=<0.05). The cerebellum was also checked for synaptophysin expression levels. The cerebellum showed significant differences between groups ($df_{treatment}3$, $df_{error}26$

f=17.157 p=5.00e-6) after 8 weeks of treatment. Compared to their respective controls, both male (p=0.003) and females (p=3.30e-5) showed a significant decrease in expression of synaptophysin. The hippocampus showed significant differences between groups ($df_{treatment}$3, df_{error}55 f=73.499 p=1.04e-18) after 8 weeks of treatment. Compared to their respective controls, both males (p=1.42e-12) and females (p=5.57e-13) showed a significant decrease in expression of synaptophysin. Significant sex difference was shown in the parietal cortex (p=0.005); while the cerebellum (p=0.465) and hippocampus (p=0.737) showed no significant sex difference.

2.3.4.2 Effect of Alcohol on Post-Synaptic Proteins at 8 Weeks

Expression of SHANK2 was checked in the four regions. The parietal cortex showed significant difference between groups ($df_{treatment}$3, df_{error}13 f=10.927 p=0.002) after 8 weeks of treatment. Compared to their respective controls, both male (p=0.026) and females (p=<0.05) showed significant differences in protein

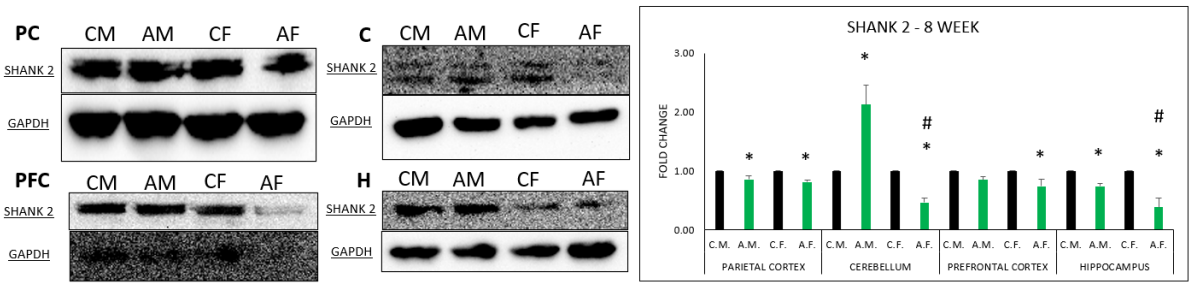


Figure 2.17 – Shank 2 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

expression. The cerebellum showed a significant difference between groups ($df_{treatment}$3, df_{error}20 f=25.590 p=2.00e-6) after 8 weeks of treatment. Compared to their respective controls, both male (p=<0.05) and female (p=<0.05) showed

significant decrease in protein expression. Hippocampus showed significant difference between groups ($df_{treatment}3$, $df_{error}25$ f=21.735 p=8.94e-7) after 8 weeks of treatment. Compared to their respective controls, both male (p=<0.05) and female (p=<0.05) groups showed significant decreases in protein expression. Sex differences was noted in the cerebellum (p=<0.05) and hippocampus (p=<0.05). Non-significant sex difference was noted in the parietal cortex (p=0.167).

Expression of PSD95 was checked in the four regions. The parietal cortex showed significant difference between the groups ($df_{treatment}3$, $df_{error}14$ f=5.497 p=0.015) after 8 weeks of expression. Compared to their respective controls, the males showed a non-significant decrease in expression (p=0.756); while the

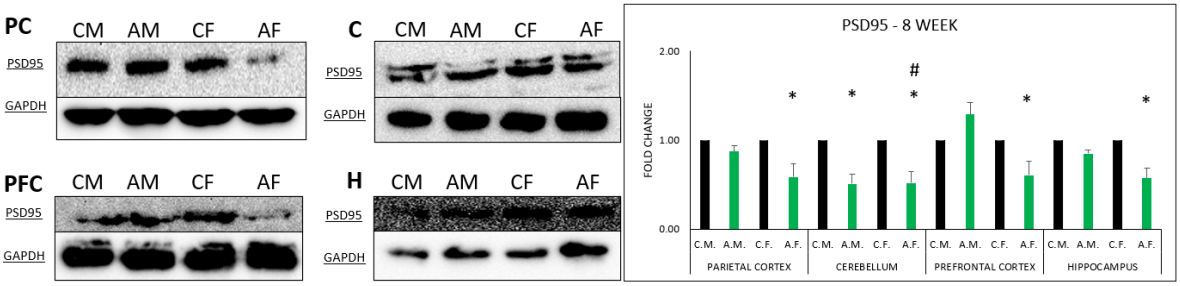


Figure 2.18 – PSD95 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

females showed a significant decrease in expression (p=<0.05). The cerebellum showed significant difference between the groups ($df_{treatment}3$, $df_{error}18$ f=18.995 p=2.30e-5) after 8 weeks of treatment. Compared to their respective controls, both males (p=<0.05) and females (p=<0.05) showed a significant decrease in protein expression. The hippocampus showed a significant difference between the groups ($df_{treatment}3$, $df_{error}17$ f=10.415 p=0.001) after 8 weeks of treatment. Compared to their respective controls, the males showed non-significant decrease in expression (p=0.477); while the females showed a significant decrease in expression

(p=0.001). Cerebellum showed sex difference in terms of expression (p=0.013); while the parietal cortex (p=0.167) and hippocampus (p=0.071) showed no significant sex difference in protein expression.

Expression of Arg 3.1 was checked in the four regions. The parietal cortex showed significant differences between the groups ($df_{treatment}$3, df_{error}35 f=11.721 p=2.40e-5) after 8 weeks of expression. Compared to their respective controls, the males a showed significant increase in expression (p=<0.05); however, the females showed a non-significant decrease in expression (p=0.187). The cerebellum showed a significant difference between the groups ($df_{treatment}$3, df_{error}21 f=11.923 p=1.56e-4) after 8 weeks of exposure. Compared to their

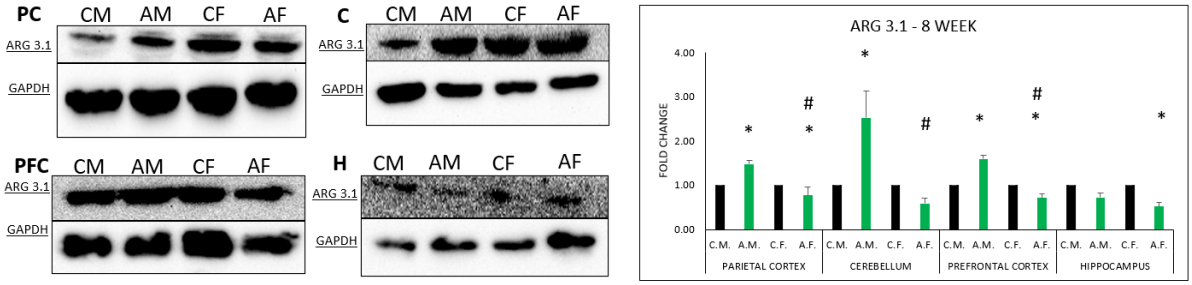


Figure 2.19 – ARG 3.1 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

respective controls, the males showed a significant increase in expression (p=<0.05); while the females showed a non-significant decrease in expression (p=0.560). The hippocampus showed significant difference between the groups ($df_{treatment}$3, df_{error}14 f=13.235 p=0.001) after 8 weeks of exposure. Compared to their respective controls, the males show a non-significant decrease in expression (p=0.069); while the females showed a significant decrease in expression (p=<0.05). Significant sex difference was noted in the parietal cortex (p=<0.05)

and cerebellum (p=<0.05), but non-significant sex differences were noted in the hippocampus (p=0.207).

2.3.4.3 Effect of Alcohol on Neurotrophins at 8 weeks

BDNF expression was checked in the four regions. The parietal cortex showed significant difference between the groups ($df_{treatment}3$, $df_{error}30$ f=10.660 p=8.50e-4) after 8 weeks of treatment. Compared to their respective controls, both males (p=<0.05) and females (p=<0.05) showed a significant increase in expression. The cerebellum showed a significant difference between groups ($df_{treatment}3$, $df_{error}18$ f=5.001 p=0.013) after 8 weeks of treatment. Compared to

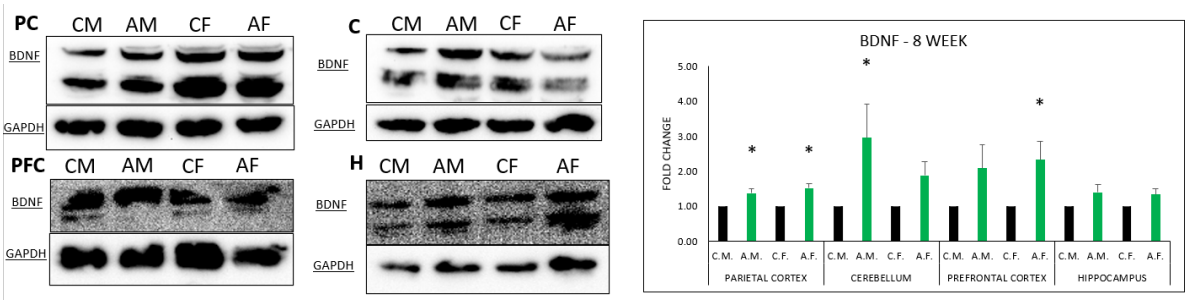


Figure 2.20 – BDNF 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

their respective controls, both male (p=<0.05); however, the females showed a non-significant increase in expression (p=0.514). The hippocampus showed a significant difference between the groups ($df_{treatment}3$, $df_{error}27$ f=4.309 p=0.014) after 8 weeks of treatment. Compared to their respective controls, both males (p=0.053) and females (p=0.121) showed non-significant increases in the expression of BDNF. Non-significant sex difference was noted in the parietal cortex (p=0.738), cerebellum (p=0.420), and the hippocampus (p=0.983).

CNTF expression was checked in the four regions. The parietal cortex showed significant differences between the groups ($df_{treatment}3$, $df_{error}25$ f=27.507 p=1.25e-7) after 8 weeks of treatment. Compared to respective controls, the males show significant increase in expression (p=<0.05); while the female show significant decrease in expression (p=<0.05). The cerebellum showed significant difference between groups ($df_{treatment}3$, $df_{error}28$ f=20.294 p=7.01e-7) after 8 weeks

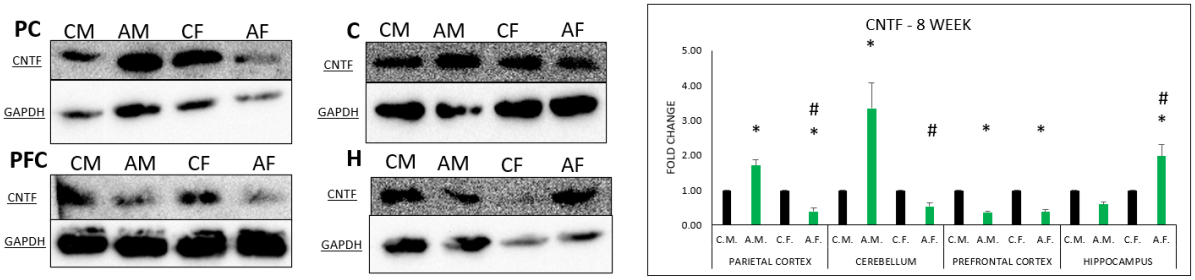


Figure 2.21 – CNTF 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

of expression. Compared to respective controls, the males showed significant increase (p=<0.05); however, the females showed non-significant decrease in expression (p=0.544). The hippocampus showed significant difference between the groups ($df_{treatment}3$, $df_{error}27$ f=14.141 p=1.60e-5) after 8 weeks of treatment. Compared to respective controls, the males showed non-significant decrease in expression (p=0.237); while the females showed significant increase in expression (p=<0.05). Significant sex difference is noted in the parietal cortex (p=<0.05), cerebellum (p=<0.05), and hippocampus (p=<0.05).

2.3.4.4 Effect of Alcohol on CamKII levels at 8 weeks

CamkII levels were checked in the three regions. The parietal cortex showed significant difference between the groups ($df_{treatment}3$, $df_{error}27$ f=27.507 p=1.25e-7) after 8 weeks of treatment. Compared to respective controls, both male (p=<0.05) and female (p=<0.05) showed significant decrease in expression.

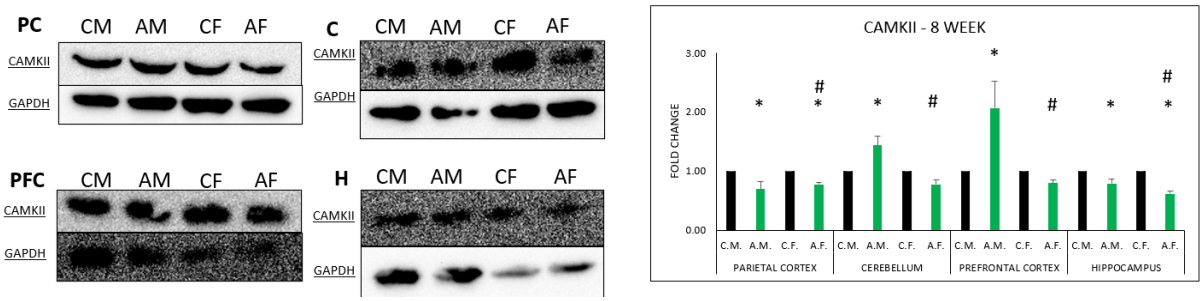


Figure 2.22 – CAMKII 8 week. The groups were n=10 male and n=10 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

The cerebellum showed significant difference between the groups ($df_{treatment}3$, $df_{error}22$ f=14.449 p=3.80e-5) after 8 weeks of treatment. Compared to respective controls, the males showed significant increase in expression (p=<0.05); however, the females showed non-significant decrease in expression (p=0.084). The hippocampus showed significant difference between the groups ($df_{treatment}3$, $df_{error}24$ f=26.391 p=2.55e-7) after 8 weeks of treatment. Compared to respective controls, both males (p=<0.05) and females (p=<0.05) showed significant decreases in expression. Sex difference was noted in the cerebellum (p=1.70e-5) and the hippocampus (p=<0.05), but not in the parietal cortex (p=0.690).

2.3.4.5 Summary of 8 Week Time Point

In the 8 week time point, the protein expression shows a trend of downregulation. The observed down regulation suggests that alcohol does cause neurodeficit, but not to the extent that is shown in the behavioral testing as shown prior. By this point of the study, we should start to see decline that would be

appreciated with further exposure to alcohol. The animals have possibly developed tolerance and further treatment would elicit a better response.

2.3.5.1 Effect of Alcohol on Pre-Synaptic Proteins at 12 Weeks

Synapsin-1 expression levels were checked after 12 weeks of treatment. In the parietal cortex, there was significant difference between the groups ($df_{treatment}3$, $df_{error}14$ f=22.531 p=5.30e-5). Compared to their respective controls, both male (p=<0.05) and female (p=<0.05) showed a significant decrease in expression. In the cerebellum, there was a significant difference between the groups ($df_{treatment}3$,

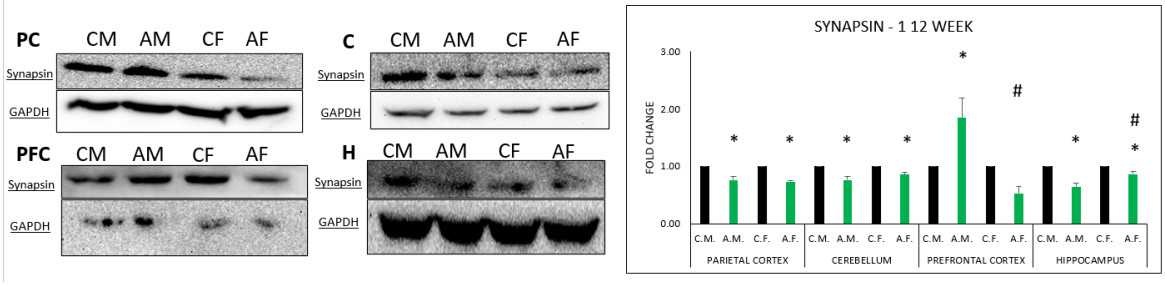


Figure 2.23 – Synapsin-1 12 week. The groups were n=8 male and n=14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

$df_{error}19$ f=15.572 p=5.30e-5). Compared to their respective controls, both male (p=<0.05) and female (p=<0.05) showed a significant decrease in expression. In the hippocampus, there was a significant difference between the groups ($df_{treatment}3$, $df_{error}27$ f=23.557 p=2.49e-7). Compared to their respective controls, both the males (p=<0.05) and the females (p=<0.05) show a significant decrease in the expression. Significant sex difference was noted in the protein expression of the hippocampus (p=<0.05), but non-significant differences were noted in the parietal cortex (p=0.893) or cerebellum (p=0.203).

Synaptophysin expression levels were checked after 12 weeks of treatment. In the parietal cortex, there was a significant difference between the

groups ($df_{treatment}3$, $df_{error}25$ f=11.058 p=1.25e-4). Compared to respective their controls, the males (p=<0.05) showed a significant increase, but the females (p=<0.05) showed a significant decrease in expression. In the cerebellum, there

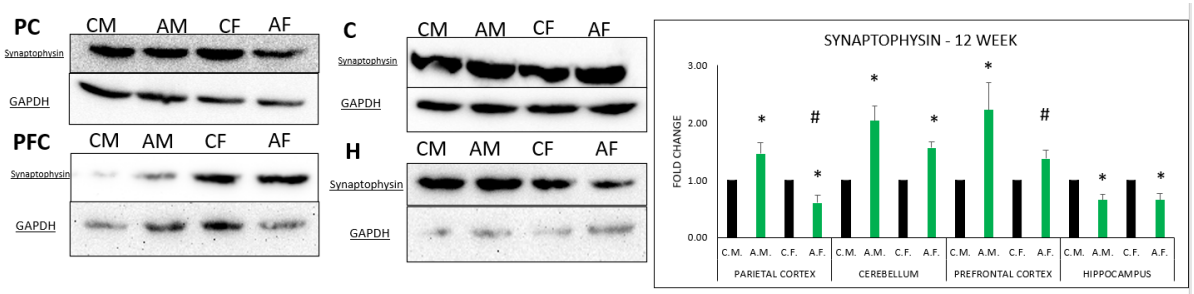


Figure 2.24 – Synaptophysin 12 week. The groups were n=8 male and n=14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

was a significant difference between the groups ($df_{treatment}3$, $df_{error}31$ f=13.022 p=1.70e-5). Compared to their respective controls, the males (p=<0.05) and the females (p=<0.05) showed a significant increase in protein expression. In the hippocampus, there was a significant difference between the groups ($df_{treatment}3$, $df_{error}27$ f=11.163 p=8.90e-5). Compared to their respective controls, both the males (p=<0.05) and females (p=<0.05) showed a significant decrease in protein expression. Significant sex difference was noted in the parietal cortex (p=<0.05), but non-significant differences were noted in the cerebellum (p=0.657) and hippocampus (p=1.000).

2.3.5.2 Effect of Alcohol on Post-Synaptic Proteins at 12 Weeks

SHANK2 levels were checked after 12 weeks of treatment. The parietal cortex showed significant difference between the groups ($df_{treatment}3$, $df_{error}33$ f=7.000 p=0.002) after 12 weeks of treatment. Compared to their respective controls, both males (p=0.066) and females showed a decrease in protein expression, but the females showed a significant decrease (p=<0.05). The cerebellum showed a significant difference between the groups ($df_{treatment}3$, $df_{error}16$ f=12.587 p=3.83e-4) after 12 weeks of treatment. Compared to respective their controls, both males (p=<0.05) and females (p=<0.05) showed a significant decrease in protein expression. The hippocampus showed a significant difference

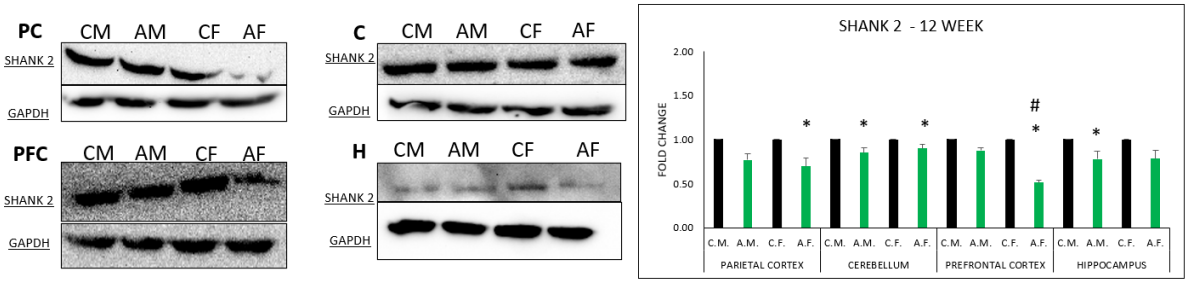


Figure 2.25 – SHANK 2 12 week. The groups were n=8 male and n=14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

between the groups ($df_{treatment}3$, $df_{error}17$ f=5.486 p=0.011) after 12 weeks of treatment. Compared to their respective control, both males and females (p=0.057) show decrease, but the males (p=<0.05) showed a significant decrease. No significant sex difference was not noted in the parietal cortex (p=0.840), cerebellum (p=0.831), and hippocampus (p=1.000) with this protein.

PSD95 levels were checked after 12 weeks of treatment. The parietal showed a significant difference between the groups ($df_{treatment}3$, $df_{error}28$ f=40.303 p=9.99e-10) after 12 weeks of treatment. Compared to their respective controls,

both males (p=<0.05) and females (p=<0.05) showed significant decrease in the protein PSD95 expression levels. The cerebellum showed a significant difference between the groups ($df_{treatment}$3, df_{error}29 f=23.523 p=1.42e-7) after 12 weeks of

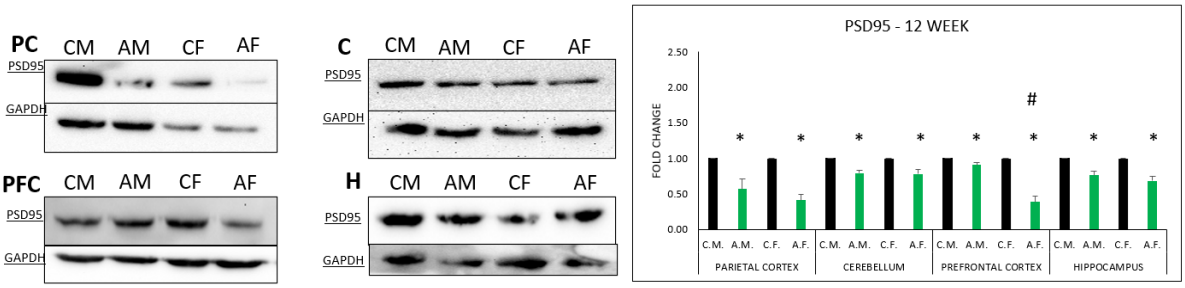


Figure 2.26 – PSD95 12 week. The groups were n=8 and n=14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

treatment. Compared to their respective controls, both males (p=<0.05) and females (p=<0.05) show a significant decrease in protein expression. The hippocampus showed a significant difference between the groups ($df_{treatment}$3, df_{error}40 f=18.115 p=2.13e-7) after 12 weeks of treatment. Compared to their respective controls, both males (p=<0.05) and females (p=<0.05) showed a significant decrease in protein expression. Sex difference in the expression was noted at a significant level in the parietal cortex (p=0.108), cerebellum (p=0.903), and hippocampus (p=0.558).

Arg 3.1 levels were checked after 12 weeks of treatment. The parietal cortex showed significant difference between the groups ($df_{treatment}$3, df_{error}18 f=10.960 p=4.57e-4) at 12 weeks of treatment. Compared to their respective controls, both male (p=0.275) and female showed a decrease in protein expression, but females showed a significant decrease (p=<0.05). The cerebellum showed a significant difference between the groups ($df_{treatment}$3, df_{error}31 f=8.766 p=2.93e-4) at 12 weeks of treatment. Compared to their respective controls, both

male (p=<0.05) and female (p=<0.05) showed a significant decrease in protein expression. The hippocampus showed a significant difference between the groups ($df_{treatment}$3, df_{error}33 f=16.793 p=1.00e-6) after 12 weeks of treatment. Compared to their respective controls, both male (p=<0.05) and females (p=<0.05) showed a

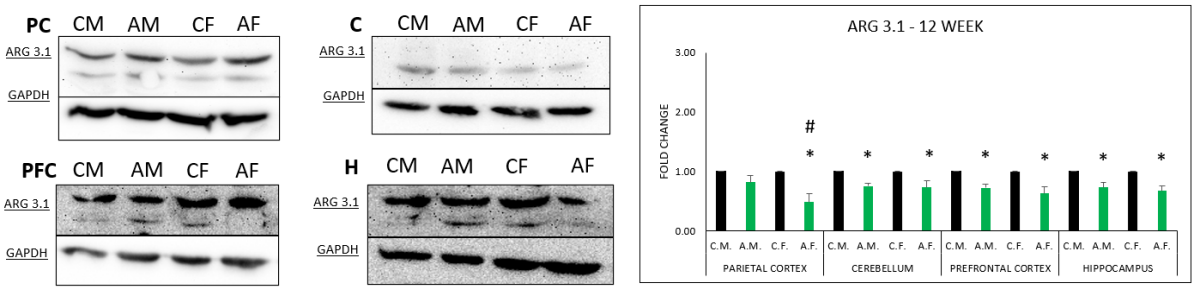


Figure 2.27 – ARG 3.1 12 week. The groups were n=8 male n=14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference

significant decrease in protein expression. Sex difference in the protein expression was noted significantly in the parietal cortex (p=<0.05), but not noted in the cerebellum (p=0.996) and hippocampus (p=0.855).

2.3.5.3 Effect of Alcohol on Neurotrophins at 12 weeks

BDNF expression levels were checked after 12 weeks of treatment. The parietal cortex showed a significant difference between the groups ($df_{treatment}$3, df_{error}21 f=3.522 p=0.036) after 12 weeks of treatment. Compared to their respective controls, both male (p=0.356) and females (p=0.058) showed non-significant increases in protein expression. The cerebellum was tested and showed significant differences between the groups ($df_{treatment}$3, df_{error}29 f=26.999 p=1.70e-5) after 12 weeks of treatment. Compared to their respective controls, the males (p=<0.05) showed a significant increase in protein expression, while the females (p=<0.05) showed a significant decrease in protein expression. The

hippocampus was tested and showed significant differences between the groups ($df_{treatment}3$, $df_{error}42$ f=6.571 p=0.001) after 12 weeks of treatment. Compared to

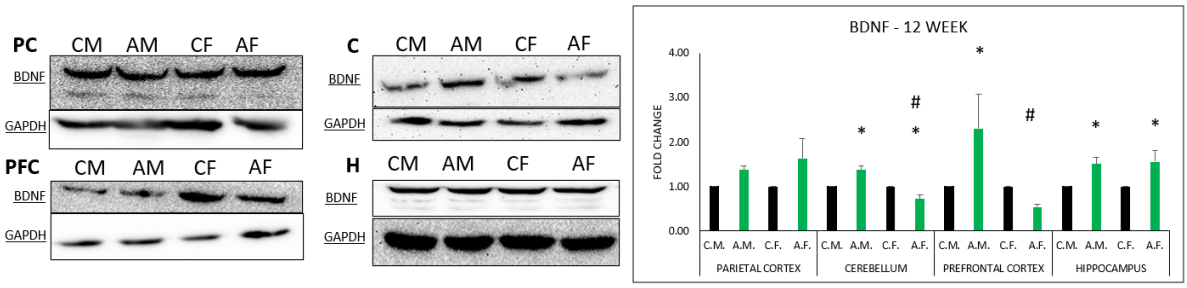


Figure 2.28 – BDNF 12 week. The groups were n=8 male and n=14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

their respective controls, both males (p=<0.05) and females (p=<0.05) show a significant increase in protein expression. Significant sex difference in protein expression was noted in the cerebellum (p=<0.05), but not noted in the parietal cortex (p=0.728) and the hippocampus (p=0.994).

CNTF expression levels were checked after 12 weeks of treatment. The parietal cortex showed significant differences between the groups ($df_{treatment}3$, $df_{error}20$ f=5.272 p=0.009) after 12 weeks of treatment. The males show a non-significant decrease in protein expression (p=0.101). The females show significant decrease in protein expression (p=<0.05). The cerebellum showed a significant

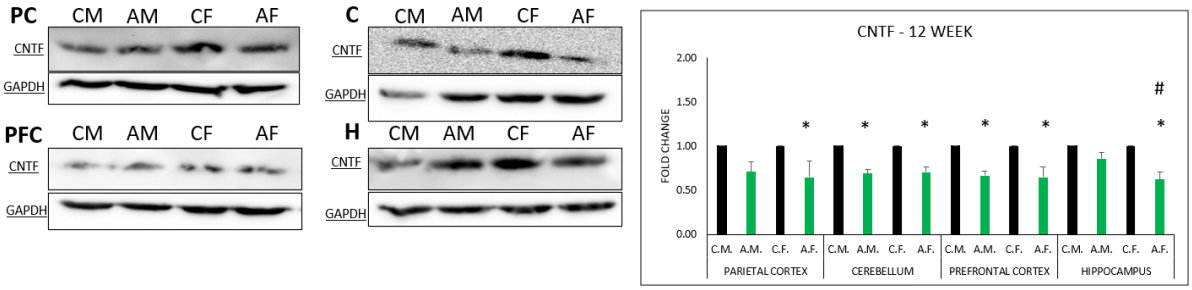


Figure 2.29 – CNTF 12 week. The groups were n=8 male and n=14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

difference between the groups ($df_{treatment}3$, $df_{error}30$ f=27.391 p=2.41e-8) after 12

weeks of treatment. Compared to their respective controls, both males (p=<0.05) and females (p=<0.05) showed a significant decrease in protein expression. The hippocampus was tested and showed significant differences between the groups ($df_{treatment}3$, $df_{error}39$ f=20.766 p=5.55e-8) after 12 weeks of treatment. The males showed a non-significant decrease in expression of the protein (p=<0.05); while the females showed a significant decrease in expression (p=<0.05). Significant sex difference in protein expression was noted in the hippocampus (p=<0.05), but non-significant sex difference is not noted in the parietal cortex (p=0.957) and the cerebellum (p=0.992).

2.3.5.4 Effect of Alcohol on CamKII levels at 12 weeks

CamkII was tested in the three different regions. The parietal cortex showed significant difference between the groups ($df_{treatment}3$, $df_{error}26$ f=26.840 p=1.08e-7) after 12 weeks of treatment. The males showed a non-significant decrease in protein expression (p=0.080). The females showed a significant decrease in protein expression (p=<0.05). The cerebellum showed significant differences between the groups ($df_{treatment}3$, $df_{error}30$ f=10.006 p=1.32e-4) after 12 weeks of treatment. Compared to their respective controls, both males (p=<0.05) and females (p=<0.05) showed a significant decrease in protein expression. The

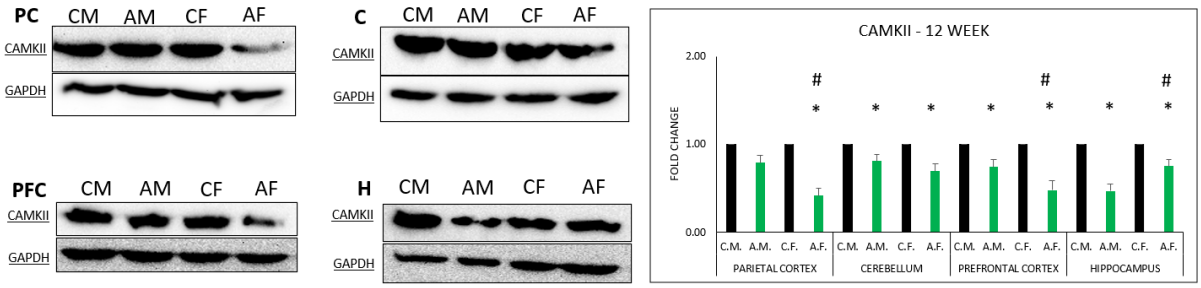


Figure 2.30 – CAMKII 12 week. The groups were n=8 male and n= 14 female. Significance is p=<0.05. * = significant change compared to control. # = significant sex difference change.

hippocampus was tested and showed significant differences between the groups ($df_{treatment}$3, df_{error}31 f=43.269 p=1.21e-10) after 12 weeks of treatment. Compared to their respective controls, both males (p=<0.05) and females (p=<0.05) showed a significant decrease in protein expression. Significant sex difference was noted in the parietal cortex (p=<0.05) and the hippocampus (p=<0.05), but a non-significant decrease was shown in the cerebellum (p=0.423).

2.3.5.5 Summary of 12 Week Time Point

The 12-week time point showed significant down-regulation in all the proteins measured, except BDNF. These results suggest that the alcohol treatment length is acceptable to study alcohol use disorder. The elevation in the BDNF protein suggests that BDNF acts as a protective mechanism for toxic insults (Alcohol in this case) (Míguez-Burbano et al. 2014; Mocchetti et al. 2014). The sex difference that has been shown in this time point is minimal with only a few regions and proteins being affected.

2.4 Discussion

Our results demonstrate that at 12 weeks of chronic treatment with alcohol treatment, we see the most productive neurodeficit when compared to previous time points. This exposure in a murine model would be comparable to an adult that drinks from age 21 to age 60 human (Dutta and Sengupta 2016). The exposure plan also shows decrease in all synaptic proteins at the 12-week time point; while at the other time points there is no general downregulation of proteins, e.g., 4 weeks vs. 8 weeks vs. 12 weeks. We also employed an alcohol administration method that mimics human consumption.

Most models use a binge model with a high dose of alcohol (Jeanblanc, et al. 2019). Our exposure plan uses a low dose of alcohol over a long period of time. Models that are used to study AUDs use different exposure methods, such as vapor chambers and injections, but are not able to mimic human usage (Spanagel 2000; Wallace 1990). We employed the use of gastric gavage because this method releases neurotransmitters and other factors, such as glucagon like peptide (GLP-1), that are released during oral administration of substances (Kruse Klausen et al. 2022).

Alcohol models focus on a specific aspect of AUDs. Our exposure plan focuses on just causing neurodeficit. Other plans that have been implemented contain deprivation phases, but our plan doesn't involve deprivation phases (Coleman, et al. 2008; Ghasemi and Dehpour 2009; Hitzemann 2000; Jeanblanc, et al. 2019; McBride and Li 1998; Spanagel 2000; Spanagel 2017; Spanagel and Hölter 2000; Sun, et al. 2012; Wallace 1990). After the 12-week pre-treatment period, alcohol dosing never stops until the end of the behavioral testing protocol. This is to mimic continued social drinking (Parsons 1998).

Some methods of studying AUDs use two-bottle choice, which means the animals are allowed free access to an alcohol solution and water for a set number of hours and days. While using two-bottle choice, the amount of alcohol solution is measured before and after the allowed drinking time period (Spanagel 2000). The draw back to these types of methods is that the investigator doesn't know how much alcohol has been consumed by each individual animal. Our method employs gastric gavage, which allows for the investigator to know how much alcohol is

consumed at one time by each animal individually. The amounts are calculated based upon weight of the animal that is taken once weekly.

Our behavioral analysis also suggests that changes in motor coordination and locomotion occur the best at 12 weeks. The other time points, 4 and 8 weeks, do not show the severe decreases as seen in the 12-week time points. Some studies have shown motor coordination is affected but do not show as to what extent this deficit exists in the population.

Our behavioral analysis suggest that 12-weeks of treatment show behavioral alteration. In the Morris Water Maze, we see a perpetual decrease in the learning curve; rather, 4 and 8 weeks we do not see the neurodeficit that is present in the 12-week time point. In the probe trial, which is the most important part of the test, shows time decrease in the southern quadrant unlike in the other time points where the decrease is not as significant.

We don't see decreases in other regions like we thought we would see at other time points, e.g., cerebellum (C), prefrontal cortex (PFC), parietal cortex (PC), and hippocampus (H). At 12 weeks in all regions, we see generalized down-regulation. This shown to be due to increased exposure to alcohol which results in cell death. The increased levels of BDNF (brain derived neurotrophin factor) acting as a safety mechanism as to protect cells from toxic insults (Logrip, Janak, and Ron 2009; Míguez-Burbano, et al. 2014; Mocchetti, et al. 2014). These changes are mimicked and seen at the 12-week time point. Overall, we established an exposure plan for a chronic low dose alcohol study. We also showed that our selected exposure plan causes desired neurodeficit at 12 weeks

of pretreatment and continuous treatment. With this study, we showed that synaptic plasticity changes occur over the period, while brain uses specific factors as protective mechanisms during the presence of toxic factors as seen in figure 2.31.

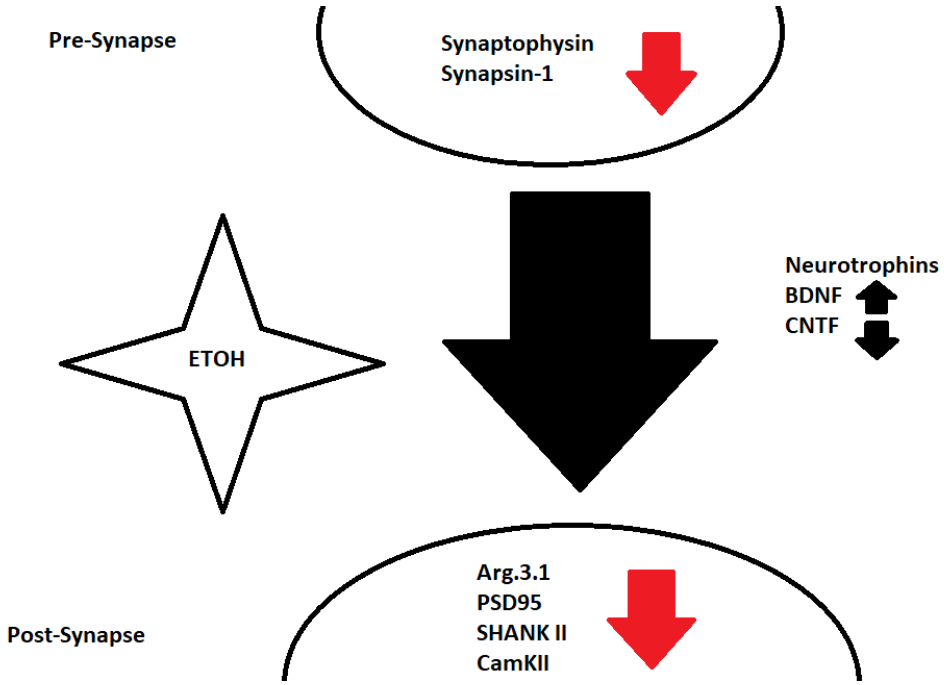


Figure 2.31 – Summary figure for specific aim 1. The result that was gained from the 12-week time point shows the downregulation of majority of the protein markers. The fact that BDNF is up regulated at this time point means that BDNF acts as a survival mechanism.

CHAPTER 3

ETOH/HIV-1 TAT INDUCED NEURODEFICIT CAUSES BEHAVIORAL, RECEPTOR, AND LIGAND CHANGES

3.1 Introduction

HIV-1 remains a problem in the United States. This condition functions by knocking down the host immune system and making the host susceptible to a host of other infections and issues (Fauci 2003; Killian and Levy 2011). This works by altering the numbers of CD4+ cells by having the population numbers being high at the start of the condition and being knocked down or completely knocked down toward the end of the condition (Vidya Vijayan, et al. 2017).

The first place that HIV-1 virus tends to go after initial exposure is the brain. The virus uses a receptor called gp120 to interact with host cells. The virus uses macrophages (CD11b+ cells) to enter the brain across the blood brain barrier (Strazza, et al. 2011; Yadav and Collman 2009). The HIV-1 virus replicates inside these cells and other cells of the brain causing cell death and releasing of viral particles and other factors being released as a result of cellular replication of the virus (Mattson, Haughey, and Nath 2005). The virus has many cofactors present to help it replicate inside the cell.

The factors and components of the HIV-1 virus, such as Tat and gp120, are known to be toxic in the neural environment (Ajasin and Eugenin 2020; Bagashev and Sawaya 2013; Hahn, et al. 2015; Haughey, et al. 2001; Jadhav and Nema 2021; Marino, et al. 2020). Tat, transactivator of transcription, is a viral component that works to regulate the transcription of the HIV-1 virus. Tat has also been used as a component to make other materials, such as drugs, available to enter cells

and cause whatever pharmacotherapy action is required (Becker-Hapak, McAllister, and Dowdy 2001).

Some disease conditions are perpetuated using substances, especially alcohol (Pandrea, et al. 2010; Silverstein and Kumar 2014). In this study, we are investigating to see whether alcohol and HIV work antagonistically, synergistically, or by additive effect. We are investigating how these two agents work to change behavioral testing, whether that be changes in spatial learning and memory or changes in search strategies. We are investigating how these agents work to change receptor/ligand constitution of the subjects and affect the neurodeficit that caused by cellular death. We will ask the following question: Do HIV-1 Tat and alcohol work cause alterations in spatial learning and memory, motor coordination, receptor constitution changes, and change in cytokine levels?

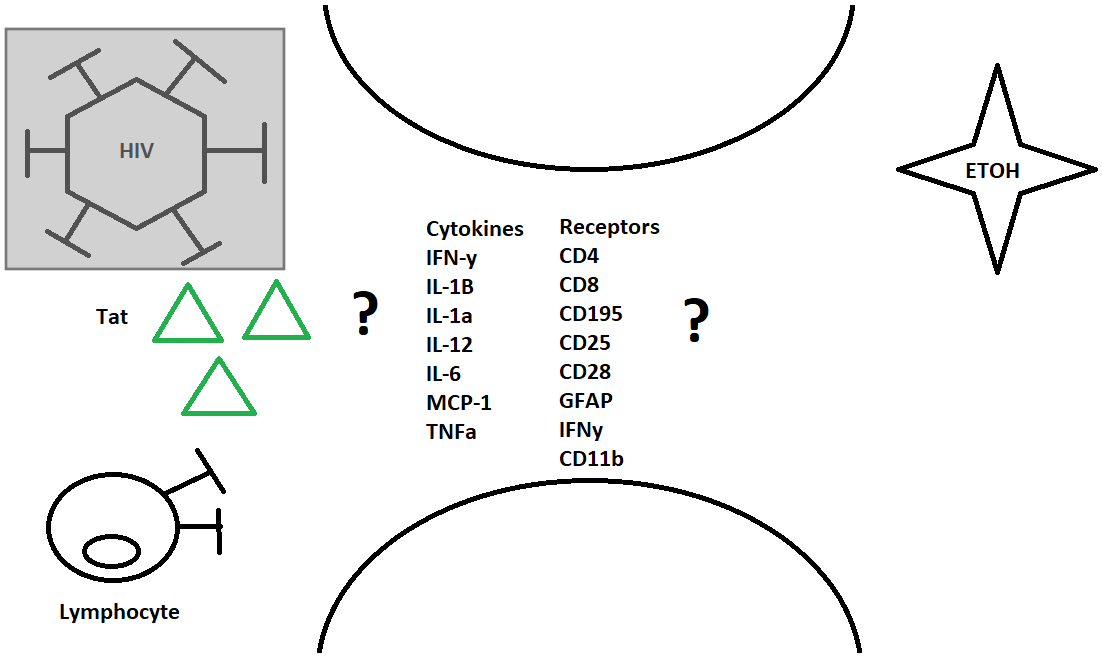


Figure 3.1 – Summary schematic for hypothesis. With addition of alcohol (ETOH) and Tat, what will happen with cytokines and receptors.

To answer this question, we make the following hypothesis: We hypothesize that with prolonged exposure HIV-1 Tat and alcohol will cause alterations and significant changes in spatial learning and memory, motor coordination, receptor constitution, and cytokines.

3.2 Materials and Methods

3.2.1 Animal Studies

3.2.1.1 Animals

Animals were obtained from the Jackson Laboratories and the colony were maintained inside the Laboratory Animal Research Core (LARC) facility. We used 8-week-old C57/BL6J mice, both male and female mice, equaling n=144. The study contained 4 groups per sex. The groups males were control n=17, ETOH n=21 Tat n=16, and Tat + EtOH n=21. The groups females were control n=17, ETOH n=18 Tat n=17, and Tat + EtOH n=17.Food and water were never restricted in this work. The mice were housed 3 to 5 animals to a cage inside the LARC facility. The animals were on a 6 am to 6 pm light and dark cycle (12-hour cycle). All behavioral testing was completed between the hours of 8 am to 5 pm till termination of the experiment. Animals were returned to the LARC at the end of the day after the experimentation was completed for the day. All procedures were approved and followed in accordance with the UMKC Institutional Animal Care and Use Committee.

3.2.1.2 Tat Induction

Tat transgenic animals were used in this study that were specifically bred with composition of Tat-tg. The animals were obtained from Dr. Kurt Hauser at

Virginia Commonwealth University. The Tat-tg is placed under a *tet-on* inducible system and GFAP promoter (Nookala, et al. 2018). The use of these mice has presented many clinical findings of HIV-1 infection that occurs but not limited to changes blood cell population, neural cell apoptosis, astrocytosis, changes in gray matter, dendritic cellular degeneration and inflammatory process (Jaeger and Nath 2012; Nookala, et al. 2018; Gorantla, Poluektova, and Gendelman 2012; Langford, et al. 2018; Sil, et al. 2021). These animals are known to show differences in learning and memory. The animals were 8 weeks old at start of induction. The animals were fed a doxycycline (DOX) laced diet for a period of 4-week pretreatment at 6 g/kg of formulated animal chow. The diet was stored in the LARC and provided by staff (Nookala, et al. 2018).

3.2.1.3 Alcohol

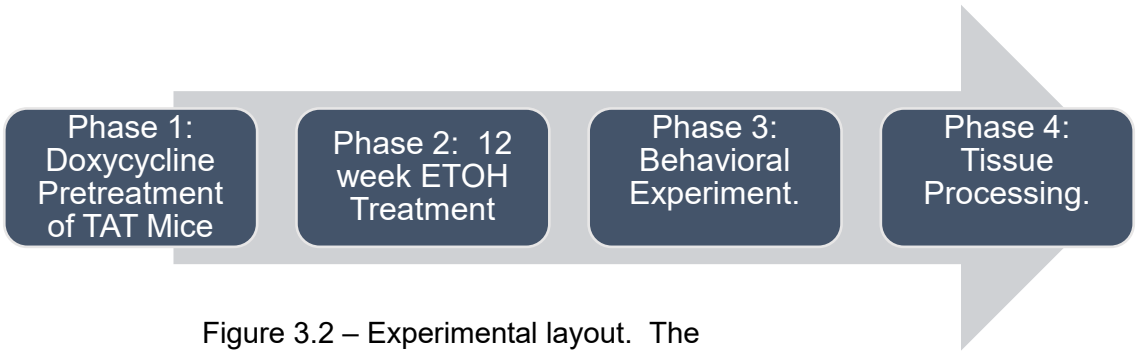


Figure 3.2 – Experimental layout. The different phases of the experimental system.

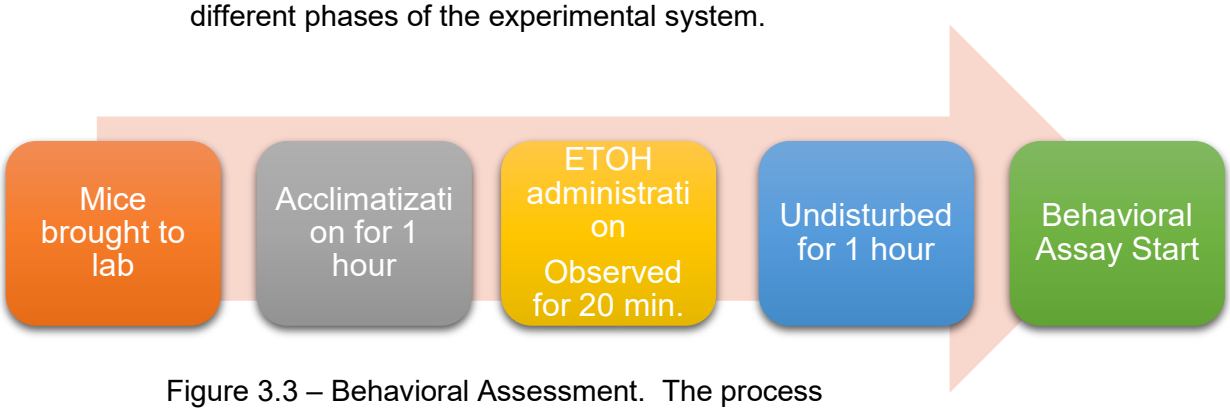


Figure 3.3 – Behavioral Assessment. The process the animals will experience once entering the lab.

The alcohol is administered the same way as outlined in chapter 2. The control and alcohol animals are handled the same way as described in chapter 2.

3.2.1.4 Rotor-Rod

The rotor-rod test is used to test the motor-coordination and locomotion. More time spent on the rod is an indication that, the animal has better motor-coordination and the more likely that locomotion is intact (Shiotsuki et al. 2010). At the start of the experiment, the animals are brought from the LARC facility. The animals complete the behavioral assessment (Fig. 3.3). The protocol calls for each animal to complete 4 trials. The speed of the rod is changed by 1 rpm every 15 seconds. During the experiment, the time spent, and distance traveled on the rod are recorded each trial.

3.2.1.5 Novel Object Recognition

The novel object recognition test is used to ascertain the animal's ability to use working memory and ascertain environmental changes (Antunes and Biala 2012; Huang and Hsueh 2014). This is a two-day assay. The animals are first transferred to the lab from the LARC facility. The animals complete the behavioral

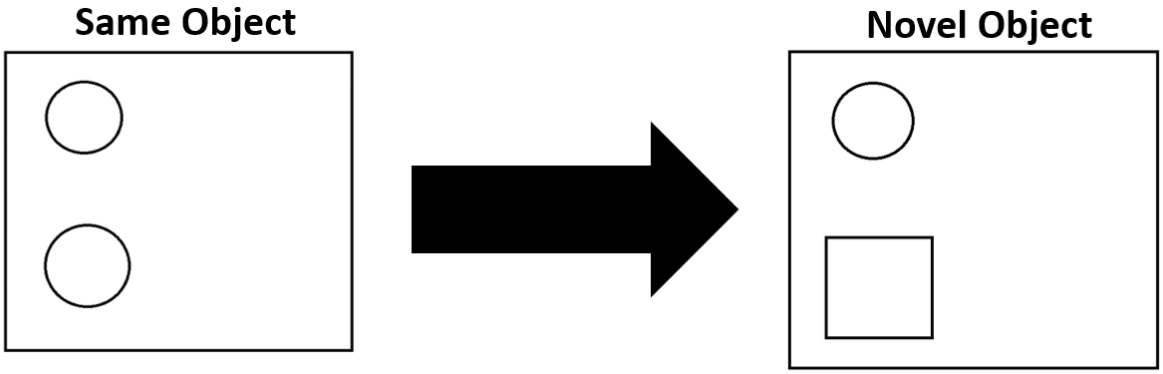


Figure 3.4 – Schematic of Novel Object Recognition Assay. The assay starts with two of the same objects (spheres) in the arena. On the second day, one of the objects (sphere) remains the same; while, the other object (cube) is swapped in the arena.

assessment once transferred from the LARC (Fig. 3.3) On the first day, the objects are the same in the experimental chamber (Fig 3.4). The animals get one trial of 5 minutes in the experimental chamber, and the animal is returned to their home cage. Once testing is completed for the first day, the animals are returned to the LARC. On the second day, the animals are treated as they were the previous day. The experimental chamber is changed by the removal of one of the objects and then a different (novel) object is placed inside the chamber (Fig 3.4). The animal is allowed another trial of 5 minutes in the experimental chamber, and then returned to the home cage. Schematic of the apparatus is shown above (Fig 3.4). Animal movements are tracked using the ANY-Maze behavioral software (version 4.99z). From this assay, we ascertain the amount of time toward the different (novel). We also ascertain the preference index between the known object vs. novel object.

3.2.1.6 Barnes Maze

The Barnes maze assay is a test of spatial learning and memory (Attar et al. 2013). The animals are transferred from the LARC to the lab. Once the animals are in the lab, the animals complete the behavioral assessment and treatment phases. To start the assay, the animals starts the assay in the middle of the apparatus. The test takes place over the course of 4 days with the test containing 3 phases. These phases to complete the Barnes maze are the habituation, training, and probe phases (Attar, et al. 2013). The habituation phase consists of one trial that lasts 2 minutes for each animal. At completion of this trial, the animals are placed back into the home cage. The training phase that has trials last two

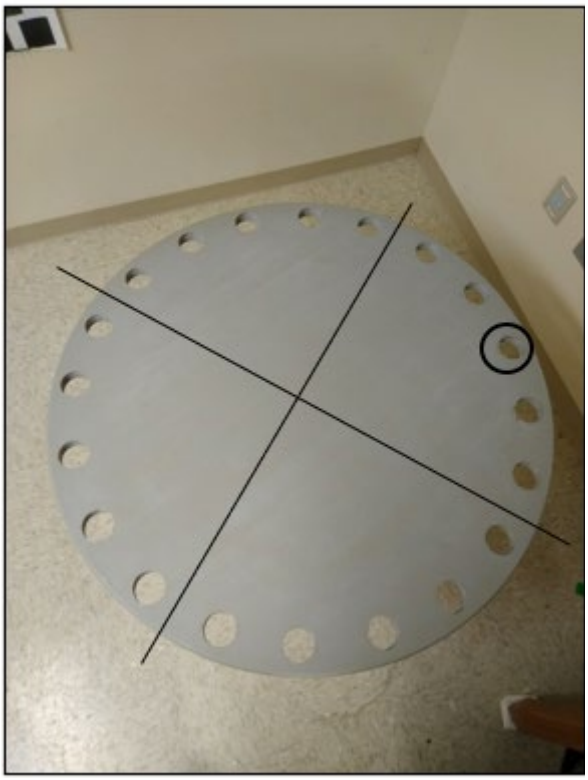


Figure 3.5 – Barnes Maze Apparatus. The table is divided into four regions. The escape hole is circled in black.

days that consist of 4 trials that are sequential for each ani mal. During the interim phase of the training trial, the animals are placed in separate cages as to not give scent cues to other animals who are completing their trials. The training trials last 2 minutes. The probe phase consists of 1 trial of 2 minutes. At the conclusion of the probe trial, the animals are placed back in their home cages. Animal movements are tracked using the ANY-Maze behavioral software (version 4.99z). This assay we analyze the time, distance, and entries into the southern region in the probe phase of the Barnes maze.

3.2.1.7 Water Maze

The water maze test is a test of spatial learning and memory (Vorhees and Williams 2006). The test takes place in a water tank. The water temperature in the water tank is regulated every day between 20 to 25 degrees Celsius. The escape island is located 1 cm below the water surface. The tank is laid out according to compass direction. On the wall of the tank, there are cues on the walls of the water tank which allows the animal to navigate the water tank. The test takes place over the course of 6 days. Days 1 thru 5 are the test period with day six being the probe trial, which is a test of reference memory (Vorhees and Williams 2006). The animals are placed in the water tank for 4 random directions. For the test period, the escape platform is left in the water tank submerged for the 5 days in the southwest quadrant. For the probe trial, the trial is 60 s per animal and the platform is removed from the southwest quadrant. The animals are tracked by the ANY-maze (version 4.99z) behavioral software. Spatial learning

and memory deficit was analyzed by analyzing the escape latency (the time it takes to get to the escape platform) and the probe trial (time spent in the target quadrant).

3.2.1.8 Sample Collection

Mice were euthanized after the behavioral experiments were completed. The mice were anesthetized using a ketamine/xylazine solution or the use of isoflurane gas mixture (McBride and Li 1998). Once the mice were sufficiently under anesthesia, the mice's heart was perfused with 30 ml of isotonic PBS. The mice brain tissue was extracted from the animal and placed on ice in a saline solution. Once in the lab, the animal tissue is divided for dissection and whole brain digestion.

3.2.1.9 Dissection:

The dissection divides the brain tissue into the following areas. The cerebellum was coronally bisected separating the medulla and the pons, and the parietal cortex was coronally bisected separating the two hemispheres of the brain (Nookala, et al. 2018). The hippocampus was dissected out from the parietal cortex. All the brain tissue was collected and, was snap frozen in liquid nitrogen. After the brain tissue was snap frozen in the liquid nitrogen, it was moved to a freezer (-80°C) for storage until testing is ready to be completed.

3.2.2. In Vitro Diagnostic Testing (IVD) Assays

3.2.2.1 Whole Brain Digestion

The brains are placed in a petri plate. The brain tissue is cut into small pieces. The cut tissue is placed in 50 ml conical tube. The tissue is added to 10 ml of trypsin and placed into a 37°C water bath for 15 minutes while gently agitating

the tube (Bilsland et al. 2006; Donnenberg 2011; Guez-Barber et al. 2012). After the 15 minutes, the tube has equal amounts, 10 mL of complete RPMI (500 ml RPMI, 50 ml fetal bovine serum (FBS), and 500 µL of penicillin/streptomycin solution). The tubes are then spun in the centrifuge for 15 min. at 3500 rpm. The tubes are then filtered 3 times using a 70 µM cell strainer (Bilsland, et al. 2006; Donnenberg 2011; Guez-Barber, et al. 2012). The samples are stored in PBS at 4°C.

3.2.2.2 Flow Cytometry

The samples were taken from the refrigerator and placed in properly labeled tubes for each animal. The sample tubes have 0.5 million cells aliquoted into each tube that are tested in triplicate. The tubes are then spun for 15 minutes at 3500 rpm to remove the saline. An antibody solution is then made for each marker that is going to be tested. Those antibodies are CD4, CD8, CD25, CD28, CD195, IFNγ (BD Pharmgen), CD11b (Cell signaling), and GFAP. The tubes have 100 µL of respective antibody of interest added to each sample and then placed on ice for 2 hours. After the 2-hour period, the samples are spun 3500 rpm for 15 minutes. The antibody solution is poured off and 1000 µL of PBS is added to each tube and then spun at 3500 rpm for 5 minutes. After each wash, the PBS is poured off and new PBS is added. This process is repeated three times. After the last wash, 1000 µL of PBS is added to each tube. The samples are then taken to the BD FACS Canto II (Franklin Lakes, New Jersey). Each run is interval gated by using an unstained tube for each antibody to ascertain positive populations during the

analysis. We also performed a 3 step validation process for our flow cytometry assays (Selliah et al. 2019).

3.2.3.3 Sample Preparation for the ELISA assay

After the dissection step, the samples are taken out of the freezer (-80°C) by brain region. The samples are then immersed in RIPA buffer and protease inhibitor solution. The amounts of the solution depend on the region that is being processed. The amount of solution for each region are the hippocampus (400 μL), prefrontal cortex (800 μL), parietal cortex (800 μL), and cerebellum (800 μL) (Nookala, et al. 2018). The samples are then homogenized using the sonicator. The samples are then spun 12000 rpm for 20 minutes. The supernatant is pipetted into a new properly labeled tube. The samples are then transferred to the freezer (4°C) until further sample processing can be completed. The samples then undergo BCA assay as to ascertain the protein content. The standards and samples (5 μL of protein with 20 μL molecular grade water) are added to a 96 well plate in duplicate. The reagent (200 μL) is added to each well. The plate is then placed in an incubator (37°C) for 30 minutes. The plates are then mixed and placed in the microplate reader (Bio-Rad, Hercules, CA). After the protein concentrations are known, the samples for each group are then pooled at 1000 μg of protein into one sample per group for a group of 4 samples. These samples are then placed in the freezer at (4°C).

3.2.3.4 Performing ELISA assay

The samples are taken from the freezer (4°C) and placed on ice on the bench. The standards are made per manufacturer instructions and are added to

the plate. The samples are made according to the manufacturer, R&D Systems (Minneapolis, MN) recommendation using a 1:1 dilution (R&D systems Minneapolis, MN). The samples are made with equal parts of pooled sample and manufacturer provided diluent then added to the kit provided 96 well plate. The prepared antibody cocktail (100 per well) is then added to each well of the plate and the plates are covered with provided adhesive foil covering. The tubes are then placed on a rotator and rocked for 2 hours at 800 rpm. After the 2-hour period, the plate is then placed on a magnet and locked in place. The plate will set on the magnet for at least 1 minute. The plate is then poured off and 100 μL of wash solution added to each well and set for 1 minute. This process takes place for three more times. After the washes are completed, the provided biotin solution is now added to each well and rocked at 800 rpm for 1 hour and 30 minutes. The washes for the plate are completed as described above. After washing, the provided streptavidin solution (50 per well) is then added to each well and rocked at 800 rpm for 30 minutes. The wash steps are completed as described above. After the wash steps, PBS (100 μL) is then added to each well. The plates are then taken to the Bio-plex (Bio-Rad Hercules, CA). The machine is setup per manufacturer recommendations. We are measuring the cytokines MCP-1, IL-1α, IL-1β, IFNγ, IL-6, and IL-12 levels in the tissue. After reading the plates are stored at 4°C.

3.2.4 Statistics

Values are reported for all behavioral assays, flow cytometry, and ELISA assay as mean ± standard error of the mean (SEM). Statistical methods that were

completed used IBM SPSS software (version 27) (IBM, Armonk, NY). Only F values that showed significant interactions are represented in the text, while non-significant F values are not represented in the text. The Tukey post-hoc test was used for further testing with a priori significance level being set at $p < 0.05$. For the behavioral assays across the time points, three-way ANOVA was used with factors being sex, ETOH exposure, and tat exposure. On the figures, significant results are indicated by a * compared to control, significant interactions compared to Tat are represented by #, significant interactions compared to EtOH by $, and to indicate sex differences are depicted by a +.

3.3 Results

3.3.1 Effect of Alcohol and Tat on Locomotion and Motor Coordination

The effects of how Tat and alcohol (ETOH) were evaluated by using the Roto-Rod apparatus. The animals were allowed to complete the assay as explained above. The male groups showed decreases in latency ($df_{latency}$ 3, df_{error} 86 f= 32.156 p = 4.91e-14) and distance ($df_{distance}$ 3, df_{error} 86 f= 22.618 p = 6.91e-11) traveled in the assay. The male groups in the latency parameter had significant interactions in the following: Ctrl vs. EtOH of p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + EtOH p=<0.05, Tat vs. Tat + EtOH p=<0.05. The male groups in the

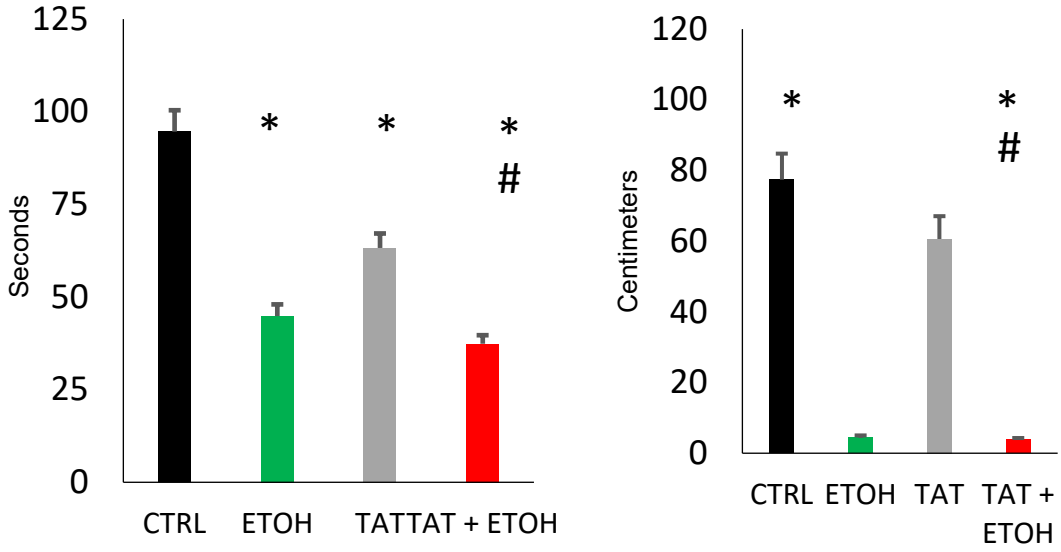


Figure 3.6 – Male Latency (left) and Distance (right). The groups for the males were Ctrl n=17, ETOH n=21, Tat n=16, and Tat + ETOH n=21. The * represents compared to control, and the # represents compared to Tat. Significance was set at p<0.05.

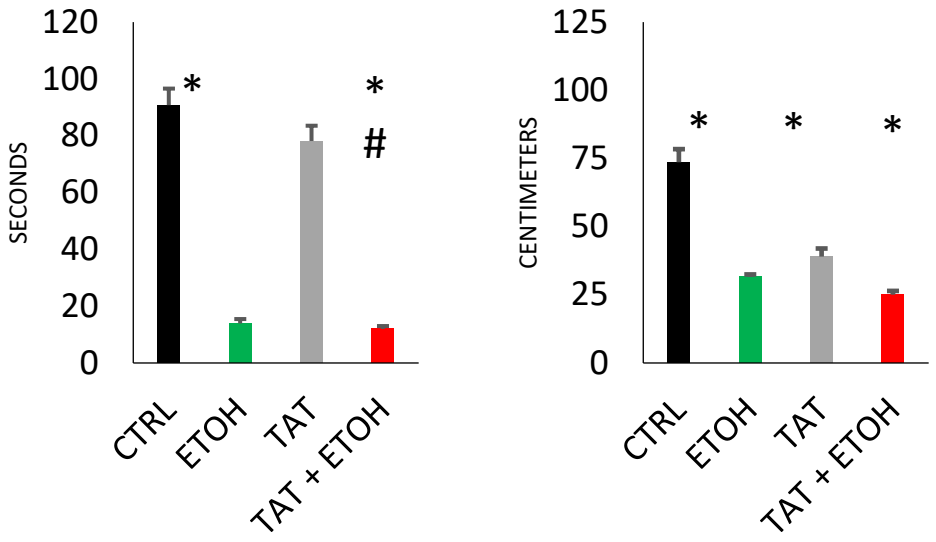


Figure 3.7 – Female Latency (left) and Distance (right). The groups for the females were Ctrl n=17, ETOH n=18, Tat n=17, and Tat + ETOH n=17. The * represents compared to control, and the # represents compared to Tat. Significance was set at p<0.05.

distance parameter had significant interactions in the following: Ctrl vs. EtOH p=<0.05, Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + EtOH p=<0.05.

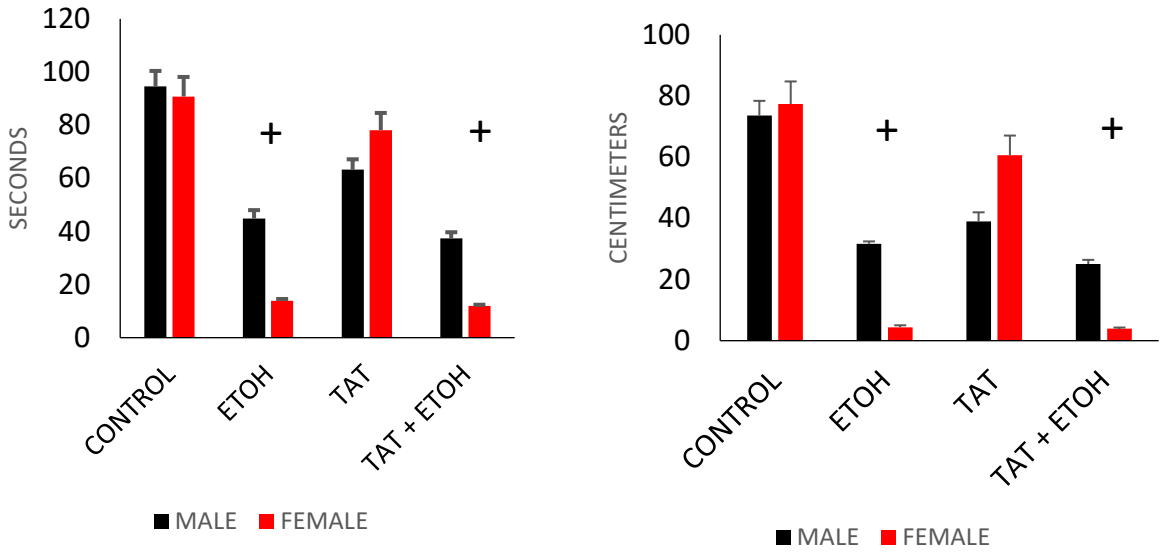


Figure 3.8 – Sex difference are seen in the Roto-Rod Assay. Latency is displayed on the left and distance traveled is displayed on the right. The + symbol represents significant sex difference. Significance was set at p<0.05.

The female groups showed decreases in latency ($df_{latency}$ 3, df_{error} 86 f= 93.047 p = 4.08e-36) and distance ($df_{distance}$ 3, df_{error} 86 f= 52.739 p = 2.28e-24).

The female groups showed significant interactions in the latency as follows: Ctrl vs. EtOH p=<0.05 and Ctrl vs. Tat + EtOH p=<0.05. We assessed sex differences between the groups, with males ($df_{latency}$ 7, df_{error} 265 f= 68.550 p = 7.18e-56) and females ($df_{distance}$ 7, df_{error} 265 f= 33.852 p = 1.71e-33). Sex differences were found to have significant interactions were in the latency parameter ETOH-male vs. ETOH-female p=<0.05 and Tat + ETOH-male vs. Tat + EtOH-female p=<0.05. Sex differences were found to have significant interaction were in the distance parameter ETOH-male vs. ETOH-female p=<0.05, Tat-male vs. Tat-female p=<0.05, and Tat + ETOH-male vs. Tat + ETOH-female p=<0.05.

3.3.2 Effect of Alcohol and Tat on Working Memory

To evaluate the effect ETOH and Tat on working memory we used the Novel Object Recognition Test. The animals completed the assay as described above. The males show significant interaction when looking at the parameters associated with the novel object. The males showed significant interactions with entrances

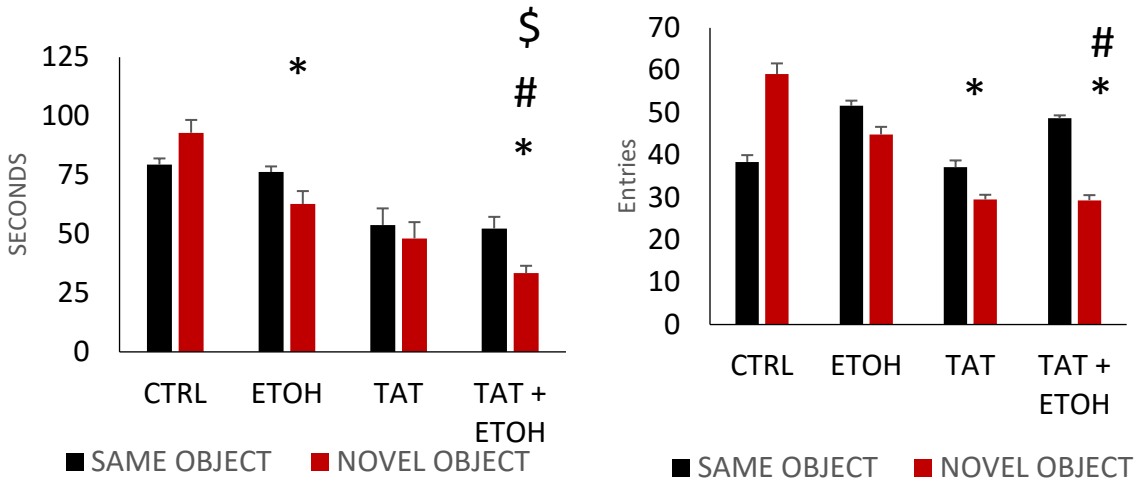


Figure 3.9 – Male time toward the novel object (left). Male entries into novel object zone (right). The groups for the males were Ctrl n=17, ETOH n=21, Tat n=16, and Tat + ETOH n=21. The * represents compared to control. The # represents compared to Tat. The $ represents compared to ETOH. Significance was set at p<0.05.

into the zone of the novel object ($df_{entriesnovelobject}$ 3, df_{error} 17 f= 8.489 p = 0.001) and time moving towards the novel object ($df_{timetowardsnovelobject}$ 3, df_{error} 17 f= 15.414 p = 4.23e-5). The entries into the novel object zone have significant interactions when compared to the following interactions: Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05 and ETOH vs. Tat + ETOH p=<0.05. The time spent in the novel object zone has significant interaction when compared to the following: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, ETOH vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05.

The females showed significant interactions with time moving towards novel object ($df_{timetowardsnovelobject}$ 3, df_{error} 40 f= 5.580 p = 0.003). The time spent in the

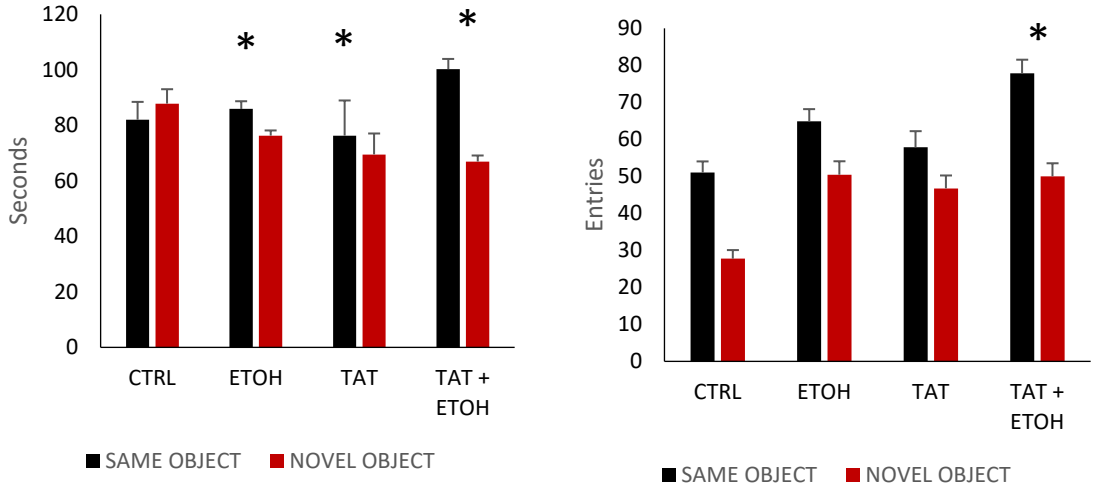


Figure 3.10 – Female time toward object (left) and entries into novel object zone (right). The groups for the females were Ctrl n=17, ETOH n=18, Tat n=17, and Tat + ETOH n=17. The * represents compared to control. Significance was set at p<0.05.

novel object zone has significant interaction when compared to the following: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + ETOH p=<0.05.

We also evaluated animal participation of both sexes for whether the animal showed preference toward the novel object versus the known object. In the males

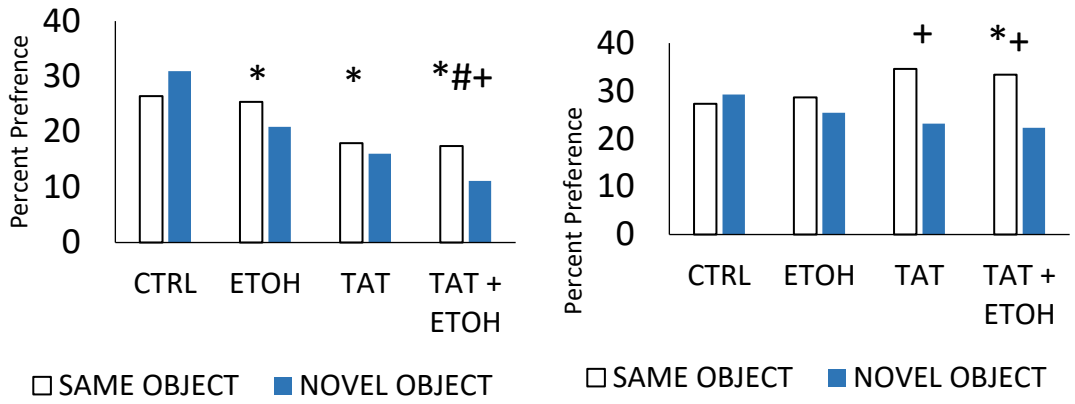


Figure 3.11 – Preference index for male (left) and female (right). The figure depicts the preference of each group toward the novel object (blue bar) compared to same object (white bar). The * represents compared to control. The # represents compared to control. The + represents significant sex difference.

there was significant decreases when the animal showed preference of the novel object ($df_{prefencenovelobject}$ 3, df_{error} 42 f= 21.807 p =1.14e-8) and the females ($df_{prefencenovelobject}$ 3, df_{error} 40 f= 3.441 p =0.026). The males showed preference for specific interactions for the following comparisons: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and ETOH vs. Tat + ETOH p=<0.05. The females showed preference for specific for just one significant interaction of Ctrl vs. Tat + ETOH p=<0.05. When investigating sex difference in the preference index, the difference exists in the significant increase between males and females ($df_{prefencenovelobject}$ 7, df_{error} 82 f= 14.152 p =6.57e-12) with only specific interaction with Tat + ETOH male vs. Tat + ETOH female p=<0.05.

3.3.3 Effects of ETOH and Tat on Spatial Learning and Memory

We used two separate assays to evaluate the effect of Tat and ETOH on spatial learning and memory. The assays that were used were the Barnes maze and the Morris water maze. We will first perform the evaluation of Tat and ETOH on spatial learning and memory by using the Barnes maze. In this assay, we evaluated four separate parameters to evaluate the changes in spatial learning and memory. The parameters that were measured are the entries, time, and distance traveled into the southern region. We also looked at the search strategies to ascertain if memory was altered.

In males, we saw significant decrease in all three parameters of entries ($df_{entries}$ 3, df_{error} 17 f=11.698 p=2.12e-4), time (df_{time} 3, df_{error} 17 f=18.348 p=1.42e-5), and distance ($df_{distance}$ 3, df_{error} 17 f=3.50 p=0.039). We saw specific significant interactions in entries: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and

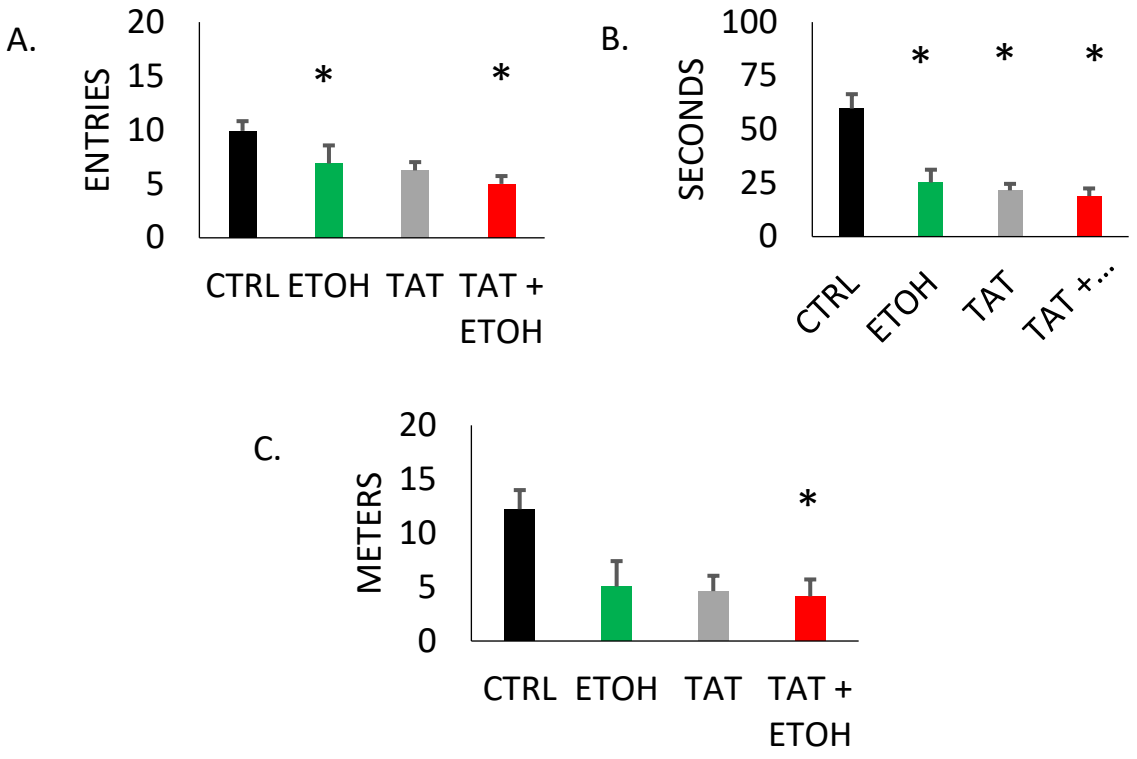


Figure 3.12 – Barnes Maze Male. The figure above represents Entries (A), Time (B), and Distance (C) for the probe trial. The groups for the males were Ctrl n=17, ETOH n=21, Tat n=16, and Tat + ETOH n=21. The * represents compared to control. Significance was set at $p<0.05$.

Ctrl vs. Tat + ETOH p=<0.05. We saw specific significant interaction in time: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05. We saw one specific interaction in the time parameter: Ctrl vs. Tat + ETOH p=<0.05.

In females, we saw significant decreases in all three parameters of entries ($df_{entries}$ 3, df_{error} 39 f=4.354 p=0.010), time (df_{time} 3, df_{error} 39 f=14.924 p=1.27e-6), and distance ($df_{distance}$ 3, df_{error} 39 f=3.109 p=0.037). We saw specific interactions in two of the three parameters. Those parameters are entries and time, respectively: Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + ETOH p=<0.05.

We investigated the changes in the search strategies were altered by the addition of Tat and ETOH using the track plots. With the males, the control groups show a systematic search strategy. The other groups (Tat, ETOH, and Tat + ETOH) show random search strategies.

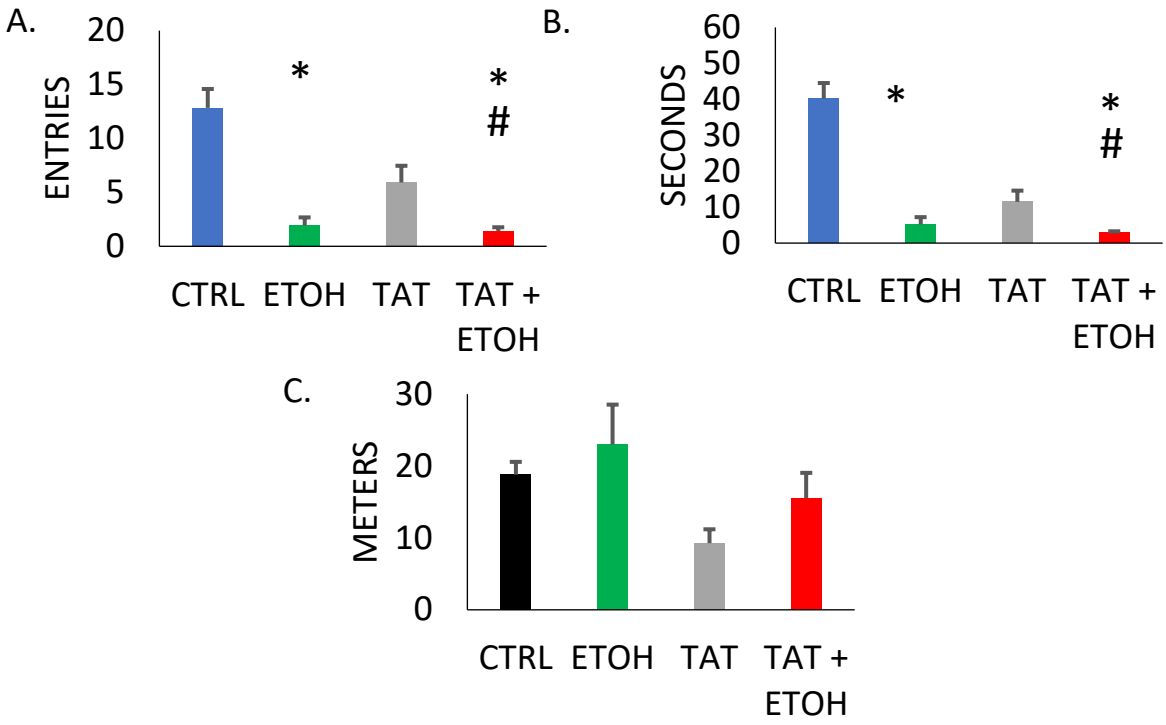


Figure 3.13 – Female Barnes Maze. The figure above represents Entries (A), Time (B), and Distance (C) for the probe trial. The groups for the females were Ctrl n=17, ETOH n=18, Tat n=17, and Tat + ETOH n=17. The * represents compared to control. The # represents compared to Tat. Significance was set at $p<0.05$.

We investigated how the search strategies are altered with addition of Tat and ETOH using the track plots. With the females, the control groups showed a systematic strategy to find the escape hole. The other groups (Tat, ETOH, and Tat + ETOH) showed random search strategies according to the track plots.

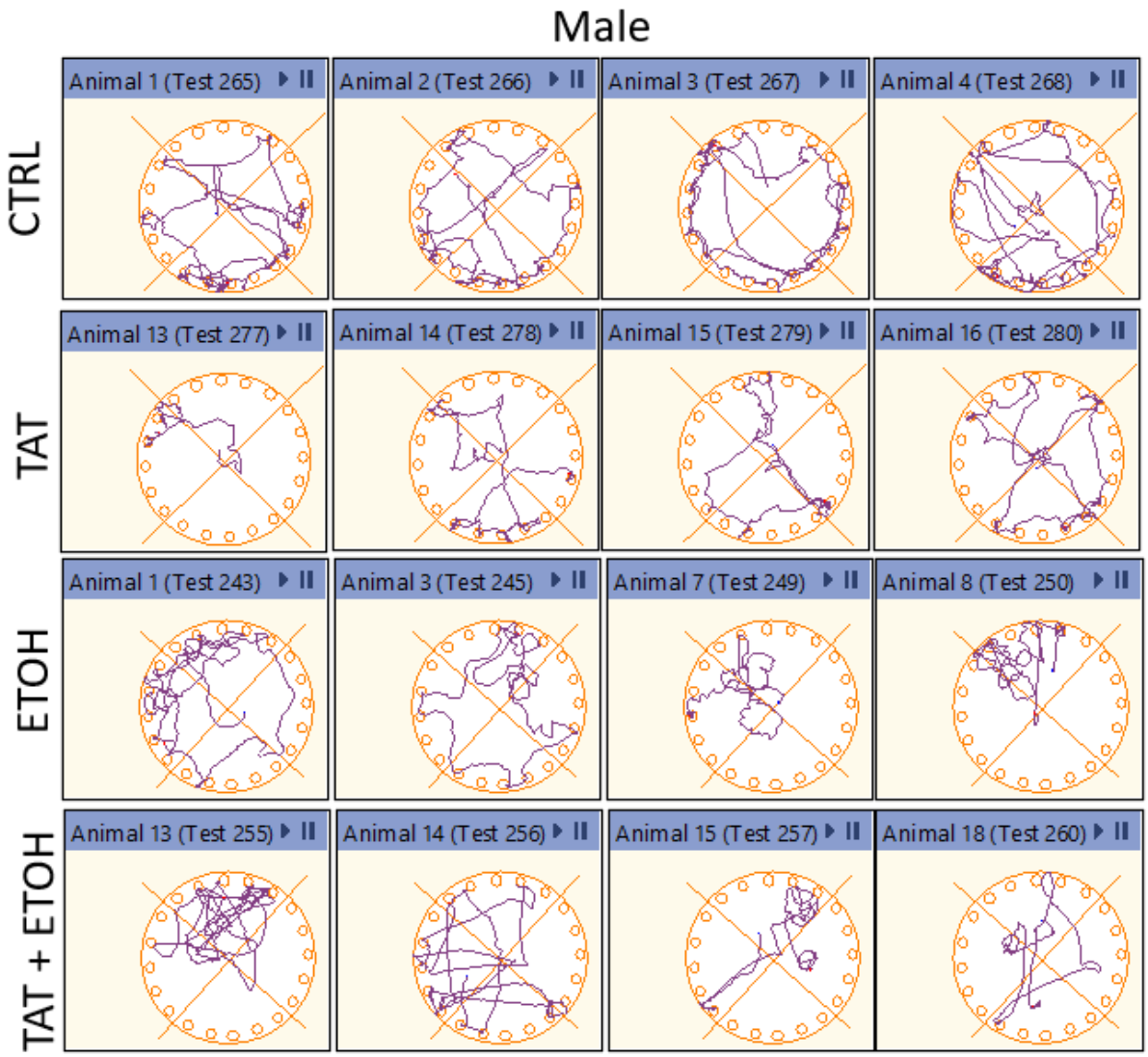


Figure 3.14 – Track plots for the Barnes maze performance of the males. Row 1 Ctrl. Row 2 Tat. Row 3 ETOH. Row 4 Tat + ETOH.

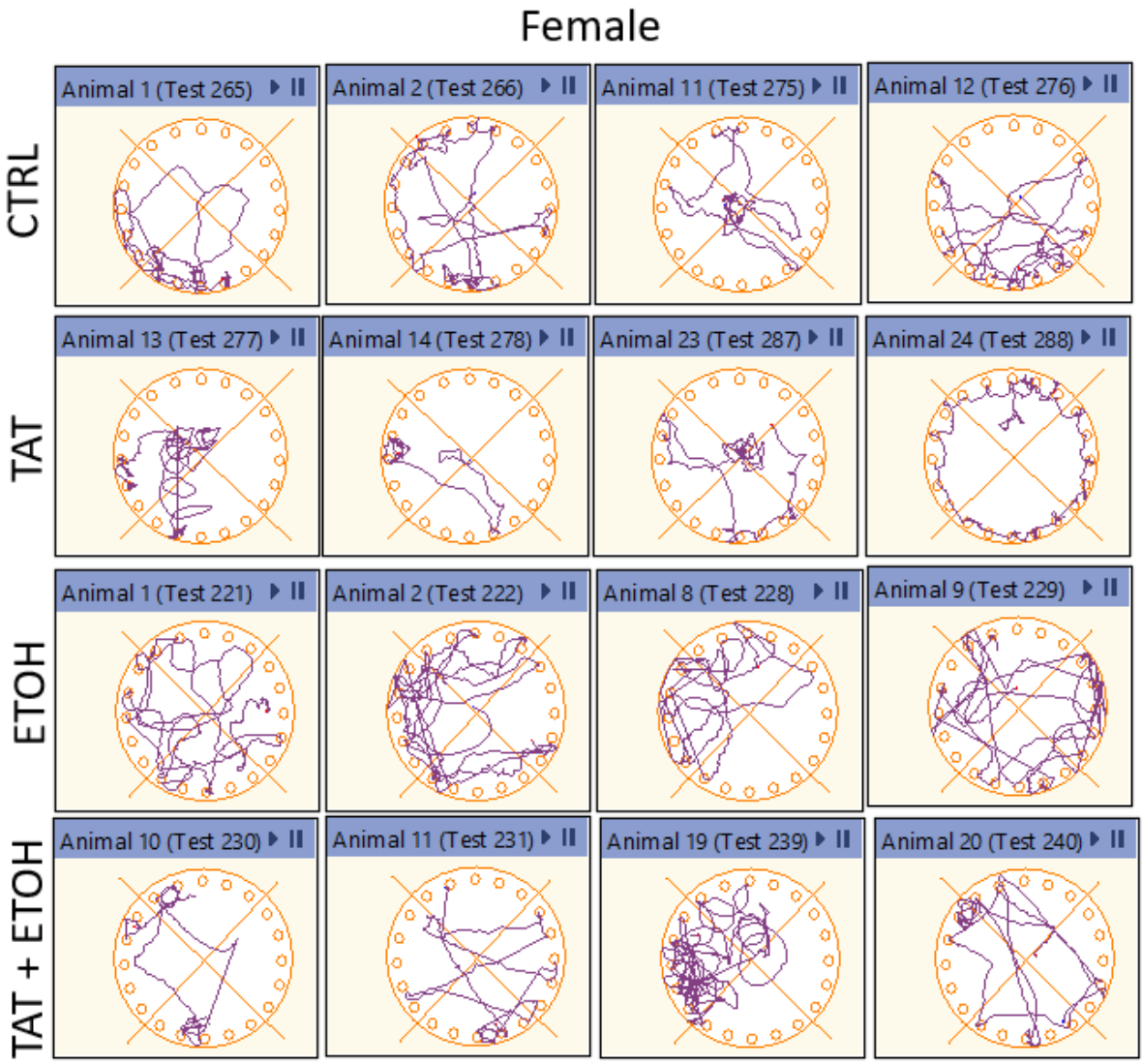


Figure 3.15 – Track plots for the Barnes maze performance of the females. Row 1 Ctrl. Row 2 Tat. Row 3 EtOH. Row 4 Tat + ETOH.

Next, we evaluated spatial learning and memory by using the Morris water maze. We observed the learning ability of animals and evaluated the extent of the learning deficit during the acquisition phase of the assay. In the males, we saw that the significant increases of all groups (df_{group} 3, df_{error} 844 f=55.899 p=5.79e-33). We saw specific interactions of the groups, and those interactions are as follows: Ctrl vs. EtOH p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat p=<0.05, EtOH vs. Tat + EtOH p=<0.05, and Tat vs. Tat + EtOH p=<0.05. We looked at the probe for the males to evaluate to the extent at which the group is learning as to where the escape platform is located. We saw a significant decrease in the ability of the animal's ability to remember where the escape platform would have been in the acquisition (df_{group} 3, df_{error} 39 f=5.602 p=0.003). We saw specific

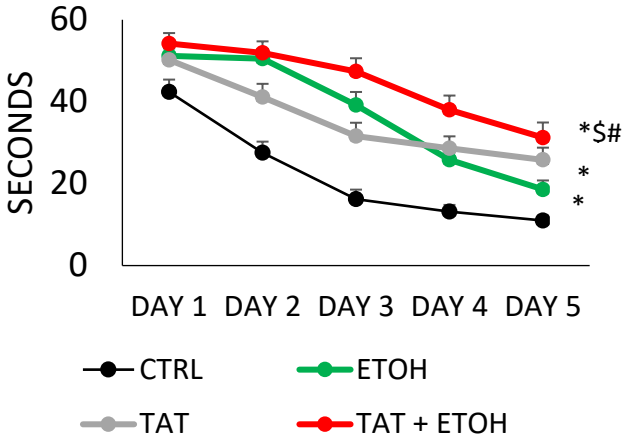


Figure 3.16 – Male Morris water maze acquisition learning curve. The groups for the males were Ctrl n=17, ETOH n=21, Tat n=16, and Tat + ETOH n=21. The * represents compared to control. The # compared to Tat. The $ compared to ETOH. Significance was set at p<0.05.

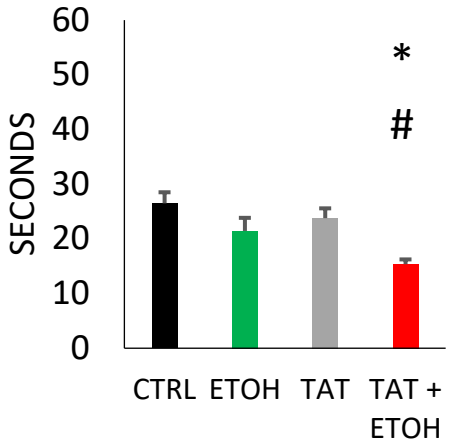


Figure 3.17 – Male probe trial. The groups for the males were Ctrl n=17, ETOH n=21, Tat n=16, and Tat + ETOH n=21. The * represents compared to control. The # represents compared to Tat. Significance was set at p<0.05.

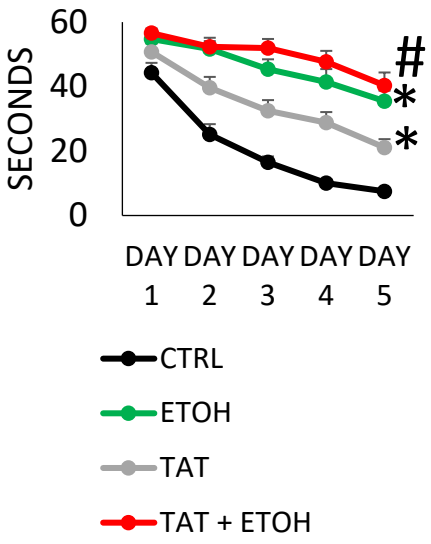


Figure 3.18 – Female Morris water maze learning curve. The groups for the females Ctrl n=17, ETOH n=18, Tat n=17, and Tat + ETOH n=17. The * represents compared to control. The # represents compared to Tat. Significance was set at p<0.05.

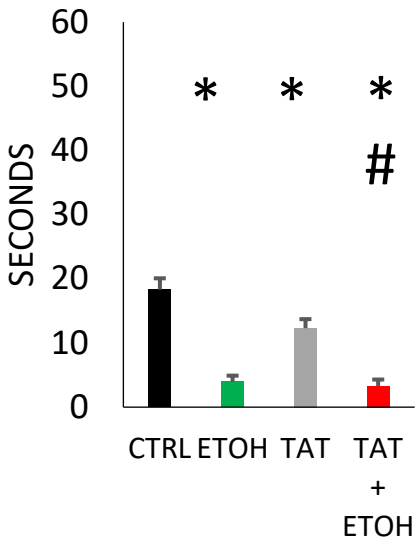


Figure 3.19 – Female Probe Trial. The groups for the females were Ctrl n=17, ETOH n=18, Tat n=17, Tat + ETOH n=17. The * represents compared to control. The # represents compared to Tat. Significance was set at p<0.05.

interactions with the males and those interactions are as follows: Ctrl vs. Tat + ETOH p=<0.05 and Tat vs. Tat + ETOH p=<0.05.

In the females, we performed the same analysis to see at what extent over the course of the acquisition phase of the assay. We saw significant increases in the ability of the female animals to reach the escape platform (df_{group} 3, df_{error} 808 f=106.997 p=2.46e-58). We saw specific interactions with the female groups and those interactions are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05. In the probe trial, the female animals showed a steep significant decrease in the ability of the animal's ability to remember where the escape platform would have been in the acquisition (df_{group} 3, df_{error} 37 f=24.548 p=6.47e-9). We saw specific interaction with the

female and those interactions are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05.

3.3.4 Receptors involved in EtOH and Tat Consumption

We investigated the effect that ETOH and Tat had on the receptor changes in the brain by looking at receptors that were relative to HIV and ETOH for these disease conditions. The markers that were interrogated were: CD4, CD8, CD11b, GFAP, CD25, CD28, CD195, and IFNγ. The CD4 marker is used for monitoring the HIV patient disease status, especially helper T cell levels (Fauci 2003; Kaul et al. 2005; Killian and Levy 2011; Liu, et al. 2009; Matavele Chissumba, et al. 2015; Vidya Vijayan, et al. 2017; Clift 2015). The CD8 marker is the principal marker of the cytotoxic T cell, which is important for monitoring pathogen state. CD11b is the marker for tissue macrophages, B cells, natural killer (NK cells), and granulocytes (Parney, Waldron, and Parsa 2009). This marker is primarily involved in and used as microglial marker in the nervous system. CD25 is also known as IL-2. This marker is available on T cells, B cell, and other myeloid precursor cells. It is also found in larger populations of resting memory T cells (Liu, et al. 2009; Matavele Chissumba, et al. 2015). CD28 marker is expressed on T cells and provides co-stimulatory signals for the activation and survival (Eylar et al. 2001). Also, known to provide potent signaling for various interleukins, e.g., IL-6. CD195 is also known as MCP-1. This marker functions as a chemokine. This marker is located on T cells, macrophages, and microglia (Clift 2015). This marker is implicated in HIV as a co-receptor to enter cells. This marker is also involved in signaling at least 3 other molecules: RANTES, MIP-1, and MCP-2 (Pharmingen).

Interferon γ (IFNγ) is a type II interferon. This receptor is responsible for activating and inducing macrophages and major histocompatibility complex II (MHC II) (Rojas et al. 2021). This factor is involved in inhibiting viral replication and is known to be secreted and located on NK cells, CD4 Th1 cells, and CD8+ cells (Clift 2015; Rojas, et al. 2021; Eylar, et al. 2001). GFAP is marker that is used to ascertain astrocyte populations. This marker is used in cellular communication and function of the Blood Brain Barrier. This marker is important in cases of central nervous system (CNS) repair in cases of CNS injury (Mehrbod et al. 2019; Sporer et al. 2004).

3.3.4.1 Cellular Populations Shifting with Addition of ETOH and Tat

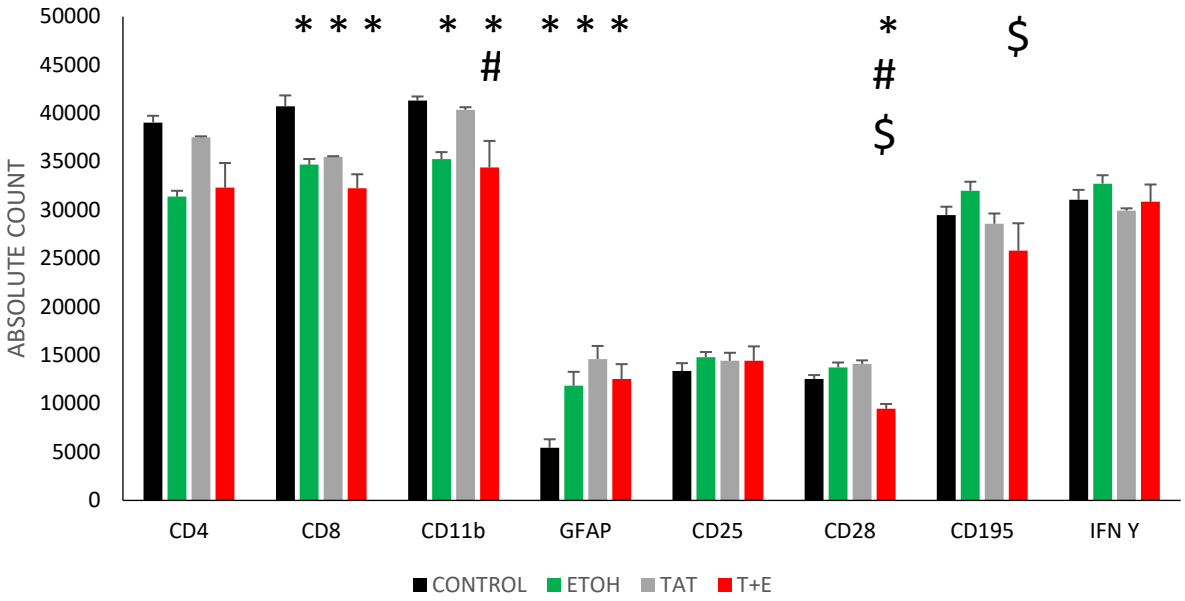


Figure 3.20 – Population shifts occur in the males in the presence of Tat and alcohol (ETOH). The responses are varied among the different receptors. The * represents compared to control. The # represents compared to control. The $ represents compared to ETOH. Significance was set at $p<0.05$.

We investigated changes in cellular population shifts due to the addition of alcohol (ETOH) and Tat. First, we investigated the males. The CD4+ populations showed non-significant decreases in the ETOH and the Tat + ETOH. The CD8+

showed significant decreases in population due to addition of ETOH and Tat (df_{group} 3, df_{error} 44 f=8.882 p=1.02e-4). We saw specific interactions with this marker and those interactions are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + ETOH p=<0.05. CD11b+ populations show significant decreases in the presence of both Tat and ETOH (df_{group} 3, df_{error} 44 f=10.101 p=3.49e-5). We saw specific significant interactions and they are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, Tat vs. Tat + ETOH p=<0.05. GFAP+ populations showed significant increase in the presence of Tat and ETOH (df_{group} 3, df_{error} 44 f=8.257 p=1.81e-4). We saw specific significant interactions as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + ETOH p=<0.05. CD25+ showed a non-significant change in the populations in the presence of Tat and EtOH. CD28+ populations showed significant decreases levels in the presence of Tat and ETOH (df_{group} 3, df_{error} 44 f=8.552 p=1.38e-4). We saw specific interactions for this population and those interactions are as follows: Ctrl vs. Tat + ETOH p=<0.05, ETOH vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05. CD195+ populations showed significant decreases in the population due to presence of ETOH and Tat (df_{group} 3, df_{error} 44 f=3.013 p=0.04). We saw specific interaction for this population and those interactions are as follows: ETOH vs. Tat + ETOH p=<0.05. IFNγ populations showed a non-significant decrease in the population in the presence of ETOH and Tat.

We investigated changes in cellular population shifts due to the addition of ETOH and Tat in the female groups. The CD4+ populations showed significant decreases in the ETOH and the Tat + ETOH (df_{group} 3, df_{error} 44 f=18.944 p=4.90E-8). We saw specific interactions with this marker, and they are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05.

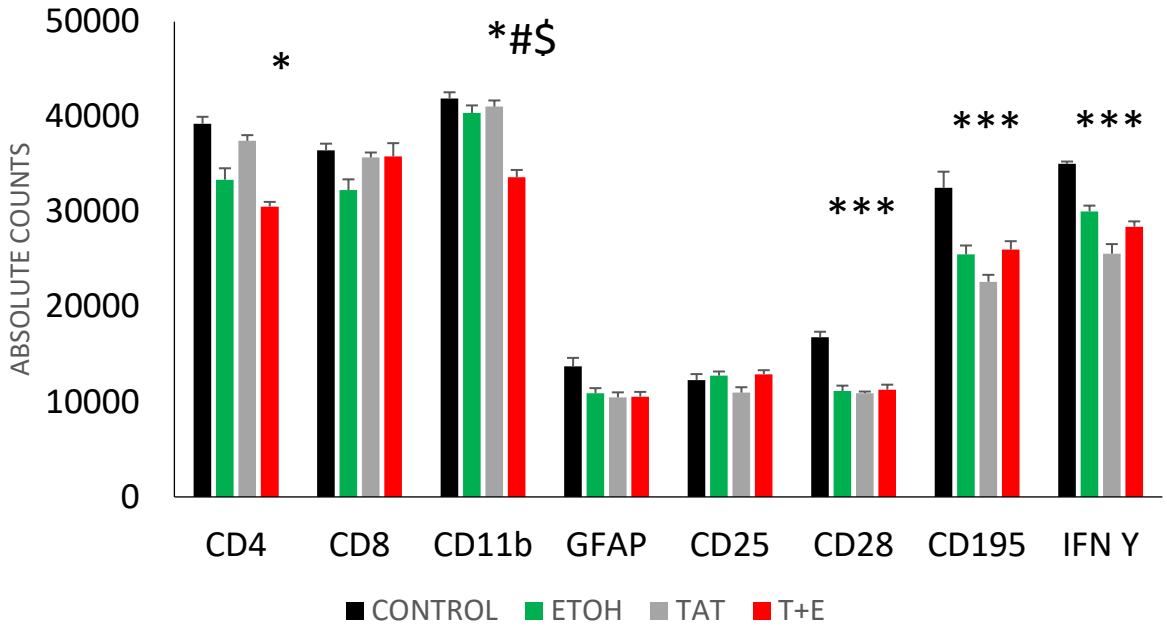


Figure 3.21 – Population shift of females in the presence of alcohol (ETOH) and Tat. The responses that were observed were varied and showed trend toward toxic environment formation. The * represents compared to control. The # represents compared to Tat. The $ represents compared to ETOH. Significance was set at p<0.05.

The CD8+ showed non-significant changes in population due to addition of ETOH and Tat. CD11b+ populations show significant decreases in the presence of both Tat and ETOH (df_{group} 3, df_{error} 44 f=23.744 p=2.71e-9). We saw specific significant interactions and they are as follows: Ctrl vs. Tat + ETOH p=<0.05, Tat vs. Tat + ETOH p=<0.05, and ETOH vs. Tat + ETOH p=<0.05. GFAP+ populations showed non-significant decreases in the presence of Tat and ETOH. CD25+ showed a non-significant change in the populations in the presence of Tat and ETOH.

CD28+ populations showed significant decreases levels in the presence of Tat and ETOH (df_{group} 3, df_{error} 44 f=8.763 p=1.14e-4). We saw specific interactions for this population and those interactions are as follows: Ctrl vs. ETOH p=8.18e-4m Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + ETOH p=<0.05. CD195+ populations showed significant decreases in the population due to presence of ETOH and Tat (df_{group} 3, df_{error} 44 f=10.156 p=3.33e-5). We saw specific interaction for this population and those interactions are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + ETOH p=<0.05. IFNγ populations showed a significant decrease in the population in the presence of ETOH and Tat (df_{group} 3, df_{error} 44 f=12.393 p=5.25e-6). We saw specific interactions for this population and those interactions are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, and Ctrl vs. Tat + ETOH p=<0.05.

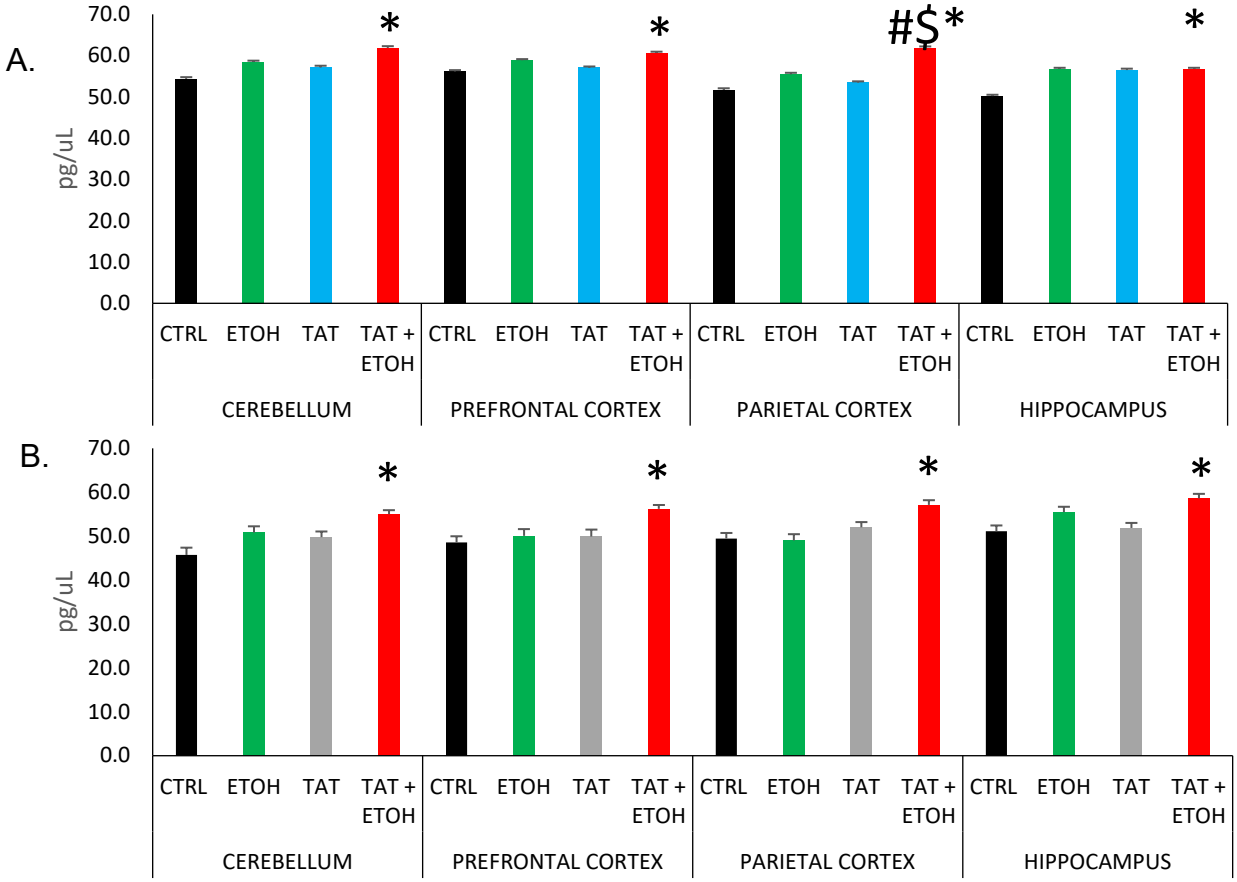
3.3.5. Ligand Changes with Alcohol and Tat Exposure

3.3.5.1 Effect of Alcohol and Tat on Ligand Constitution

We were interested in how both alcohol (ETOH), and Tat worked to either cause increases in decreases in ligand (cytokine) constitution. We are also interested in how these changes are affected with respect to sex difference with exposure to Tat and ETOH. We have evaluated each cytokine levels for each brain region.

We first investigated the changes in levels of **MCP-1**. MCP-1 is known to be a co-receptor for entry into a host cell by HIV-1. MCP-1 for males shows significant increase in the combo group (Tat + ETOH) (df_{group} 3, df_{error} 144 f=3.110 p=0.028). The specific significant comparison of control vs. Tat + ETOH p=<0.05.

MCP-1 for the females show significant increases again in the combo group and in the parietal cortex (df_{group} 3, df_{error} 144 f=35.476 p=3.15e-17). We saw significant interactions for the females are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, ETOH vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05. We also investigated at how the cytokines performed by region in both sexes. In males, this cytokine performed at a non-significant level; while the females performed showing significant increase across all the regions (df_{group} 3, df_{region} 144 p=4.10e-6). We saw significant specific interactions when looking at the increases in some regions. In the females these values are as follows: parietal cortex p=<0.05 and hippocampus p=<0.05. We also looked at sex difference between males and females. The MCP-1 levels showed significant sex difference (df_{group} 7, df_{error} 288 f= 7.739 p=1.40e-8). We did not see any specific sex difference interactions as pertain to the groups. When looking at the regions,



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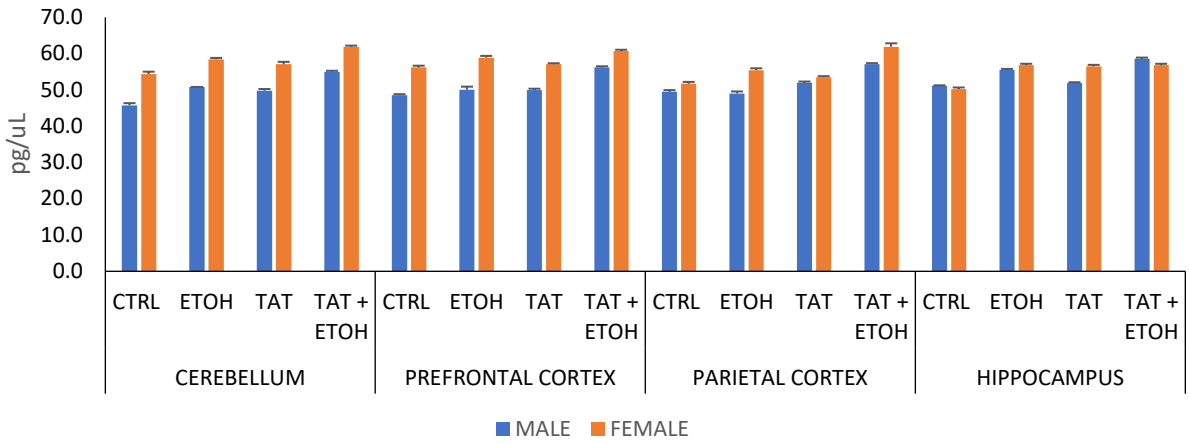
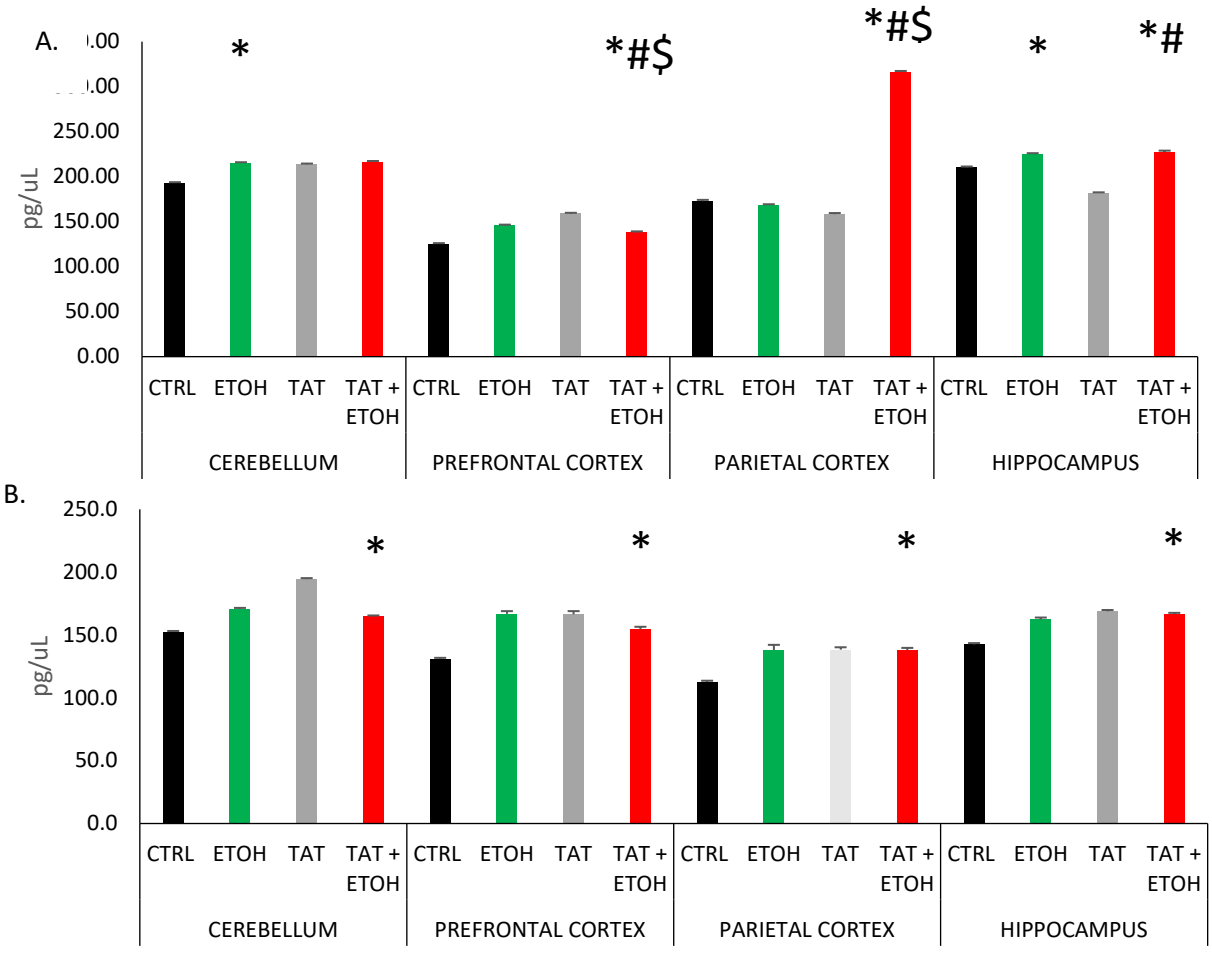


Figure 3.22 – Levels of MCP-1. A. Male MCP-1 levels. B. Female MCP-1 levels. C. MCP-1 levels by sex. The levels of MCP-1 show increases in both sexes. There was no significant sex difference detected. The * represents compared to control. The # represents compared to Tat. The $ represents compared to ETOH. Significance was set at $p<0.05$.

we did not see significant sex differences, or any specific interactions as pertains to regions.

Next, we looked at the levels of **IL-1α**. When looking at the levels of IL-1α, we saw significant changes in the males for the level of IL-1α (df_{group} 3, df_{error} 144 f=235.94 p=2.30e-55) and significant difference in the level of regions IL-1α (df_{region} 3, df_{error} f=509.39 p=1.95e-76). The levels of IL-1α showed significant specific interactions and are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, ETOH vs. Tat + ETOH p=<0.05, and Tat + Tat + ETOH p=<0.05. The levels of IL-1α showed significant specific interactions in the following regions: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=0.004. In the females, the levels of IL-1α showed significant differences (df_{group} 3, df_{error} 144 f=28.025 p=2.47e-14) and significant difference were seen when looking at the regions (df_{region} 3, df_{error} 144 f=39.441 p=1.15e-18). The females showed specific interactions for the levels of IL-1α are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs.

Tat + ETOH p=<0.05. The females showed specific interactions with the females when looking at the regions are as follows: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05. The levels of IL-1α showed sex difference in the groups (df_{group} 3, df_{error} 288 f=151.31 p=1.39e-92) and regions ($df_{regions}$ 3, df_{error} f=160.58 p=3.56e-61). The levels of IL-1α showed specific interactions as pertains to sex difference for the groups are as follows: ETOH-M vs. ETOH-F p=<0.05, Tat-M vs. Tat-F p=<0.05, and Tat + ETOH-M vs. Tat + ETOH-F p=<0.05. The level showed IL-1α showed specific interactions as pertains to sex difference for the regions are as follows: cerebellum p=<0.05, parietal cortex p=<0.05, prefrontal cortex p=<0.05, and hippocampus p=<0.05.



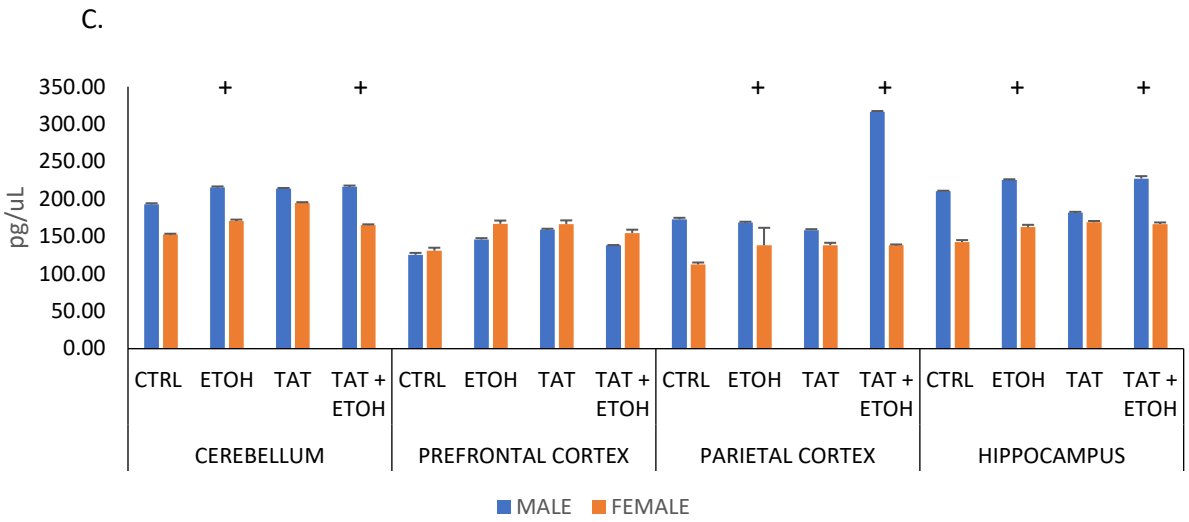


Figure 3.23 – Levels of IL-1α. A. Male levels IL-1α. B. Female levels IL-1α. C. IL-1α levels by sex. The levels of IL-1α show increases in both sexes. There was significant sex difference detected in the levels of IL-1α. The * represents compared to control. The # represents compared to Tat. The $ represents compared to ETOH. The + represents significant sex difference. Significance was set at $p<0.05$.

The next cytokine we looked at is **IL-6**. IL-6 is a potent inflammatory signaling cytokine, especially relating to most inflammatory processes. The levels of IL-6 in the males showed significant changes in the groups (df_{group} 3, df_{error} 144 f=16.54 p=2.74e-9) and in the regions (df_{region} 3, df_{error} 144 f=55.72 p=5.71e-24). The levels of IL-6 showed specific interactions pertaining to the groups themselves are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05. The levels of IL-6 showed significant changes in the regions and those are as follows: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05. The female levels of IL-6 showed significant differences in the groups (df_{group} 3, df_{error} 144 f=39.09 p=1.52e-18) and region (df_{region} 3, df_{error} 144 f=138.15 p=3.46e-42). The levels of IL-6 showed

significant interactions and those are as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs.

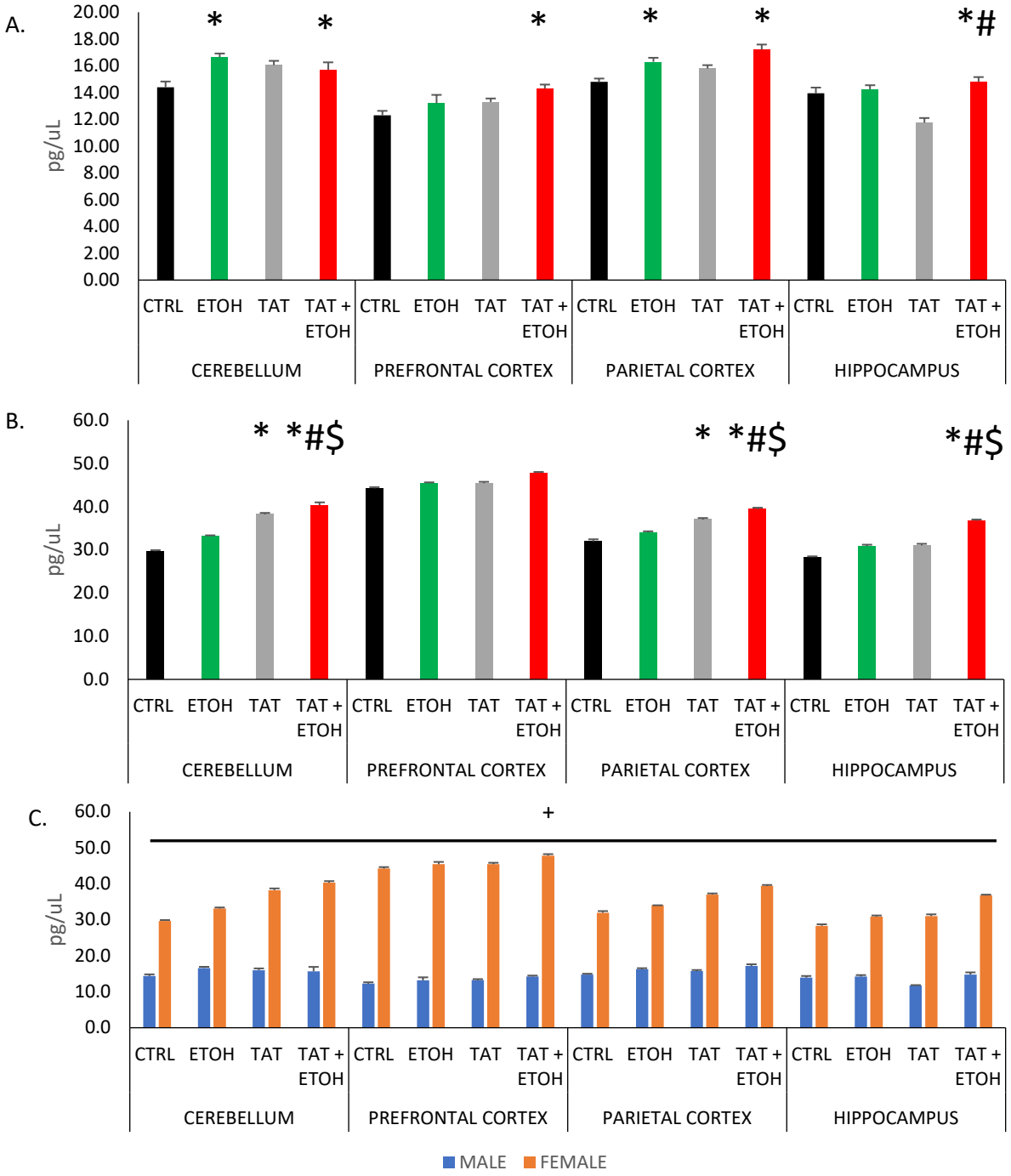


Figure 3.24 – Levels of IL-6. A. Male levels of IL-6. B. Female levels of IL-6. IL-6 levels. The levels of IL-6 by sex increases with exposures to both Tat and ETOH. There was significant sex difference detected across all areas of the brain. The * represents compared to control. The # represents to Tat. The $ represents compared to ETOH. The + represents significant sex difference. Significance was set at p<0.05.

Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, ETOH vs. Tat + ETOH p=<0.05, and

Tat vs. Tat + ETOH p=<0.05. The levels of IL-6 showed significant changes in each region: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, hippocampus p=<0.05. Looking at the sex difference between groups and regions, there is significant difference between group (df_{group} 3, df_{error} 288 f=68.18 p=1.83e-57) and region (df_{region} 3, df_{error} 288 f= 56.35 p=1.09e-28). The specific significant sex differences between groups are as follows: ETOH-M vs. ETOH-F p=<0.05, Tat-M vs. Tat-F p=<0.05, and Tat + ETOH-M vs. Tat + ETOH-F p=<0.05. The specific significant sex differences between regions are as follows: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05.

The next cytokine we examined was **TNF-α**. The males exhibited significant levels of TNF-α in groups (df_{group} 3, df_{error} 144 f=3.55 p=0.016). The levels of TNFα in the brain regions of the males did not show significant differences. The males showed specific interactions pertaining to the comparison of the groups and they are as follows: Ctrl vs. Tat + EtOH p=<0.05. The females showed significant changes in levels of TNF-α in groups (df_{group} 3, df_{error} 144 f=5.394 p=1.51e-3) and brain regions (df_{region} 3, df_{error} 144 f=16.81 p=2.05e-9). The groups showed specific significant interactions for comparisons of the groups and those are as follows: Ctrl vs. Tat p=<0.05 and Tat vs. Tat + ETOH p=<0.05. The brain regions saw significant differences in the levels of TNF-α and they are as follows: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05. The groups in both male and females showed sex difference in both groups (df_{group} 7, df_{error} 288 f=3.968 p=3.70e-4) and regions (df_{region} 3, df_{error} 288

f=3.134 p=2.59e-2). The groups showed no specific significant interactions when it came to comparing the groups for sex difference; while, the brain regions showed specfic significant interactions when comparing the groups for sex difference and

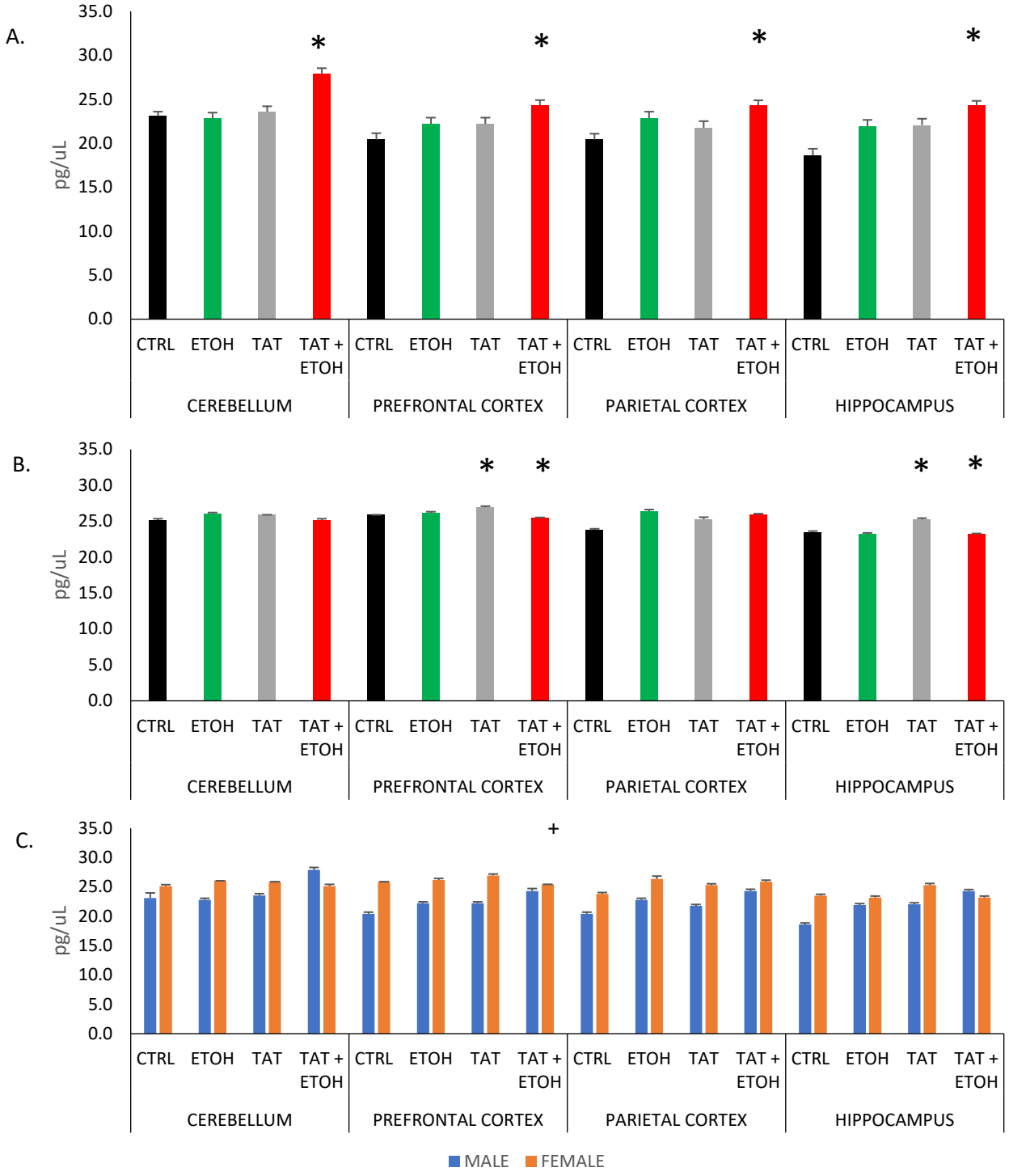


Figure 3.25 – Levels of TNFα. A. Male levels of TNFα. B. Female levels of TNFα. C. Level of TNFα by sex. The levels of TNFα by sex increases with exposures to both Tat and ETOH. There was significant sex difference detected across all areas of the brain. The * represents compared to control. The # represents to Tat. The $ represents compared to ETOH. The + represents significant sex difference. Significance was set at p<0.05.

they are as follows: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex

p=<0.05, and hippocampus p=<0.05.

The next cytokine we analyzed was **IFNγ**. The males exhibited significant levels of IFNγ when comparing the groups (df_{group} 3, df_{error} 144 f=3.612 p=0.003). and the males exhibited significant levels of IFNγ in the different brain regions (df_{region} 3, df_{error} 144 f=4.815 p=0.003). The males showed specific interaction in the groups only in the comparison: Ctrl vs. Tat + ETOH p=<0.05. The males showed specific interactions only in the parietal cortex, p=<0.05. The females exhibites significant changes in both group (df_{group} 3, df_{error} 144 f=51.198 p=1.39e-22) and region (df_{region} 3, df_{error} 144 f=16.805 p=4.07e-76). The females showed specific significant interaction and those are noted as follows: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, and p=<0.05. The females showed specific significant interaction for the regions and those are noted as follows: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05. We next looked at sex difference as related to groups (df_{group} 3, df_{error} 288 f=9.57 p=1.06e-10) and regions (dfregion 3, dferror 288 f=40.93 p=4.64e-22), which showed significant interactions. The groups showed significant interaction and those are shown: ETOH-M vs. ETOH-F p=<0.05 and Tat-M vs. Tat-F p=<0.05. The regions showed specific significant interactions and those are shown below: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05.

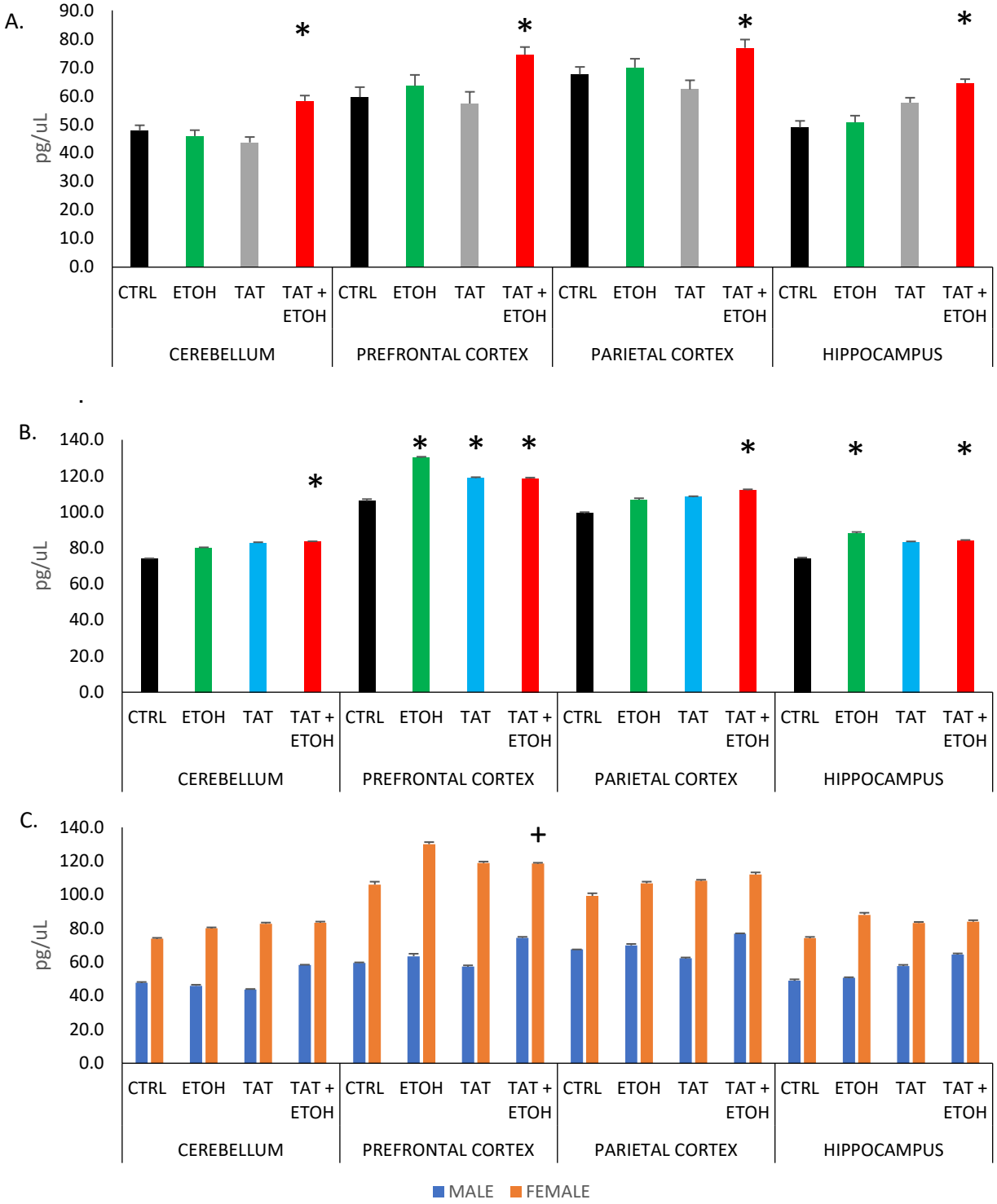


Figure 3.26 – Levels of IFNγ. A. Male IFNγ level. B. Female IFNγ level. C. Sex based levels of IFNγ. The levels of IFNγ show increases by sex. There is significant sex difference being observed across the brain. The * represents compared to control. The + represents significant sex difference. Significance was set at $p<0.05$.

Next, we analyzed the levels of **IL-1β** that are affected by presence of Tat and EtOH. The males showed significant interactions in the amount of IL-1β in the tissue pertaining to groups (df_{group} 3, df_{error} 144 f=12.334 p=3.15e-7) and regions (df_{region} 3, df_{error} 144 f=15.24 p=1.16e-8). The males showed specific interactions in the groups and those interactions are shown below: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05. The brain regions showed specific interactions and those are shown below: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05. The females showed significant specific interactions with presence of IL-1β in the tissue for groups (df_{group} 3, df_{error} 144 f=43.93 p=3.22e-20) and regions (df_{group} 3, df_{error} 144 f=83.75 p=2.06e-31). The females showed significant specific interactions with respect to group comparisons and those are shown below: Ctrl vs. ETOH p=<0.05, Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, and Tat vs. Tat + ETOH p=<0.05. The females showed significant specific interactions with respect to brain regions and those are shown below: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05. We next looked at whether sex difference exists between groups and brain regions. Significant sex difference was observed in both groups (df_{group} 3, df_{error} 288 f=21.46 p=3.06e-23) and regions (df_{region} 3, df_{error} 288 f=36.58 p=4.63e-20). The groups showed significant sex difference and those comparisons are showed below: ETOH-M vs. ETOH-F p=<0.05, and Tat + ETOH-M vs. Tat + ETOH-F p=<0.05. The brain regions showed specific significant intercations with respect to sex

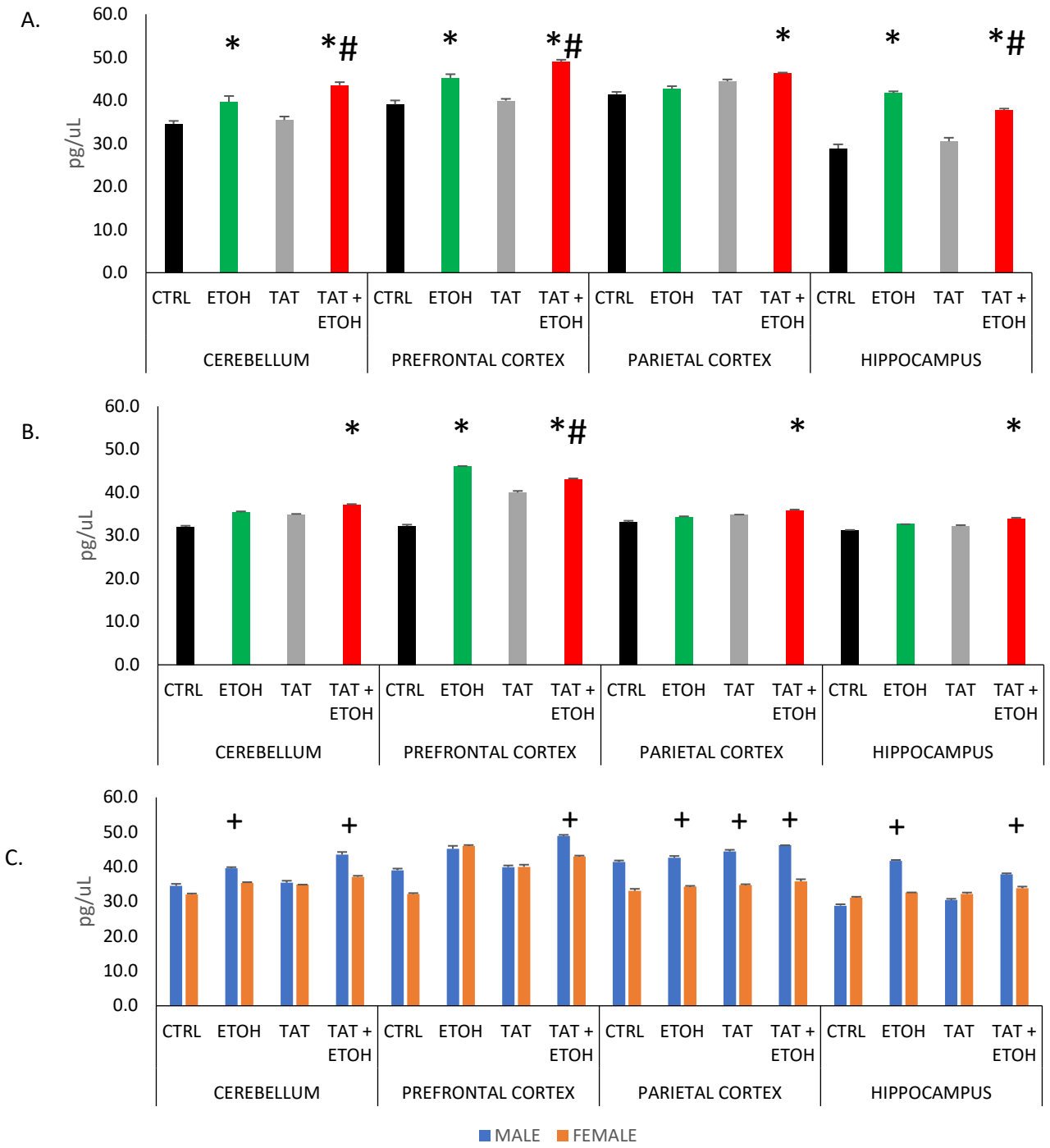


Figure 3.27 – Levels of IL-1β. A. Male levels of IL-1β. B. Female levels of IL-1β. C. Levels of IL-1β by sex. The levels of IL-1β show increases across the brain. There is significant sex difference seen in all areas of the brain. The * represents compared to control. The # represents compared to Tat. The $ represents compared to ETOH. The + represents significant sex difference. Significance was set at p<0.05.

difference and those are shown below: cerebellum p=<0.05, prefrontal cortex

p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05.

Lastly, we anaylzed the levels of **IL-12** in the tissue. The males showed significant interactions pertaining to groups (df_{group} 3, df_{error} 144 f=12.98 p=1.49e-7), but not brain regions which were non-significant. The groups showed specific significant interactions and those are listed below: Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + ETOH p=<0.05, Tat vs. Tat + ETOH p=<0.05, and EtOH vs. Tat + ETOH p=<0.05. The females showed significant interactions with respect to group (df_{group} 3, df_{error} 144 f=10.54 p=2.60e-6) and brain regions (df_{region} 3, df_{error} 144 f=41.47 p=2.23e-19). The groups showed specific significant interactions as pertaining to group and those interactions are shown below: Ctrl vs. Tat + ETOH p=<0.05, ETOH vs. Tat + ETOH p=<0.05, Tat vs. Tat + ETOH p=<0.05. The brain regions showed specific significant interactions and those are shown below: cerebellum p=<0.05, prefrontal cortex p=<0.05, parietal cortex p=<0.05, and hippocampus p=<0.05. We looked at significant interactions as pertaining to sex difference. We saw significant interactions in sex difference as pertaining to groups (df_{group} 3, df_{error} 288 f=56.61 p=1.52e-50) and brain regions (df_{region} 3, df_{error} 288 f=3.210 p=2.34e-2). The groups showed specific significant interactions and those are shown below: ETOH-M vs. ETOH-F p=<0.05, Tat-M vs. Tat-F p=<0.05, and Tat + ETOH-M vs. Tat + ETOH-F p=<0.05. The brain regions showed significant specific interactions and those are shown below: cerebellum p=<0.05, prefrontal cortex p=<0.05, and parietal cortex p=<0.05.

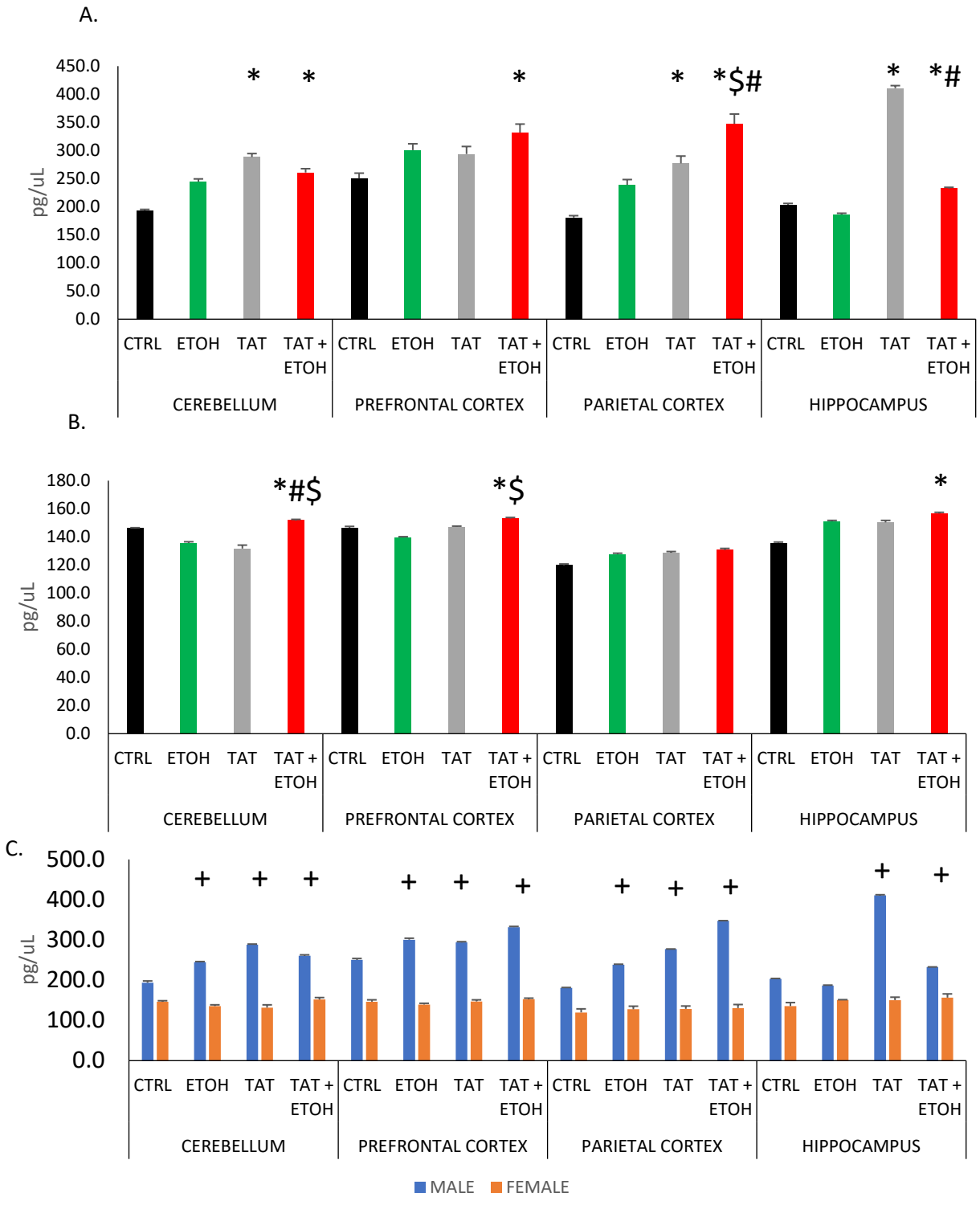


Figure 3.29 – Levels of IL-12. A. Male levels of IL-12. B. Female levels of IL-12. C. Levels of IL-12 by sex. The levels of IL-12 show increases across the brain. There is significant sex difference seen in all areas of the brain. The * represents compared to control. The # represents compared to Tat. The $ represents compared to ETOH. The + represents significant sex difference. Significance was set at $p<0.05$

3.4 Discussion

We have completed a study regarding the combined effects of alcohol and Tat that shows a predominatly synergistic effect between these agents, and further shows significant differences in behavioral parameters and, protein markers; as well as sex differences within treatment classes. These changes are reflected especially in the Barnes maze, water maze, flow cytometry, and ELISA assay. While previous literature has displayed some of these differences in behavior and within the *in vitro* assays, the current study is the first to examine these agents together using a rodent model designed specifically to allow us to study both behavioral and protein expression differences in long-term alcohol exposure that mimics chronic human drinking.

The treatment method that we have selected for this study simulates social drinking behavior over the adult lifetime of legal drinking age to later on in adulthood (age 21 to 60s) (Dutta and Sengupta 2016). There are subtle differences between binges and chronic treatment. Our exposure protocol replicates the difference that are seen in chronic drinking models. The liquid diet has trouble with administration of alcohol to the animal in that the amount of alcohol is not truly known because we don't know how much is being consumed by individual animals. The gastric gavage that we are using is the ability to give individualized doses that are adjusted to animal weights every week. Our methods reflect human consumption and is beneficial to understanding the effect of alcohol and Tat exposure.

Alcohol has also been documented to show modification change in behavioral testing, cytokine, and receptor modifications (Crews, et al. 2006; Crews, et al. 2017; Erickson, et al. 2019; Flora, et al. 2005; Harper 2007; Hauser and Knapp 2014; Hidalgo, Atluri, and Nair 2015; Kelley and Dantzer 2011; Maubert et al. 2015; Neupane, et al. 2016). The changes that are seen with alcohol in the presence of Tat are important because of HIV infected individuals have attendency to take part in substance abuse. This can complicate testing, treatment, and a variety of other concerns for these indivduals that might make the disease worse (Pandrea, et al. 2010). The addition of using Tat transgenic animals and administering alcohol shows synergistic effect in both behavioral assays and in vitro testing (flow cytometry and ELISA). These two agents do work together to cause drug effects that are uniform between the two sexes as well as in the different regions of the brain.

This study, looked at how the component of HIV-1, Tat, causes neurodeficit. This study has shown that Tat causes a decrease in motor coordination and gait, and both working memory and spatial learning and memory (Ajasin and Eugenin 2020; Carey et al. 2012; Jadhav and Nema 2021; Jaeger and Nath 2012; Kaul, et al. 2005; Rice 2017). Tat has been known to cause neurodeficit with destruction of cells releasing cofactors and other debris that are known to be toxic and can cause cell death. Tat has also been well documented in the literature and is known to cause receptor and ligand modifications. Expression of cytokines (e.g. IL-6) have been known to vary previously (Ajasin and Eugenin 2020; Bagashev and Sawaya 2013; Carey, et al. 2012; Jadhav and Nema

2021; Jaeger and Nath 2012; Kaul, et al. 2005; Rice 2017). However, the present study clearly examines Tat expression in all rain regions with concomittant long-term EtOH use, and this study documented modification of cytokines in these brain regions and noted sex differences that were no previously described..

We have looked for literature that describes the shared effects of Tat and alcohol to examine the combined drug (or agent) effects. It is important to understand how disease agents and drugs work together in an organism in order to ascertain their ability to cause protective effects, no effects, or detrimental effects. It has been documented in the literature that HIV-1 Tat can interact with a variety of agents, including alcohol, to cause toxic effects on the neural environment (Ajasin and Eugenin 2020; Bagashev and Sawaya 2013; Carey, et al. 2012; Jadhav and Nema 2021; Jaeger and Nath 2012; Kaul, et al. 2005; Rice 2017). The selection of these markers and cytokines were made because of the fact that these are common to alcohol use disorder and HIV.

The fact that the receptors show down regulation in the presence of alcohol and Tat demonstrate that there is marked shift toward a toxic environment forming and has the ability to perpetuate disease and other concerns. The fact that the cytokines show up regulation stipulate that the formation of the toxic envronment is ensured because of the down regulation of the receptors which will perpertuate the disease processes and further toxicity occurring. The other fact of these findings when dealing with the receptors and cytokines is that these effects are no uniform across both sexes, also which was unexpected, and has not been noted in the literature. These findings further make the case that alcohol and Tat cause

formation of toxic environments. The present study clearly shows that HIV-1 Tat and alcohol either work together synergistically or work by additive effect. In the *in vitro* testing, we see detrimental effects across a variety of brain regions, and within measured behaviors.

Our behavioral assays are selected and are scaled to reflect methods performed in literature for alcohol. The roto-rod (motor coordination and gait), novel object recognition (NORT) (working memory), Barnes maze (spatial learning and memory), and Morris water maze (spatial learning and memory) are all designed to and measure of various brain functions (Antunes and Biala 2012; Attar, et al. 2013; Huang and Hsueh 2014; Vorhees and Williams 2006). Our assays show that neurodeficit is present with alcohol and Tat treatment, and these agents are working together.

Overall, we have performed a study to show that these two agents (alcohol and Tat) are neurotoxic in two different ways. The first is that we have shown that together these agents cause modification of behavioral processes. This modification comes in three separate ways. The first is changes in motor coordination and gait. The second is loss of ability to recognize objects and showing anxiety to new surroundings. The third and last behavioral modification is changes in the ability to learn spatially and changes of how systematic an individual is about learning. The second way we have been able to show neurotoxic effect in this study is that we have demonstrable cellular and receptor modification. This modification in our study shows to be synergistic or additive in effect. We also see some instances of antagonistic effect being present.

This study has shown that 1. that these two agents will work together to change behavioral outcomes and will cause significant neurodeficit, and 2. that these two agents will work together to cause significant changes in receptor and ligand constitution to perpetuate that neurodeficit. On balance, the interaction of these agents is synergistic

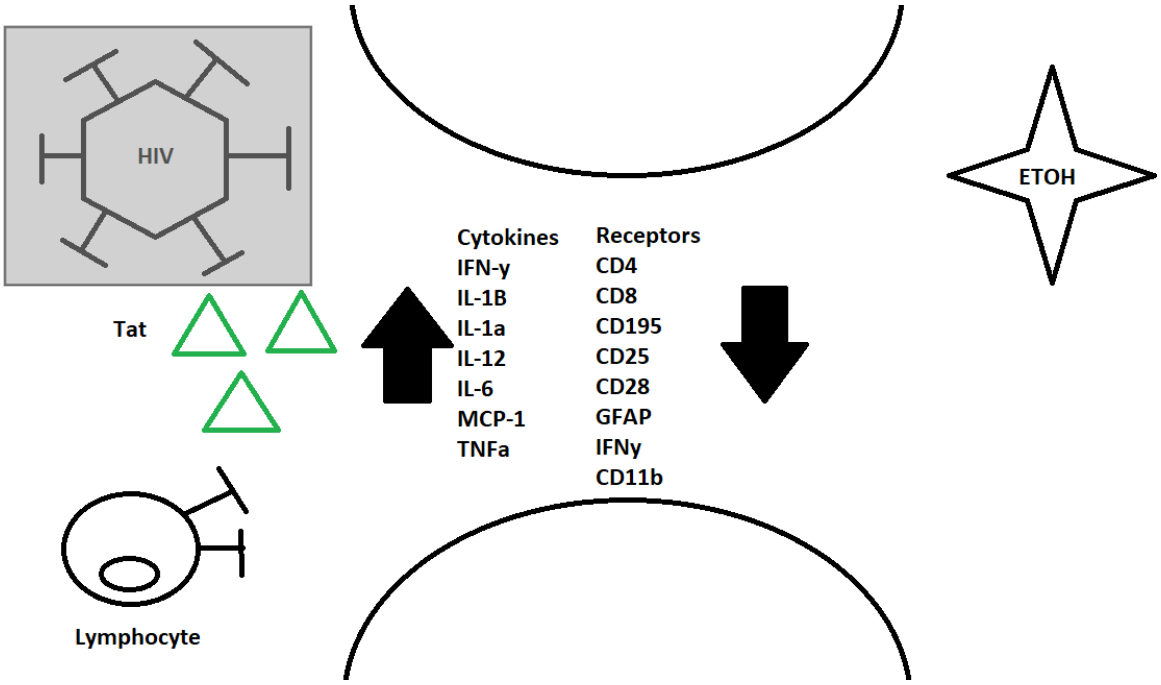


Figure 3.29 – Summary Figure for Specific Aim 2. There are significant changes in the receptors and cytokines in the presence of Tat and ETOH.

CHAPTER 4
PPAR γ AGONIST HELPS AMELIORATE HIV-1 TAT INDUCED NEURODEFICIT

4.1 Introduction

Human Immunodeficiency Virus-1 (HIV-1) remains a constant problem globally and in our society. Globally, there are 37.7 million people that live with HIV with 36 million adults and 1.7 million children ("Global Statistics"). HIV-1 infection has been known to lead to other diseases and opportunistic infections. Infection with HIV-1 has been known to cause impaired judgement, delays in testing, and compromise of other body functions (Fauci 2003; Killian and Levy 2011).

It has been documented that different components of the HIV virus cause toxicity inside the neural environment (Kaul, et al. 2005; Rice 2017; Ajasin and Eugenin 2020; Porter and Sutliff 2012; Mehrbod, et al. 2019; Avdoshina, Bachis, and Mocchetti 2013; Fitting, et al. 2013). Tat and gp120 are documented in the literature to cause neurotoxicity, and among other things such as cell death, oxidative stress, and a decrease in synaptic plasticity (Atluri, et al. 2015; Bagashev and Sawaya 2013; Green et al. 2019; Hahn, et al. 2015; Jadhav and Nema 2021; Rice 2017). Tat, which stands for trans activator of transcription, is a co-factor that is known to control the transcription and enhance the replication process of the HIV virus (Ajasin and Eugenin 2020; Bagashev and Sawaya 2013; Flora, et al. 2005; Jadhav and Nema 2021; Krogh, Green, and Thayer 2014; Marino, et al. 2020; Meyaard, et al. 1992; Rice 2017; Saro, et al. 2021). Tat itself can help the virus enter host cells and has been used for other applications to help facilitate

moving drugs and other compounds into cells (Becker-Hapak, McAllister, and Dowdy 2001).

Since the discovery of the HIV virus and the first care for human patients, treatment strategies have evolved. Infected individuals are now living longer than at the start of the HIV epidemic, and in some cases patients may live with disease for 30 years or even more (Fauci 2003; Killian and Levy 2011). Each treatment strategy and pharmacotherapy has their own draw backs, causing neurodeficit, cell death, and other issues (Shah et al. 2016; Behl, et al. 2021; Huang, et al. 2014; W. Huang et al. 2009; Wen Huang, et al. 2009; Huang, et al. 2015; Huang et al. 2008; Justin, et al. 2020; Labandeira, et al. 2022; Layrolle, Payoux, and Chavanas 2021; Ogura and Yamaguchi 2022; Omeragic, et al. 2017; Sagheddu, et al. 2021; Shou et al. 2022; Sola, et al. 2022; Sundararajan, et al. 2006; Tufano and Pinna 2020; Villapol 2018; Wallace 2022; Zamanian, et al. 2022). The use of other drugs (including recreational drugs like alcohol) can cause changes in how treatment strategies work but has had the consequence of leading to promiscuous behavior which has been a long issue of causing treatment strategies and regimens. This consequence has caused increases viral loads and decreased CD4+ counts which alters the course of the disease.

The literature suggests that treatment with peroxisome proliferator receptor γ (PPAR) drugs allow persons affected by neurocognitive disorders to show recovery of function (Behl, et al. 2021; Huang, et al. 2014; W. Huang, et al. 2009; Wen Huang, et al. 2009; Huang, et al. 2015; Huang, et al. 2008; Justin, et al. 2020; Labandeira, et al. 2022; Layrolle, Payoux, and Chavanas 2021; Ogura and

Yamaguchi 2022; Omeragic, et al. 2017; Sagheddu, et al. 2021; Shou, et al. 2022; Sola, et al. 2022; Sundararajan, et al. 2006; Tufano and Pinna 2020; Villapol 2018; Wallace 2022; Zamanian, et al. 2022). This has been reported in people affected by Alzheimer's disease. The PPAR family of receptors is known to be located primarily in the nucleus of cells, and are known to be in adipose tissue, macrophages, and in the large intestine. PPAR drugs also have action in various organs, such as the liver and muscle tissue (Bosse 2011; Katzung 2015).

We are using a drug out of this class called rosiglitazone (Rosi) (Avandia), which belongs to a specific class of diabetic drugs. This drug was discovered and marketed in 1999 by GlaxoSmithKline (Khalaf and Taegtmeyer 2012). Rosiglitazone specifically belongs to the class of drugs call thiazolidinedione drugs. These drugs typically work in the presence of any types of insulin. The benefits of taking these drugs are that is they help with decreasing lipid concentrations, increasing the ability of insulin to act, and increases insulin sensitivity (Bosse 2011; Katzung 2015). These drugs have been used to help with glycemic control in diabetics who have type II Diabetes Mellitus (Bosse 2011; Katzung 2015; Olefsky and Saltiel 2000; Company).

In this study, we are interested to see how these drugs/agents work antagonistically, synergistically, or by additive effect to elicit their effect. We are also interested as to what degree we see protection from neurotoxic effects with addition of rosiglitazone. We will examine the combined drug effect of TAT, alcohol, and rosiglitazone by performing behavioral testing, and examine changes in spatial learning and memory or changes in search strategies. We are also

interested in how these agents work to maintain or change receptor/ligand constitution of the subjects and affect the neurodeficit that is caused by cellular death. With the interests being noted the following question has been asked: Does the addition of Rosiglitazone to HIV-1 Tat exposed animals cause circumvention of neurodeficit with respect to spatial learning and memory, alterations in motor coordination, receptor constitution changes, and change in cytokine levels? The following hypothesis has been made to help answer this question: We hypothesize that the addition of rosiglitazone to HIV-1 Tat exposed animals will circumvent the neurodeficit and associated changes in spatial learning and memory, motor coordination, receptor constitution, and cytokines (Fig 4.1).

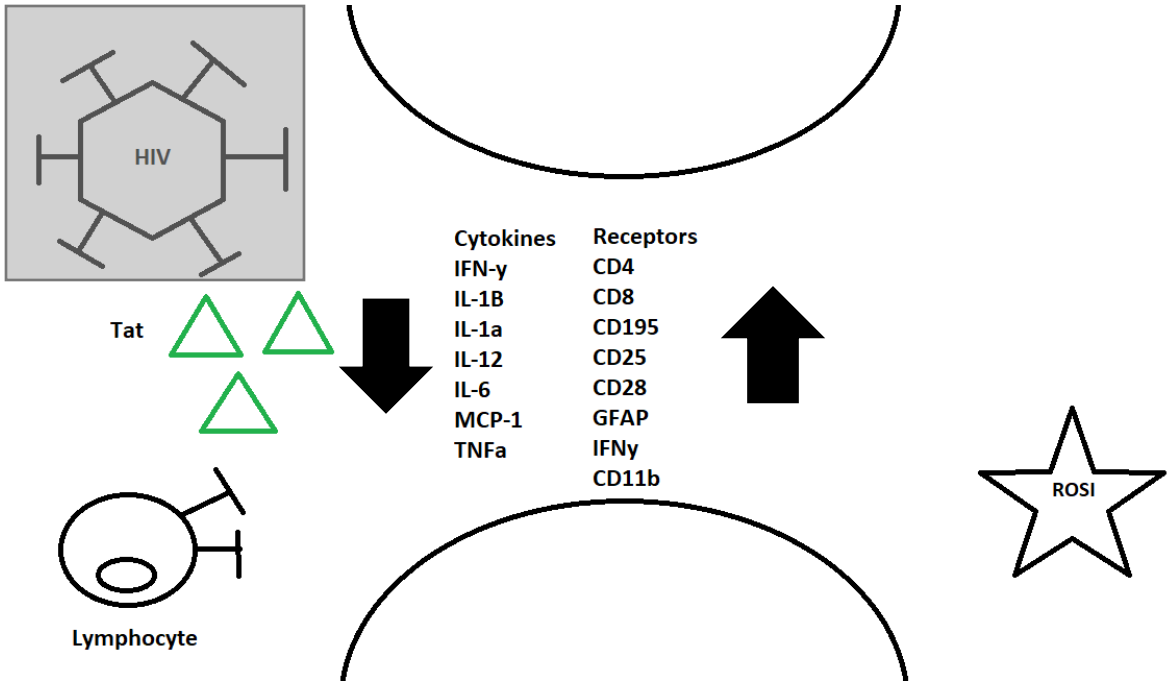


Figure 4.1 – Summary schematic hypothesis. The presence of ETOH, Tat, and Rosi might change the constitution of both the receptors and cytokines.

4.2 Materials and Methods

4.2.1 Animal Studies

4.2.1.1 Animals

Animals were obtained from the Jackson Laboratories and the colony were maintained inside the Laboratory Animal Research Core (LARC) facility. We used 8-week-old C57/BL6J mice, both male and female mice, equaling n=64. The study contained 4 groups per sex. The male groups were control n = 5, Rosi n = 10, Tat n = 5, and Tat + Rosi = 8 The female groups were control n=5, Rosi n= 13, Tat n=5, and Tat + Rosi = 13. Food and water were never restricted in this work. The mice were housed 3 to 5 animals to a cage inside the LARC facility. The animals were on a 6 am to 6 pm light and dark cycle (12-hour cycle). All behavioral testing was completed between the hours of 8 am to 5 pm till termination of the experiment. Animals were returned to the LARC at the end of the day after the experimentation was completed for the day. All procedures were approved and followed in accordance with the UMKC Institutional Animal Care and Use Committee.

4.2.1.2 Tat Induction

Tat transgenic animals were used in this study that were specifically bred with composition of Tat-tg. The animals were obtained from Dr. Kurt Hauser at Virginia Commonwealth University. The Tat-tg is placed under a *tet-on* inducible system and GFAP promoter (Nookala, et al. 2018). The use of these mice has presented many clinical findings of HIV-1 infection that occurs but not limited to changes blood cell population, neural cell apoptosis, astrocytosis, changes in gray matter, dendritic cellular degeneration, and inflammatory process (Jaeger and Nath 2012; Langford, et al. 2018; Nookala, et al. 2018; Sil, et al. 2021; Gorantla,

Poluektova, and Gendelman 2012). These animals are known to show differences in learning and memory. The animals were 8 weeks old at start of induction. The animals were fed a doxycycline (DOX) laced diet for a period of 4-week pretreatment at 6 g/kg of formulated animal chow. The diet was stored in the LARC and provided by staff (Nookala, et al. 2018).

4.2.1.3 Rosiglitazone Treatment

Animals were dosed with rosiglitazone morning and evening. The rosiglitazone was dissolved in dimethyl sulfoxide (DMSO) and housed in a 0.5% carboxymethylcellulose (CMC) solution (Company). The animals were weighed at the beginning of the experiment and weighted once a week till termination of the experiment. Weighing the animals every week allowed us to adjust the amount rosiglitazone the animals received at dosing. When giving the animals the assigned rosiglitazone dose, we followed the following dosing rules. 1. Animals that weighed between 22.5 grams to 27.5 grams, mean weight = 25 grams, were given 0.5 mg of rosiglitazone per day. 2. Animals that weighed between 27.6 grams to 32.5 grams, mean weight = 30 grams, were given 0.6 mg per day. These dosing rules continued the duration of the treatment period and the behavioral testing. The control and Tat animals were administered sterile 0.5% CMC solution at the same time that treated animals received doses. Below, is the experimental format for the treatment and the sequence of events.

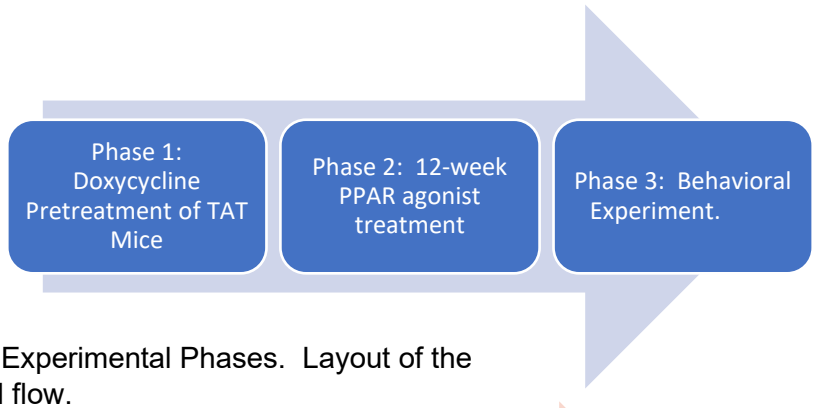


Figure 4.2 – Experimental Phases. Layout of the experimental flow.

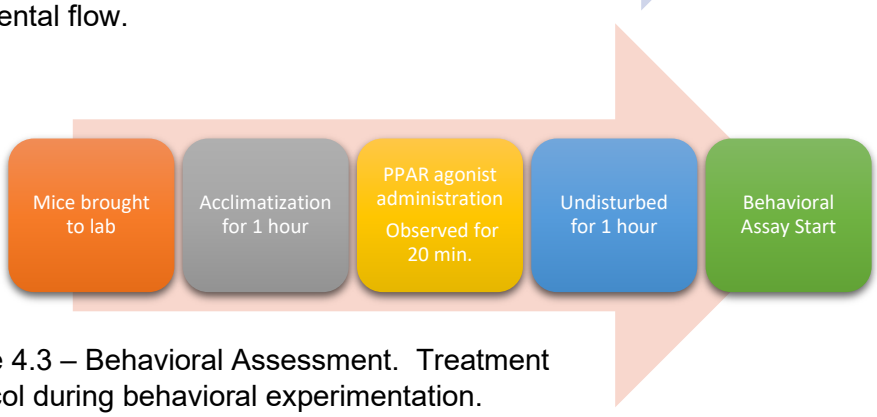


Figure 4.3 – Behavioral Assessment. Treatment protocol during behavioral experimentation.

4.2.1.4 Behavioral Testing

4.2.1.4.1 Rotor-Rod

The test was performed as described in the previous chapters.

4.2.1.4.2 Novel Object Recognition

The test was performed as described in the previous chapter.

4.2.1.4.3 Barnes Maze

The test was performed as described in the previous chapter.

4.2.1.4.4 Water Maze

The test was performed as described in the previous chapters.

4.2.1.4.5 Sample Collection

The procedure was performed as described in previous chapters.

4.2.1.4.6 Dissection:

The dissection procedure is performed as described in previous chapters.

4.2.2 In Vitro Diagnostic Testing (IVD) Assays

4.2.2.1 Whole Brain Digestion

The whole brain digestion procedure was performed as described in previous chapter.

4.2.2.2 Flow Cytometry

The flow cytometry procedure was performed as described in previous chapter.

4.2.2.3 Sample Preparation for the ELISA assay

The sample preparation procedure for the ELISA assay was performed as described in the previous chapter.

4.2.2.4 Performing ELISA assay

The ELISA was performed as described in the previous chapter.

4.2.4 Statistics

The statistical analysis was performed as described in the previous chapter.

4.3 Results

4.3.1 Evaluating Tat and Rosiglitazone Impact on Motor Coordination and Gait

The effects of Tat and Rosi on motor coordination and gait are evaluated using the roto-rod assay as described above. The males completed the assay showing significant interactions in both escape latency ($df_{escapelatency}$ 3, df_{error} 108 f=12.842 p=3.07e-7) and distance ($df_{distance}$ 3, df_{error} 108 f=10.285 p=5.00e-6). The males showed specific significant interactions in the following comparisons for escape latency: Ctrl vs. Tat p=<0.05 and Tat vs. Tat + Rosi p=<0.05. The males also showed specific interactions in the following comparisons for distance parameter: Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05.

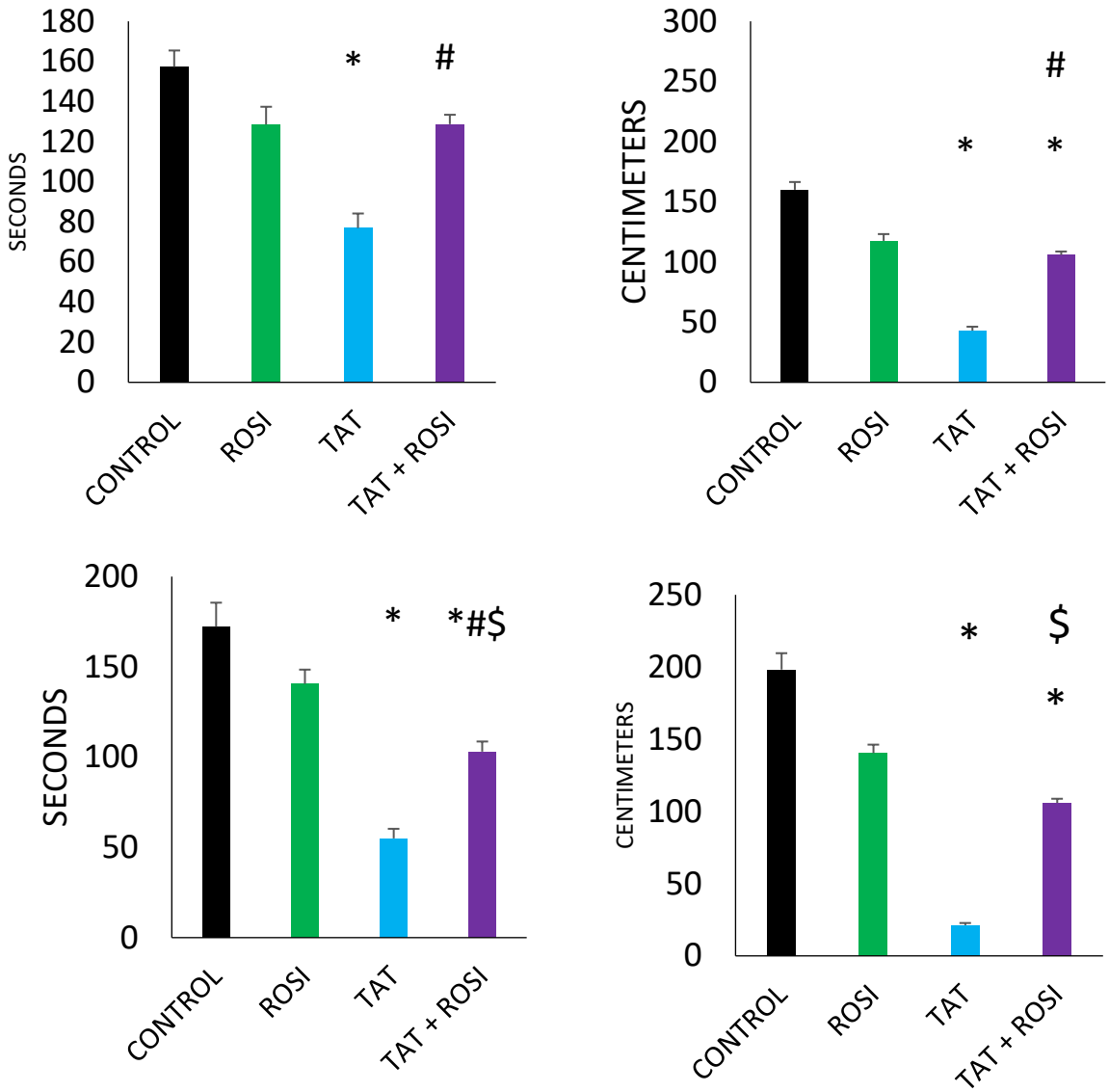


Figure 4.4 – Roto-rod male (top) and female (bottom). The escape latency (left) and distance traveled (right). In these figures, there is a recovery of motor function when rosiglitazone is used as treatment for the animals. The groups for the males were Ctrl n=5, Rosi n=10, Tat n=5, and Tat + Rosi n=10. The groups for the females were Ctrl n=5, Rosi n=13, Tat n=5, and Tat + Rosi n=13. The * represents compared to control. The # represents compared to Tat. The $ represents compared to Rosi. Significance was set at $p<0.05$.

We investigated the same effects in the females as well. The females completed the assay showing significant interactions in escape latency ($df_{escapelatency}$ 3, df_{error} 140 f=25.231 p=4.13e-13) and distance ($df_{distance}$ 3, df_{error} 140 f=18.805 p=2.63e-10). The females showed specific significant interaction in the

following comparisons for escape latency: Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + Rosi p=<0.05, Tat vs. Tat + Rosi p=<0.05, and Rosi vs. Tat + Rosi p=<0.05. The females showed specific significant interaction in the following comparisons for the distance parameter: Ctrl vs. Tat p=<0.05, Ctrl vs. Tat + Rosi p=<0.05, and Rosi vs. Tat + Rosi p=<0.05. We looked at how the sexes compared to each other throughout completion of this assay. We saw no significant sex difference interactions in both escape latency and distance traveled in the assay.

4.3.2 Effects Tat and Rosiglitazone on Working Memory

To evaluate the effectiveness of Rosi on the ability to help alleviate neurodeficit on working memory. To perform this evaluation, we performed the Novel Object Recognition Test (NORT). This test is commonly used to evaluate working memory and indirectly anxiety-like behavior. We measured several different parameters including time toward same object, time toward novel object, entries into novel zone, and entries into same zone. The animals showed non-significant interactions toward the same object. However, the animals showed significant interactions for the parameter of time toward novel object (df_{toward} 3, df_{error} 56 f=2.962 p=0.010). No specific significant interactions were noted using this parameter. The animals showed significant interactions with respect to parameter entries novel object zone ($df_{entriesnov}$ 3, df_{error} 56 f=5.026 p=1.82e-4). Specific interactions were noted as follows: Control – M vs. Tat – M p=4.54e-4 and Tat – M vs. Tat – F p=<0.05. The animals showed significant interactions

about the entry's same parameter ($df_{entriessame}$ 3, df_{error} 56 f=3.644 p=0.003). The animals showed specific significant interactions and are showed as follows: Control – M vs. Tat + Rosi -M p=<0.05, Rosi – M vs. Tat + Rosi – M

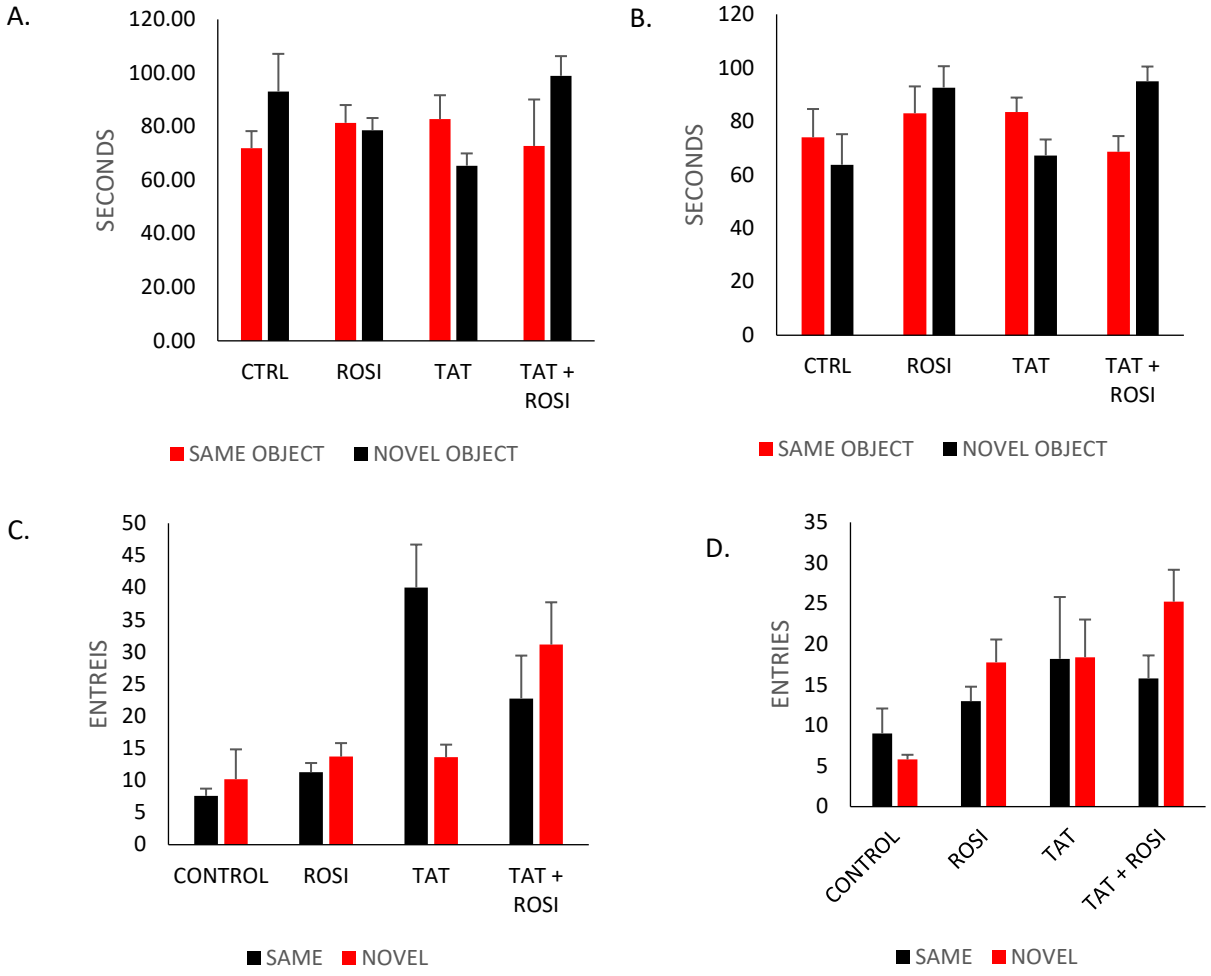


Figure 4.5 - Time toward object – Male (A) and Female (B). Entry zone of object – Male (C) and Female (D). The combination group (Tat + Rosi) shows increased time spent with the novel object, which also shows decrease in anxiety-like behavior. Significance was set at p<0.05.

p=<0.05, and Control – F vs. Tat + Rosi – F p=<0.05.

We also looked at the preference of the objects based upon the groups. The combo (Tat + Rosi) treatment groups show strong preference for the novel objects in the males at 32.98% vs. the same object at 24.25%, and the females showed the same preference at 31.66% vs. the same object at 22.86%. The Tat groups show strong preference for the same object with males showing at 27.59% vs. the novel object at 21.79%, and the females showed preference for the same object at 27.82% vs. the novel object at 22.41%.

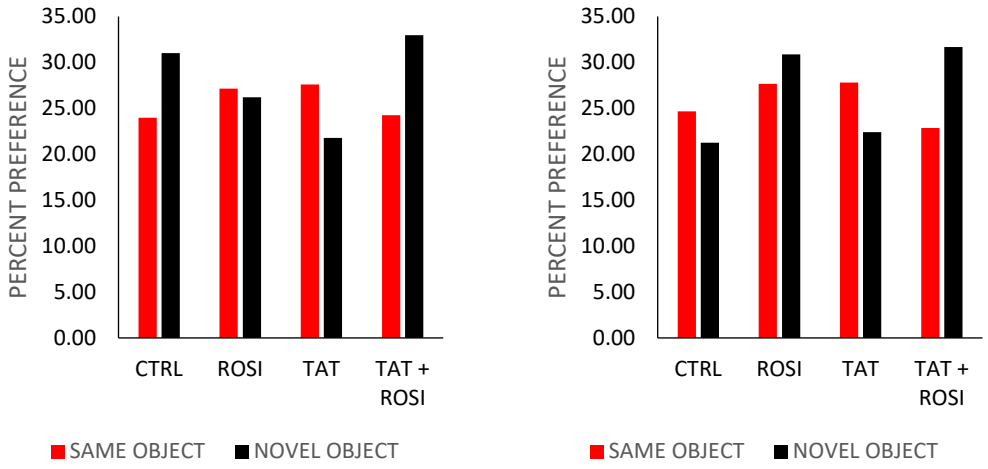


Figure 4.6 – Preference index male (left) and female (right). The combination group (Tat + Rosi) show increased time spent with the novel object (black bar), while the Tat group spent more time with the same object (red bar). This means their recovery of function pertaining to working memory. Significance was set at $p<0.05$.

4.3.3 Effects of Rosiglitazone and on Spatial Learning and Memory

We used two separate assays to evaluate the effect Tat and Rosi on spatial learning and memory. The assays that were used were the Barnes maze and the Morris water maze. We will first perform the evaluation of Tat and Rosi on spatial learning and memory by using the Barnes maze. In this assay, we evaluated four separate parameters to evaluate the changes in spatial learning and memory. The parameters that were measured are the entries, time, and distance traveled into

the southern region. We also looked at the search strategies to ascertain if memory was altered.

In the Barnes maze, the males showed significant interactions in entries ($df_{entries}$ 3, df_{error} 26 f=8.037 p=0.001) and time (df_{time} 3, df_{error} 26 f=27.60 p=8.44e-8). The males showed non-significant interactions with respect to distance parameter. The males showed no specific significant interactions in the entry parameter but showed specific interactions in the time parameter: Control – M vs. Rosi – M p=<0.05, Control – Male vs. Tat – M p=<0.05, Control – M vs. Tat +

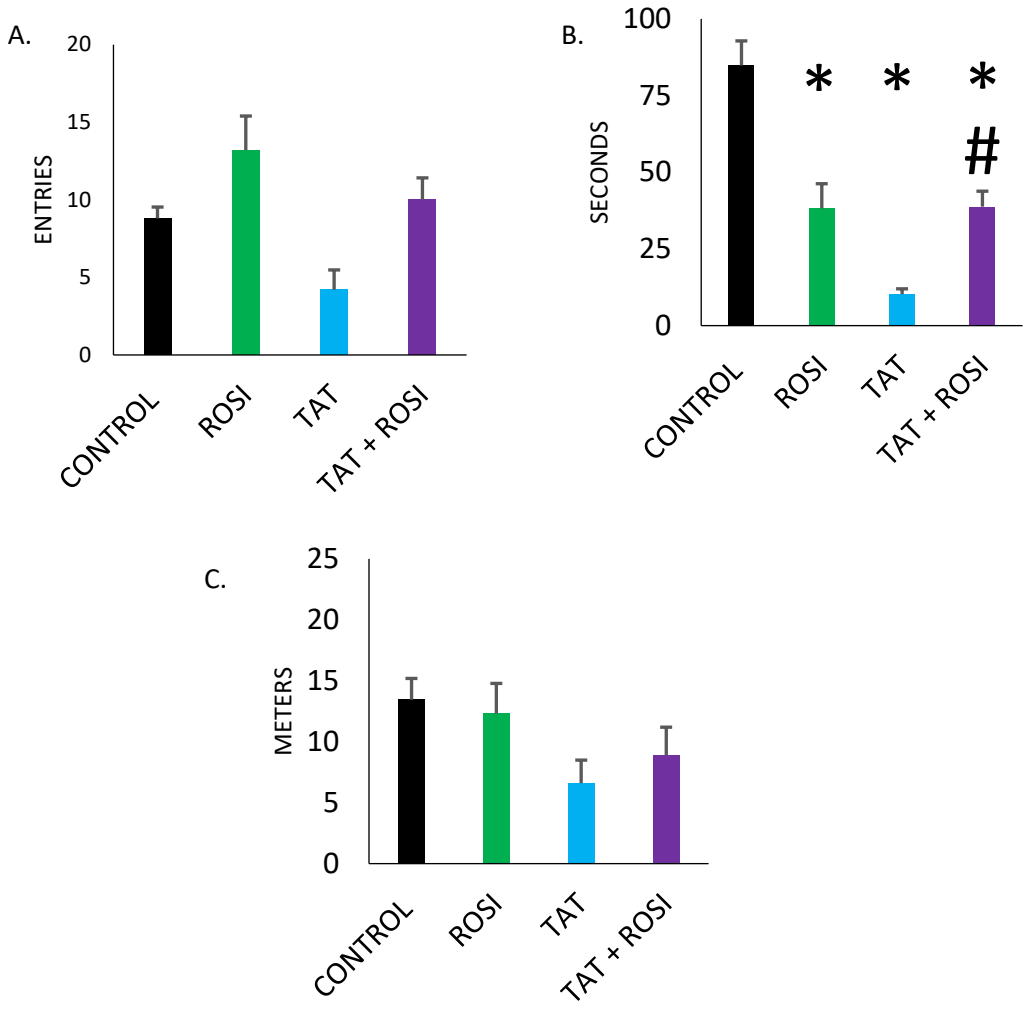


Figure 4.7 – Male parameters Entries (A), Time (B), and Distance (C). The males show recovery of function when treated with rosiglitazone. The male groups were Ctrl n= 5, Rosi n=10, Tat n=5, and Tat + Rosi n=8. The * represents compared to control. The # represents compared to Tat. Significance was set at $p<0.05$

Rosi – M $p=<0.05$, and Tat – M vs. Tat + Rosi – M $p=<0.05$.

The females performed in the Barnes maze showed significant interactions in all three parameters entries ($df_{entries}$ 3, df_{error} 34 f=7.179 p=0.001), time (df_{time} 3, df_{error} 34 f=4.815 p=0.007), and distance ($df_{distance}$ 3, df_{error} 34 f=7.176 p=0.001). The females showed specific interactions in the entry parameter: Control – F vs. Tat – F p=<0.05 and Rosi – F vs. Tat + Rosi – F p=<0.05. The females showed

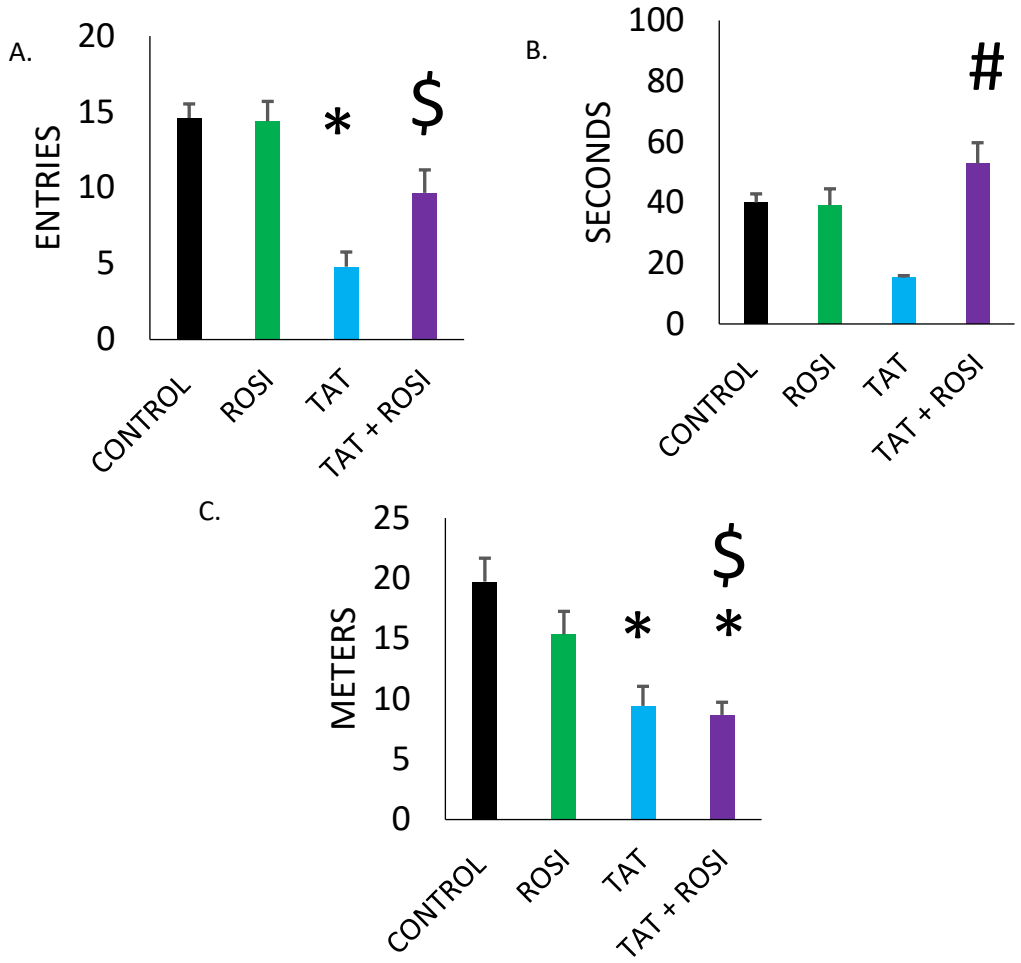


Figure 4.8 – Female parameters Entries (A), Time (B), and Distance (C). The females show recovery of function when treated with rosiglitazone. The female groups were Ctrl n=5, Rosi n=13, Tat n=5, and Tat + Rosi n=13. The * represents compared to control. The # represents compared to Tat. The $ represents compared to Rosi. Significance was set p<0.05.

specific interactions in the time parameter: Tat – F vs. Tat + Rosi – F p=<0.05. The females showed specific interaction in the distance parameter: Control – F vs. Tat – F p=<0.05, Control – F vs. Tat + Rosi – F p=<0.05, and Rosi – F vs. Tat + Rosi – F p=<0.05.

We next looked at how the animals performed according to sex difference. The animals showed significant interactions in all three parameters entries ($df_{entries}$ 3, df_{error} 54 f=13.459 p=1.00e-6), time (df_{time} 3, df_{error} 54 f=15.916 p=1.55e-7), and distance ($df_{distance}$ 3, df_{error} 54 f=8.632 p=8.90e-5). The animals showed no specific significant interactions when looking at each parameter as shown above.

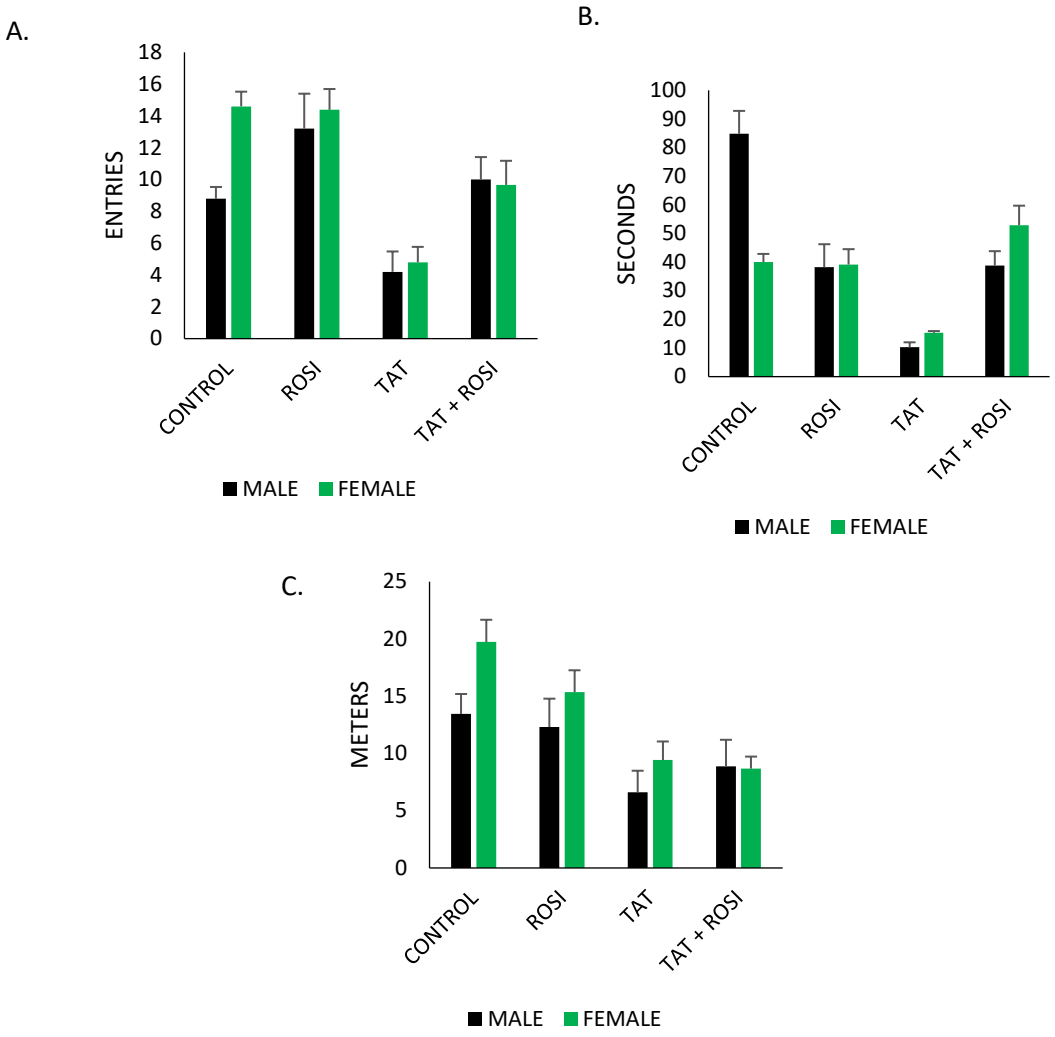
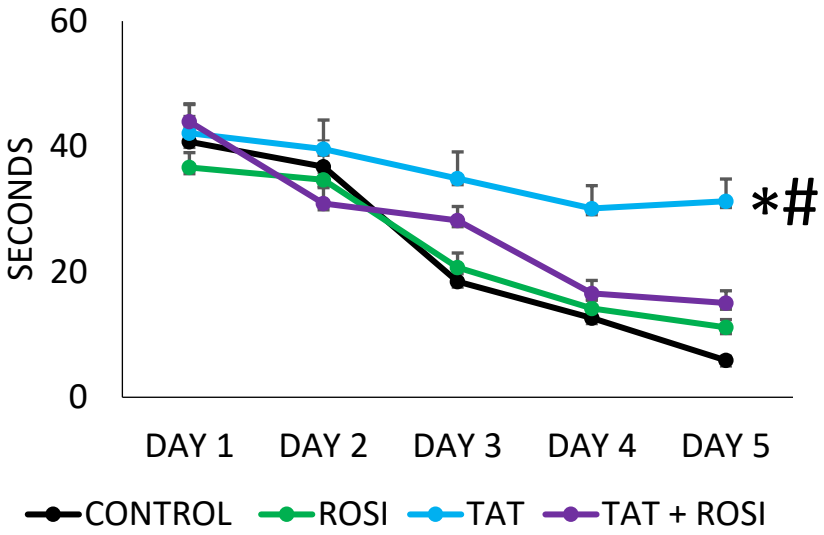


Figure 4.9 – Male and Female parameters Entries (A), Time (B), and Distance (C). The males and females show effect with time spent in the target quadrant; while, in other parameters the effect is not as pronounced as it would thought to have been.

We analyzed the search strategies of the animals to determine the effect that the drug combination had on the animals. We noticed that the control animals had a systematic strategy to find the escape hole. Meaning, they either explored the apparatus and found the hole or they found the hole and stayed at the hole. We noticed changes in search strategies in the Tat groups. The search strategies for these groups are random in this case. The Rosi groups show similar search strategies to the control groups. The combo groups show recovery of function when compared to Tat groups when looking at search strategies.

Next, we evaluated spatial learning and memory for the Morris Water Maze assay. In the males, the males showed significant interactions throughout the learning phase of the test ($df_{treatment}$ 3, df_{error} 520 f=24.796 p=5.22e-15). The males showed specific significant interactions as follows: Control – M vs. Tat – M p=<0.05 and Tat – M vs. Tat + Rosi – M p=<0.058. The females showed significant interactions throughout the learning phase of the test ($df_{treatment}$ 3, df_{error} 680 f=15.529 p=2.24e-10). The females showed specific significant interactions as follows: Control – F vs. Tat – F p=<0.05 and Tat – F vs. Tat + Rosi – F p=<0.05.

A.



B.

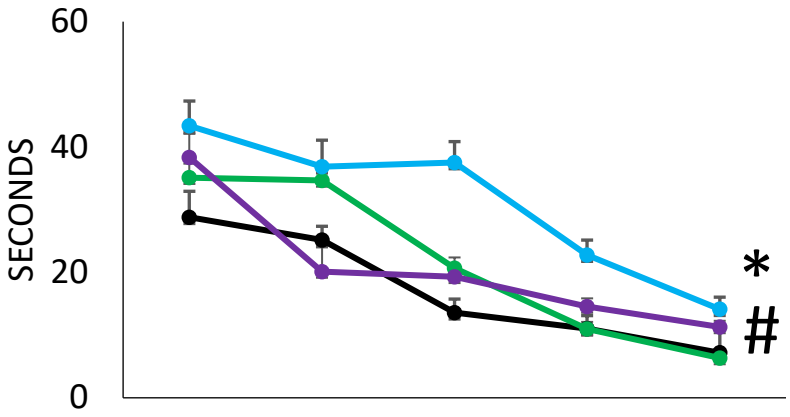


Figure 4.10 – Learning phase of Morris Water Maze. Male (A) and Female (B). Both male and female groups show significant improvement in ability to spatially learn and navigate when treated with rosiglitazone. The groups for the males were Ctrl n=5, Rosi n=10, Tat n=5, and Tat + Rosi n=8. The groups for the females were Ctrl n=5, Rosi n=13, Tat n=5, and Tat + Rosi n=13. The * represents compared to control. The # represents compared to Tat. Significance was set at $p<0.05$.

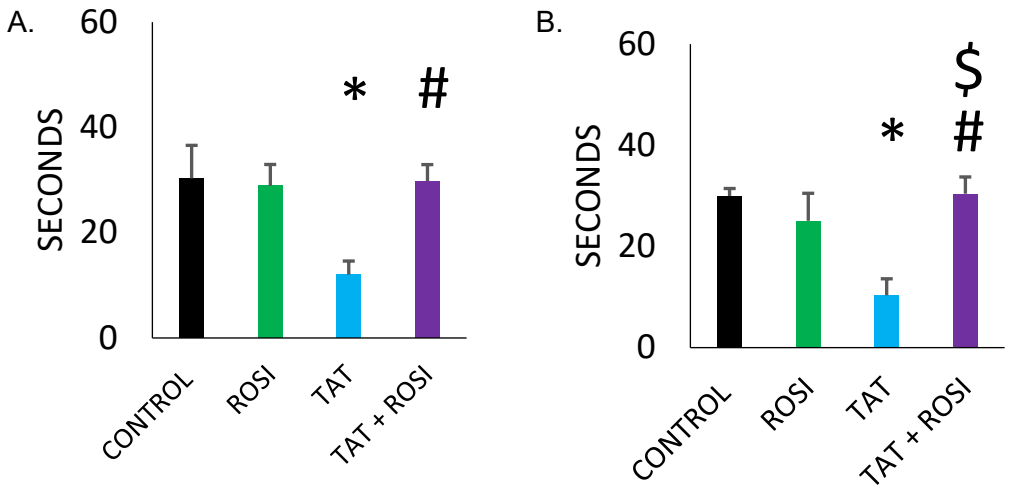


Figure 4.11 – Probe Trial. Male (A) and Female (B). Both male and female animals show ability to remember where the target island was in the water tank. This is a marker of recovery of function. The groups for the males were Ctrl n=5, Rosi n=10, Tat n=5, and Tat + Rosi n=8. The groups for the females were Ctrl n=5, Rosi n=13, Tat n=5 and Tat + Rosi n=13. The * represents compared to control. The # represents compared to Tat. The $ represents compared to Rosi. Significance was set at $p<0.05$.

We next analyzed the probe trial. This is where during the learning phase the escape island is placed in the southwest region of the tank. The probe trial removes that island and looks at whether the animal remembers where the escape island was located. In the males, the animals showed significant interactions in the probe trial ($df_{treatment}$ 3, df_{error} 23 f=24.365 p=2.52e-7). The females showed significant interactions in the probe trial ($df_{treatment}$ 3, df_{error} 31 f=30.242 p=2.46e-9). The males had specific significant interactions as follows: Control – M vs. Tat – M p=<0.05 and Tat-M vs. Tat + Rosi – M p=<0.05. The females showed specific significant interactions as follows: Control – F vs. Tat – F p=<0.05, Tat – F vs. Tat + Rosi – F p=<0.05, and Rosi – F vs. Tat + Rosi – F p=<0.05.

4.3.4 Receptors Change with Combination of Tat and Rosi

We analyzed the effect that Rosi and Tat had on the receptor changes in the brain. We analyzed markers that were relevant to HIV and Rosi for the disease

conditions. We analyzed levels of CD4, CD8, CD11b, GFAP, CD25, CD28, CD195, and IFNγ. The CD4 marker is used for monitoring the HIV patient disease status, especially helper T cell levels (Fauci 2003; Kaul, et al. 2005; Killian and Levy 2011; Liu, et al. 2009; Matavele Chissumba, et al. 2015; Vidya Vijayan, et al. 2017; Clift 2015). The CD8 marker is the principal marker of the cytotoxic T cell, which is important for monitoring pathogen state. CD11b is the marker for tissue macrophages, B cells, natural killer (NK cells), and granulocytes (Parney, Waldron, and Parsa 2009). This marker is primarily involved in and used as microglial marker in the nervous system. CD25 is also known as IL-2. This marker is available on T cells, B cell, and other myeloid precursor cells. It is also found in larger populations of resting memory T cells (Liu, et al. 2009; Matavele Chissumba, et al. 2015). CD28 marker is expressed on T cells and provides co-stimulatory signals for the activation and survival (Eylar, et al. 2001). Also, known to provide potent signaling for various interleukins, e.g., IL-6. CD195 is also known as MCP-1. This marker functions as a chemokine. This marker is located on T cells, macrophages, and microglia (Clift 2015). This marker is implicated in HIV as a co-receptor to enter cells. This marker is also involved in signaling at least 3 other molecules: RANTES, MIP-1, and MCP-2 (Pharmingen). Interferon γ (IFNγ) is a type II interferon. This receptor is responsible for activating and inducing macrophages and major histocompatibility complex II (MHC II) (Rojas, et al. 2021). This factor is involved in inhibiting viral replication and is known to be secreted and located on NK cells, CD4 Th1 cells, and CD8+ cells (Clift 2015; Rojas, et al. 2021; Eylar, et al. 2001). GFAP is marker that is used to ascertain astrocyte populations.

This marker is used in cellular communication and function of the Blood Brain Barrier. This marker is important in cases of central nervous system (CNS) repair in cases of CNS injury (Mehrbod, et al. 2019; Sporer, et al. 2004).

4.3.4.1 Receptor Constitution Changes with Addition of Rosi

We analyzed receptor changes inside the brain with regards to administration of Tat and Rosi. We will first analyze the changes in the males. For the CD4+ cellular population, the males showed a significant change in the population (df_{group} 3, df_{error} 80 f=42.150 p=1.93e-16). The males showed specific significant interactions as follows: Control vs. Tat p=<0.05 and Tat vs. Tat + Rosi p=<0.05. The CD8+ cellular population showed a significant change in the population (df_{group} 3, df_{error} 80 f=29.340 p=6.78e-13). The males showed specific significant interactions as follows: Control vs. Tat p=<0.05 and Tat vs. Tat + Rosi p=<0.05. For the CD11b+ population showed significant differences in the expressing population (df_{group} 3, df_{error} 80 f=41.994 p=2.11e-16). The males showed specific significant interactions as follows: Control vs. Rosi p=<0.05, Control vs. Tat + Rosi p=<0.05, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The CD25+ populations showed significant changes in the population expression (df_{group} 3, df_{error} 80 f=36.609 p=5.39e-15). The males showed specific significant interaction as follows: Control vs. Tat p=<0.05, Control vs. Tat + Rosi p=<0.05, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The males showed significant interactions with expression of CD28+ population (df_{group} 3, df_{error} 80 f=378.613 p=3.73e-47). The males showed specific significant interactions as follows: Control vs. Rosi p=<0.05, Control vs. Tat

p=<0.05, Control vs. Tat + Rosi p=<0.05, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The males showed significant changes in the GFAP+ population (df_{group} 3, df_{error} 80 f=150.602 p=8.25e-33). The males showed specific significant interactions as follows: Control vs. Tat p=<0.05, Control vs. Tat + Rosi p=<0.05, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The males showed significant changes when surveying the CD195+ population (df_{group} 3, df_{error} 80 f=118.587 p=2.34e-29). The males showed specific significant interactions when measuring expression in the population as follows: Control vs. Rosi p=<0.05, Control vs. Tat p=<0.05, Control vs. Tat + Rosi p=<0.05, Rosi vs. Tat + Rosi p=<0.05, Tat vs. Tat + Rosi p=<0.05. The males showed significant interactions when measuring the interferon γ population (df_{group} 3, df_{error} 80

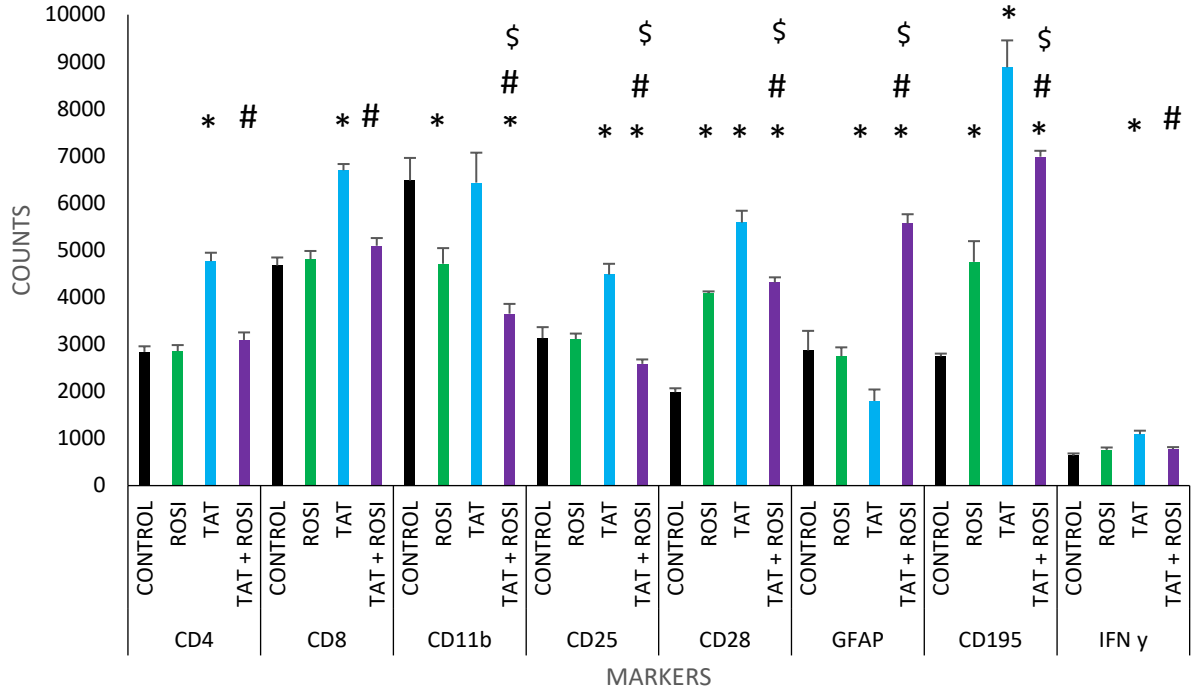


Figure 4.12 – Receptor expression in the male animals. Across the different markers, there is recovery of the population in the presence of rosiglitazone. In majority of markers, the combination group is highly upregulated. The * represents compared to control. The # represents compared to Tat. The $ represent compared to Rosi. Significance was set at p<0.05.

f=13.758 p=2.55e-7). The males showed specific significant interactions when measuring this population as follows: Control vs. Tat p=<0.05 and Tat vs. Tat + Rosi p=<0.05.

We next analyzed the females for population changes. The CD4+ population showed significant changes in the representative population (df_{group} 3, df_{error} 95 f=20.531 p=2.45e-10). The females showed specific significant changes as follows: Control vs. Tat p=<0.05 and Tat vs. Tat + Rosi p=<0.05. The females showed significant receptor shift in the CD8+ population (df_{group} 3, df_{error} 95 f=18.175 p=2.12e-9). The females showed specific significant interactions: Control vs. Tat p=<0.05 and Tat vs. Tat + Rosi p=<0.05. The females showed significant changes in receptor constitution in the population of CD11b+ cells (df_{group} 3, df_{error} 95 f=30.476 p=6.86e-14). The females showed specific significant interactions as follows: Control vs. Rosi p=<0.05, Control vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The females showed significant changes in receptor constitution in the population of CD25+ cells (df_{group} 3, df_{error} 95 f=42.010 p=2.27e-17). The females showed specific significant interactions as follows: Control vs. Rosi p=<0.05, Control vs. Tat p=<0.05, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The females showed significant changes in receptor constitution in the population of CD28+ cells (df_{group} 3, df_{error} 95 f=231.965 p=1.41e-43). The females showed specific significant interactions as follows: Control vs. Rosi p=<0.05, Control vs. Tat p=<0.05, Control vs. Tat + Rosi p=9.97e-8, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The females showed significant changes in the receptor constitution in the population GFAP+

cells (df_{group} 3, df_{error} 95 f=216.870 p=2.31e-42). The females showed specific significant interactions as follows: Control vs. Rosi p=<0.05, Control vs. Tat p=<0.05, Control vs. Tat + Rosi p=<0.05, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The females showed significant change in the receptor constitution of CD195+ cells (df_{group} 3, df_{error} 95 f=94.718 p=1.91e-28). The females showed specific significant interactions as follows: Control vs. Rosi p=<0.05, Control vs. Tat p=<0.05, Rosi vs. Tat + Rosi p=<0.05, and Tat vs. Tat + Rosi p=<0.05. The females showed significant changes in the receptor constitution of interferon γ (+) cells (df_{group} 3, df_{error} 95 f=8.308 p=5.79e-5). The females showed specific significant interactions as follows: Tat vs. Tat + Rosi p=<0.05.

Next, we analyzed how the receptor constitution changed with exposure to Tat and the combination group (Tat + Rosi) with respect to sex differences. First, we analyzed how the CD4 marker was affected. The CD4 marker showed significant interaction with respect to population change (df_{group} 7, df_{error} 175 f=29.273 p=1.73e-26). The sexes should significant interaction in the rosiglitazone groups p=<0.05. The CD8 marker showed significant interaction with respect to population change (df_{group} 7, df_{error} 175 f=23.514 p=2.45e-22). The sexes showed

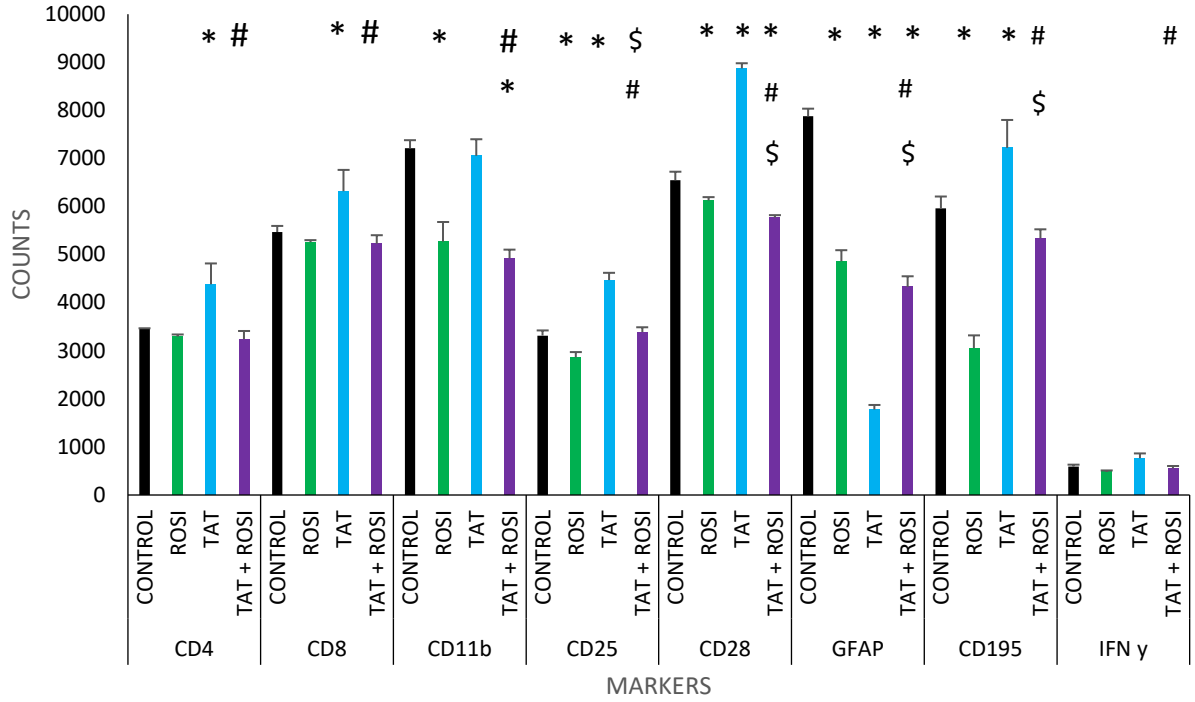


Figure 4.13 – Receptor expression in female animals. In the presence of rosiglitazone, there is recovery of population of the receptors. There is upregulation of the receptors in the combination group (Tat + Rosi). The * represents compared to control. The # represents compared to Tat. The $ represents compared to Rosi. Significance was set at p<0.05.

significant interaction in the rosiglitazone groups p=<0.05. The CD11b marker showed significant interaction with respect to population change (df_{group} 7, df_{error} 175 f=34.608 p=5.67e-30). The sexes showed significant interaction in the combination groups (Tat + Rosi) p=<0.05. The CD25 marker showed significant

interaction with respect to population change in receptor expression (df_{group} 7, df_{error} 175 f=35.017 p=3.16e-30). The sexes showed significant interaction in the combination groups (Tat + Rosi) p=<0.05. The CD28 marker showed significant interaction with respect to population change in receptor expression (df_{group} 7, df_{error} 175 f=570.464 p=6.74e-117). The sexes showed significant interaction in the Rosi group p=<0.05, Tat groups p=<0.05, and the combination groups (Tat + Rosi) p=<0.05. The GFAP marker showed significant interaction with respect to population change in expression (df_{group} 7, df_{error} 175 f=174.375 p=2.04e-75). The sexes showed specific significant interactions in the Rosi groups p=<0.05 and the combination groups (Tat + Rosi) p=<0.05. The CD195 marker showed significant interaction with respect to population change expression (df_{group} 7, df_{error} 175 f=97.869 p=4.02e-57). The sexes showed specific significant interactions in the Rosi groups p=<0.05, Tat groups p=<0.05, and the combination groups (Tat + Rosi) p=<0.05. Lastly, the interferon γ showed significant interaction with respect to population change in expression (df_{group} 7, df_{error} 175 f=19.199 p=6.48e-19). The sexes showed specific significant interactions in the Rosi groups p=<0.05, Tat groups p=<0.05, and the combination group (Tat + Rosi) p=<0.05.

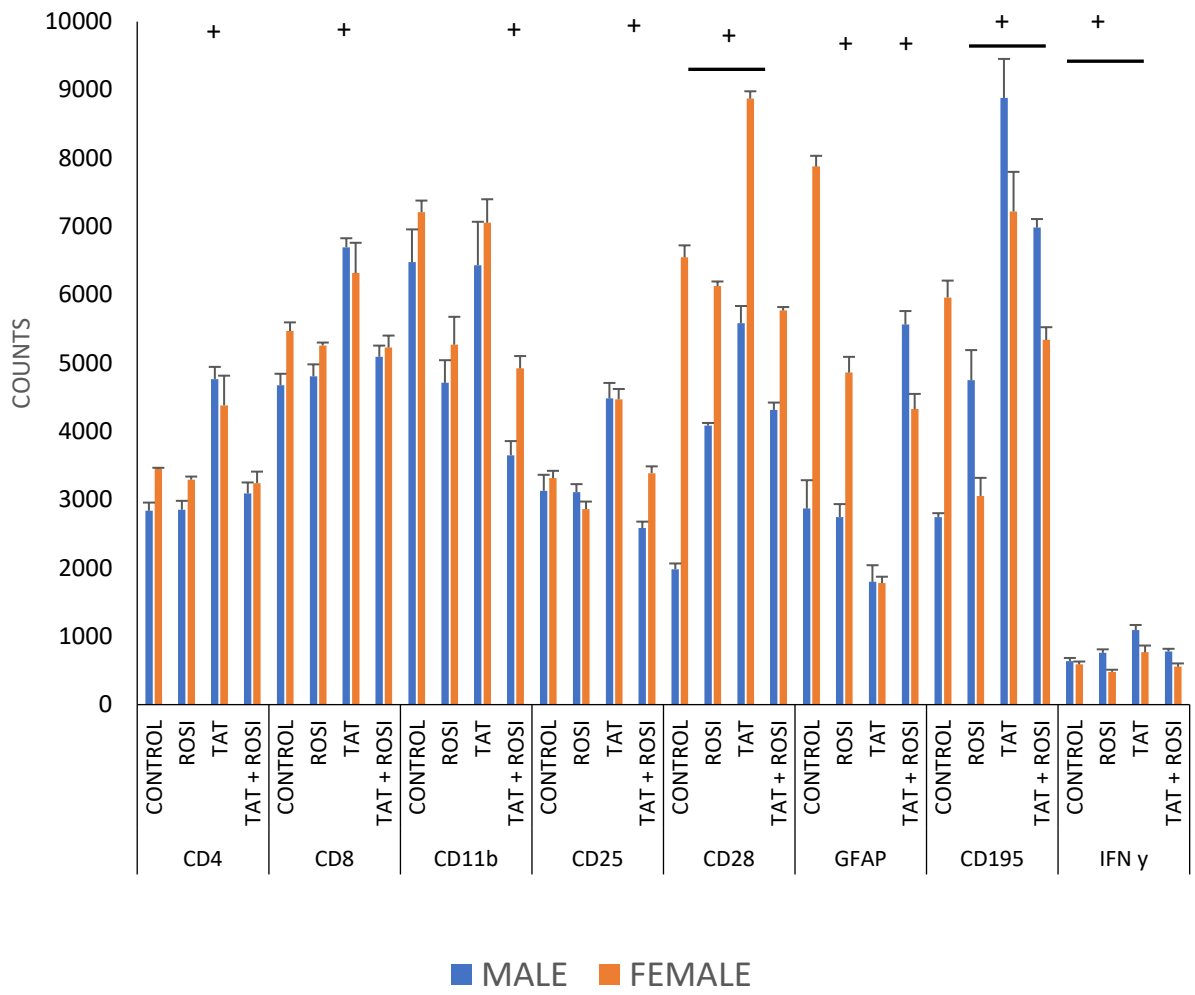


Figure 4.14 – Receptor expression between the sexes. There is significant sex difference noted between the sexes in the various markers that were analyzed. There is significant upregulation of the receptors in presence of rosiglitazone. The solid black bars represent sex difference that is noted within the four treatment groups. The + represents the comparison between the sexes. Significance was set $p<0.05$.

Markers	Significant Sex Difference
CD4	TAT
CD8	ROSI
CD11b	TAT + ROSI
CD25	TAT + ROSI

CD28	CONTROL, ROSI, TAT, & TAT + ROSI
GFAP	CONTROL, ROSI, & TAT + ROSI
CD195	CONTROL, ROSI, TAT, & TAT + ROSI
IFNγ	CONTROL, ROSI, TAT, & TAT + ROSI

Table 4.1 – Sex difference depiction of receptor expression between the sexes. This table depicts the comparisons denoted in figure 4.14.

4.3.5 Ligand Changes with addition of Tat and Rosiglitazone

4.3.5.1 Cytokine Changes with Addition of Rosi

We have examined the effect of addition of rosiglitazone on Tat treated animals on the cytokine expression. We examine these levels based upon sex, region, and sex difference. First, we analyzed the changes in the MCP-1 cytokine.

The males showed significant interactions with addition of rosiglitazone with

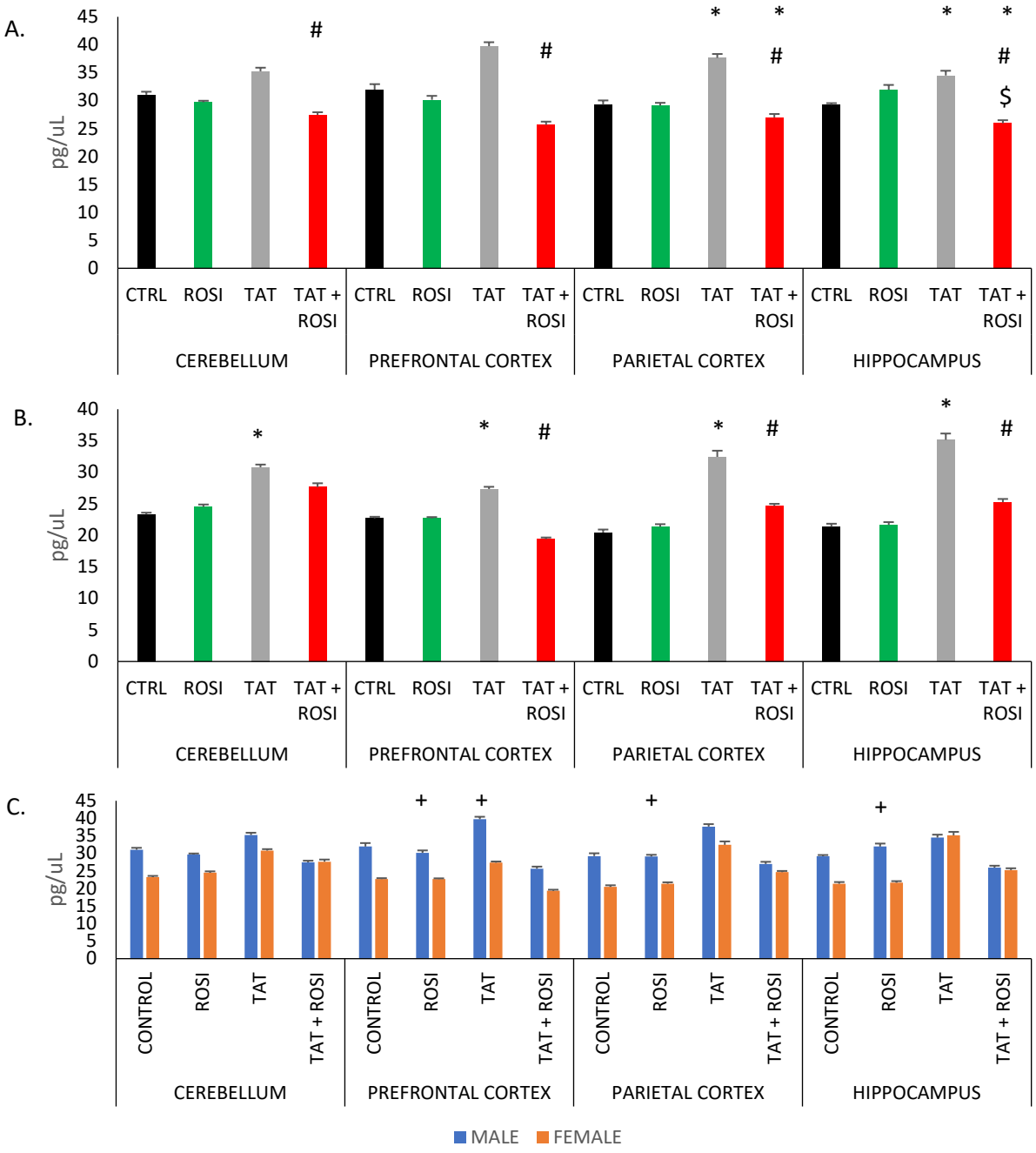
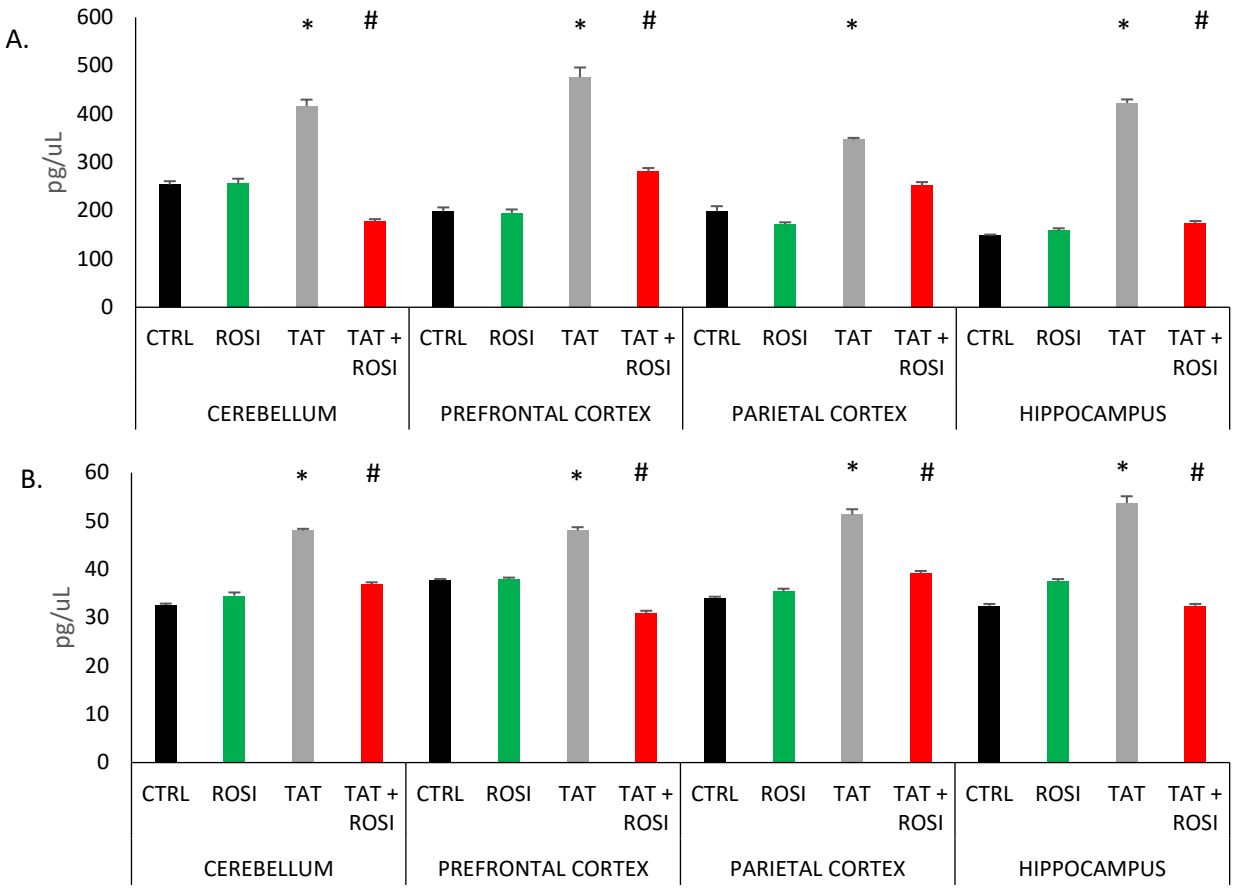


Figure 4.15 – A. Male B. Female C. Combined figures for MCP-1 Levels. There is significant downregulation of MCP-1 levels in the presence of Rosiglitazone. There is also significant down regulation of MCP-1 when the compared sexes are compared. The * represents compared to control. The # represents compared to Tat. The $ represents compared to Rosi. The + represents difference between sexes. Significance was set $p<0.05$.

respect MCP-1 cytokine (df_{group} 3, df_{error} 144 f=18.620 p=2.91e-10). The males

showed specific significant interactions in changes of MCP-1 cytokine as follows: Ctrl-M vs. Tat-M p=<0.05, Ctrl-M vs. Tat+Rosi-M p=<0.05, Rosi-M vs. Tat+Rosi-M p=<0.05, and Tat-M vs. Tat + Rosi-M p=<0.05. The females showed significant interaction with the addition of rosiglitazone with respect to MCP-1 cytokine (df_{group} 3, df_{error} 144 f=31.67 p=8.81e-16). The females showed specific significant interactions in changes of MCP-1 cytokine as follows: Ctrl-F vs. Tat-F p=<0.05 and Tat-F vs. Tat + Rosi-F p=<0.05. We examined the expression for changes of MCP-1 with the addition of rosiglitazone to both sexes (df_{group} 7, df_{error} 288 f=33.157 p=1.141e-33). The sexes showed specific significant interactions as follows: Rosi-M vs. Rosi-F p=<0.05 and Tat-M vs. Tat-F p=<0.05. The groups showed no specific interactions in the regions between sexes with respect to MCP-1

Next, we analyzed the effect on the IL-1α cytokine. The males showed a significant change in levels of IL-1α in the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=63.332 p=3.60e-26). The males showed specific significant interactions as follows: Ctrl-M vs. Tat-M p=<0.05 and Tat-M vs. Tat + Rosi-M p=<0.05. The females showed significant alterations in levels IL-1α in the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=70.362 p=4.47e-28). The females showed specific significant interactions as follows: Ctrl-F vs. Tat-F p=<0.05 and Tat-F vs. Tat + Rosi-F p=<0.05. The sexes showed significant interactions when compared



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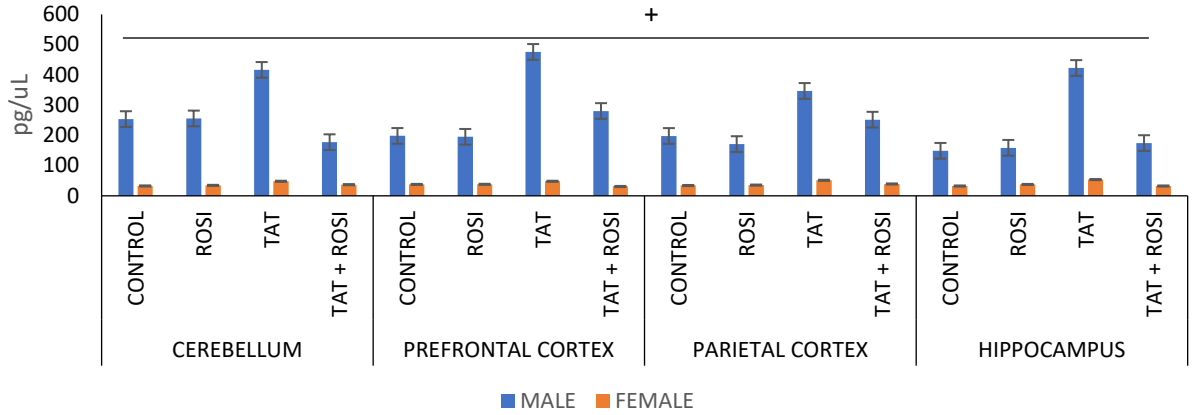


Figure 4.16 – A. Male B. Female C. Combined for IL-1α. There is significant down regulation of IL-1α in the presence of Rosiglitazone. There is also significant sex difference. The * represents compared to control. The # represents compared to Tat. The + represents significant sex difference. Significance was set at p<0.05.

to each other (df_{group} 7, df_{error} 288 f=208.535 p=8.494e-109). The sexes showed specific significant interactions in the groups are as follows: Rosi-M vs. Rosi-F p=<0.05, Tat-M vs. Tat-F p=<0.05, and Tat + Rosi-M vs. Tat + Rosi-F p=<0.05. The sexes showed significant interactions in the regions with respect to levels of IL-1α (df_{region} 7, df_{error} 288 f=4.442 p=4.529e-3). The sexes showed specific significant interactions as follows: cerebellum vs. hippocampus p=<0.05 and prefrontal cortex vs. hippocampus p=<0.05.

The levels of IL-6 were analyzed. The males showed significant interactions in the levels of IL-6 with the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=34.386 p=8.05e-17). The males showed specific significant interactions as follows: Ctrl-M vs. Tat-M p=<0.05 and Tat-M vs. Tat + Rosi-M p=<0.05. The females showed significant changes in the levels of IL-6 in the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=38.791 p=1.957e-18). The females showed specific significant interactions as follows: Ctrl-F vs. Tat-F p=<0.05 and Tat-F vs. Tat + Rosi-F

p=<0.05. The sexes showed significant changes in the levels of IL-6 in the presence of rosiglitazone (df_{group} 7, df_{error} 288 f=103.025 p=1.279e-74). The sexes showed specific significant interactions as follows: Rosi-M vs. Rosi-F p=<0.05, Tat-M vs. Tat-F p=<0.05, Tat + Rosi-M vs. Tat + Rosi-F p=<0.05. The sexes

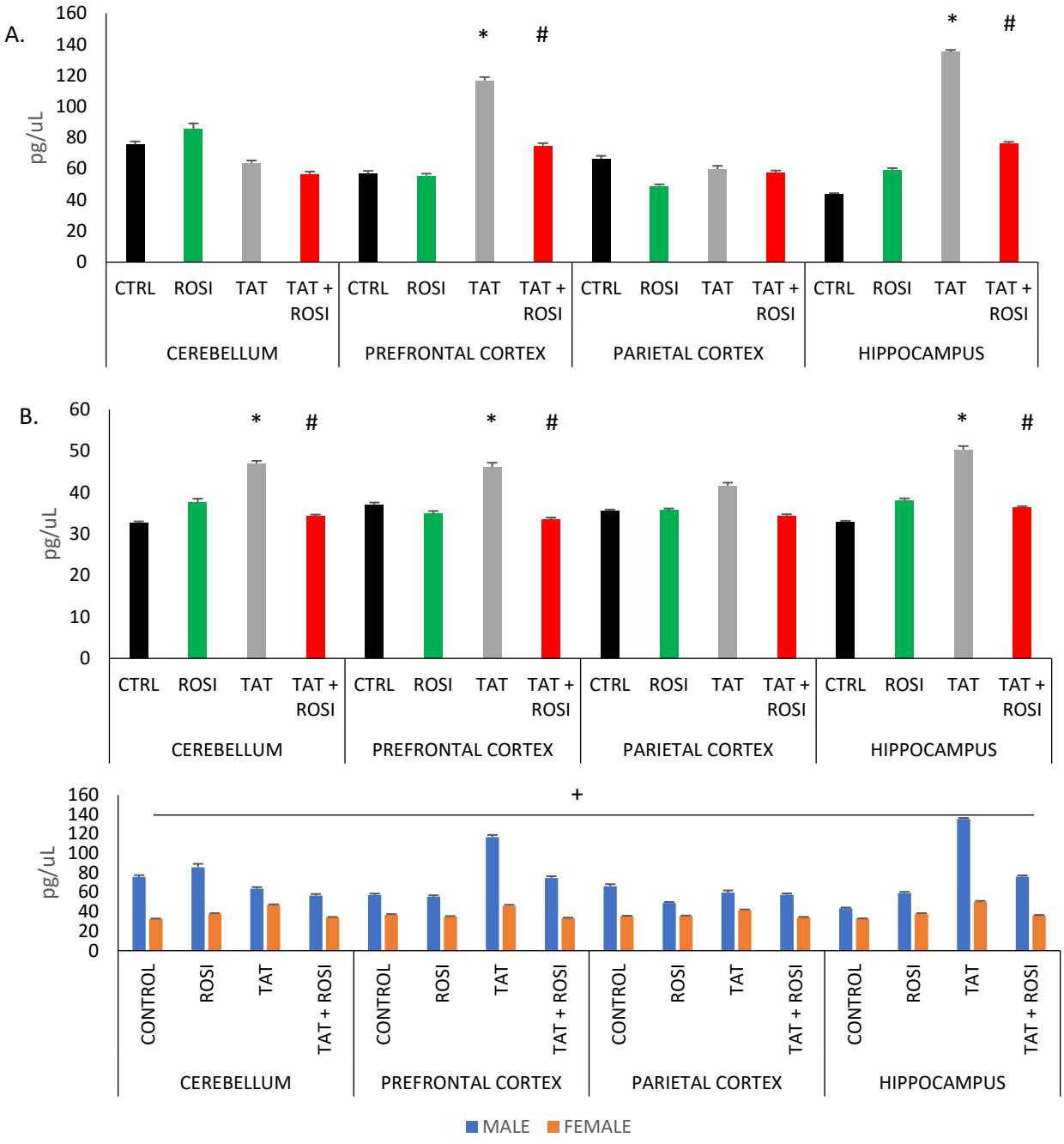
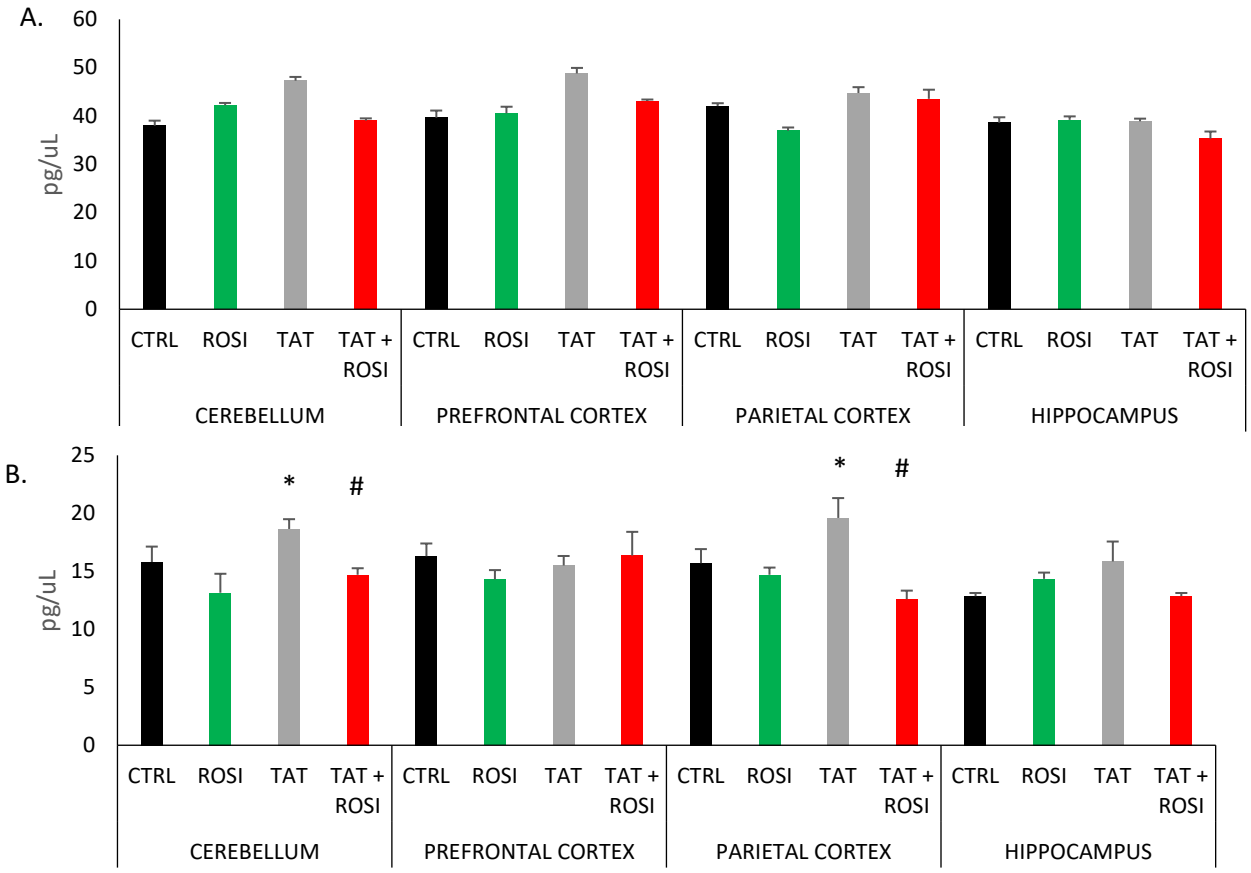


Figure 4.17 – A. Male B. Female C. Combined figures for IL-6 Levels. There is significant down regulation of IL-6 in the presence of Rosiglitazone. There significant sex difference across all of the regions being measured. The * represents compared to control. The # represents compared to Tat. The + represents compared to sexes. Significance was set at p<0.05.

showed significant interactions in the regions with IL-6 (df_{region} 7, df_{error} 288 f=12.851 p=6.647e-8). The sexes showed specific significant interactions as follows: cerebellum vs. parietal cortex p=<0.05, prefrontal cortex vs. parietal cortex p=<0.05, and parietal cortex vs. hippocampus p=<0.05.

The levels of TNF-α were measured. The males showed significant interactions in the levels of TNF-α in the presence of rosiglitazone (df_{group} 3, df_{error}



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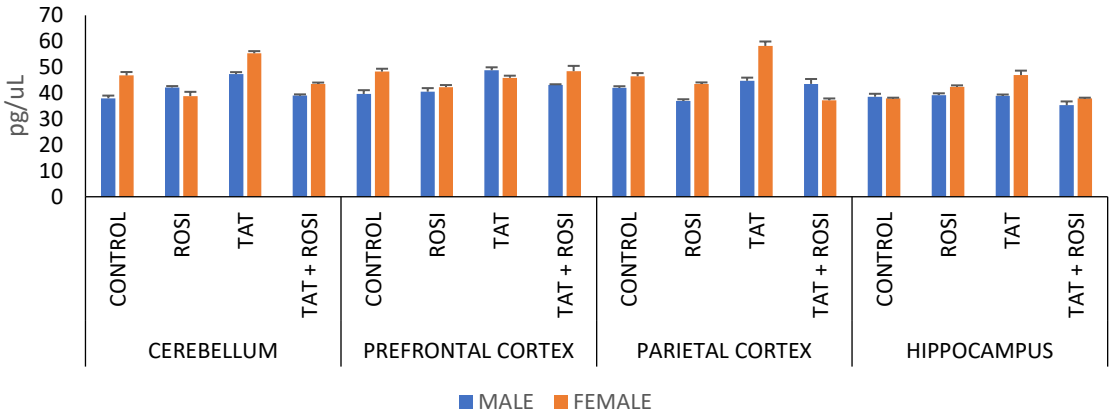


Figure 4.18 – A. Male B. Female C. Combined figures for TNF-α levels. The males show nonsignificant decreases in levels of TNF-α levels in the presence of Rosiglitazone. The females show significant difference in the presence of Rosiglitazone. There are nonsignificant sex differences seen in the presence of Rosiglitazone. The * represents compared to control. The # represents compared to Tat. Significance was set at $p<0.05$.

144 f=2.578 p=5.61e-2). The males showed no specific significant interactions when comparing groups. The females showed significant interactions in the levels of TNF-α in the presence of TNF-α in the presence of rosiglitazone df_{group} 3, df_{error} 144 f=7.291 p=1.38e-4). The females showed specific significant interactions as follows: Ctrl-F vs. Tat-F p=<0.05 and Tat-F vs. Tat + Rosi-F p=<0.05. The sexes showed significant changes in the levels of TNF-α in the presence of rosiglitazone (df_{group} 7, df_{error} 288 f=5.802 p=2.635e-6). The sexes showed no specific significant interactions when comparing group to group. The sexes showed significant interactions with respect to TNF-α (df_{region} 7, df_{error} 288 f=3.300 p=2.080e-2). The sexes showed specific significant interactions as follows: prefrontal cortex vs. hippocampus p=<0.05.

The levels of interferon γ (IFNγ) were analyzed. The males showed significant interactions in the levels of IFNγ in the presence of rosiglitazone (df_{group}

3, df_{error} 144 f=75.869 p=1.72e-29). The males showed specific significant interactions as follows: Ctrl-M vs. Tat-M p=<0.05 and Tat-M vs. Tat + Rosi-M p=<0.05. The females showed significant interaction in the levels of IFNγ in the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=22.530 p=5.110e-12). The females showed specific significant interactions as follows: Ctrl-F vs. Tat-F p=<0.05and Tat-F vs. Tat + Rosi-F p=<0.05. The sexes showed significant interactions in the levels of IFNγ in the presence of rosiglitazone (df_{group} 7, df_{error} 288 f=99.566 p=4.122e-73). The sexes showed specific interactions when comparing groups as follows: Rosi-M vs. Rosi-F p=<0.05, Tat-M vs. Tat-F p=<0.05, and Tat + Rosi-M vs. Tat + Rosi-F p=<0.05. The sexes showed significant interactions in the regions (df_{region} 7, df_{error} 288 f=12.067 p=1.834e-7). The sexes showed specific significant interactions in the region comparisons: cerebellum vs. prefrontal cortex p=<0.05,

cerebellum vs. hippocampus p=<0.05, prefrontal cortex vs. parietal cortex p=5.701e-6, and parietal cortex vs. hippocampus p=<0.05.

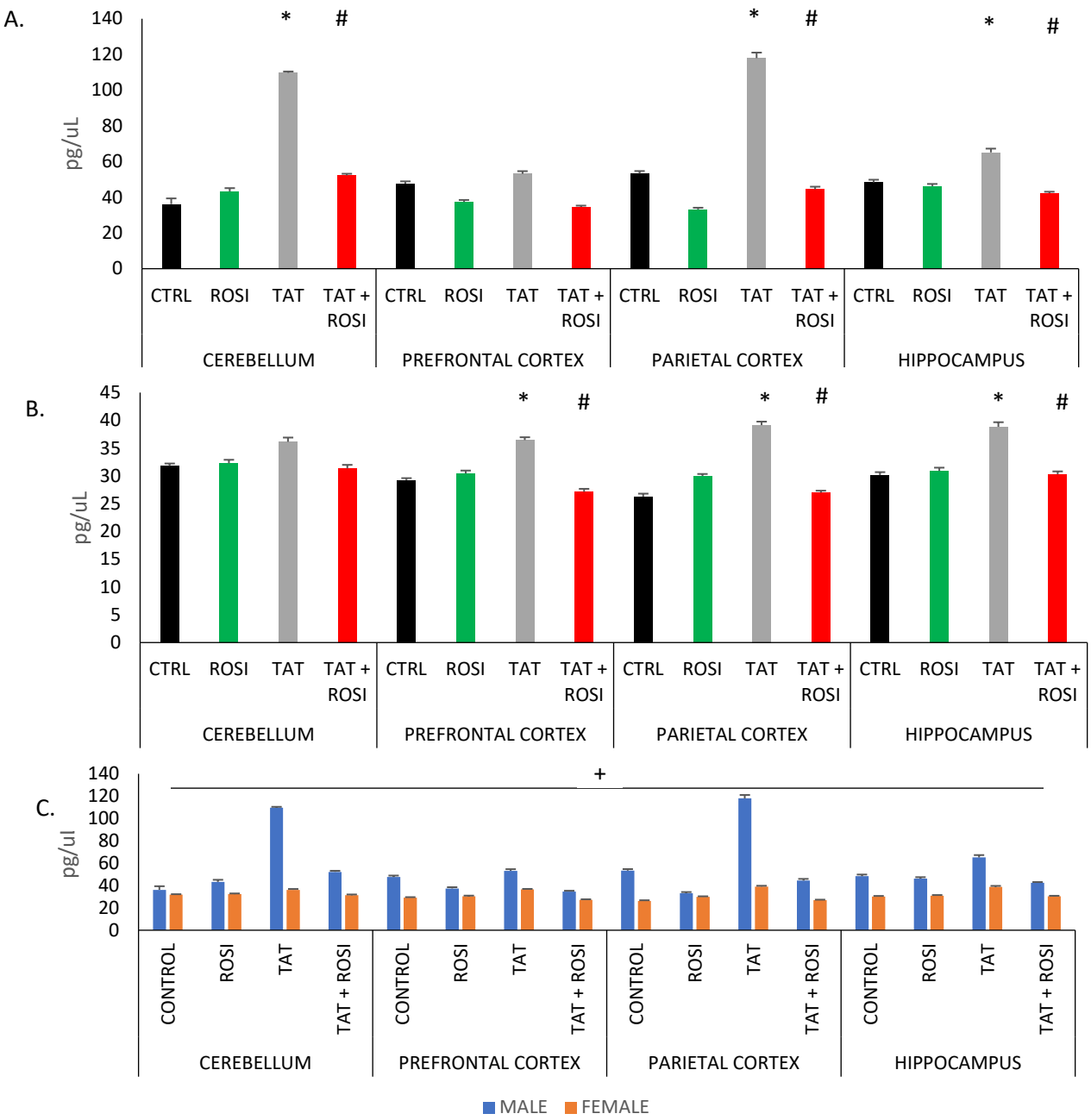
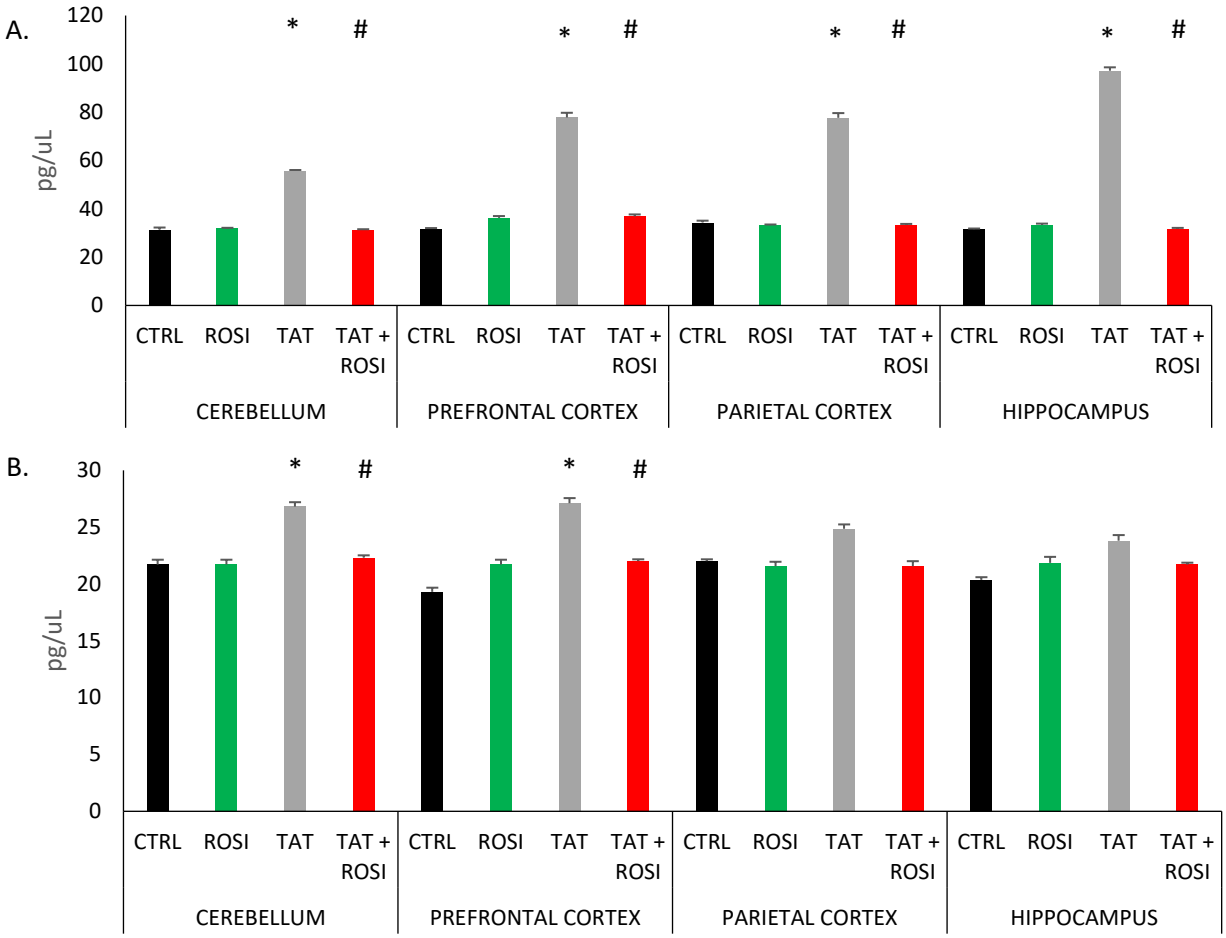


Figure 4.19 – A. Male B. Female C. Combined figures for IFNγ Levels. The levels of IFNγ for the males show significant down regulation in all regions of the brain. The levels of IFNγ for the females show significant down regulation in three regions. There is significant sex difference seen with the levels of IFNγ. The * represents compared to control. The # represents compared to Tat. The $ represents significant sex difference. The + represents significant sex difference. Significance was set at p<0.05.

The levels of IL-1β were analyzed. The males showed significant interactions in the levels of IL-1β with the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=256.277 p=1.59e-57). The males showed specific significant interactions as follows: Ctrl-M vs. Tat-M p=<0.05 and Tat-M vs. Tat + Rosi-M p=<0.05. The females showed significant interactions in the levels of IL-1β with the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=13.126 p=1.261e-7). The females showed specific significant interactions as follows: Ctrl-F vs. Tat-F p=<0.05 and Tat-F vs. Tat + Rosi-F p=<0.05. The sexes showed significant interactions in the levels of



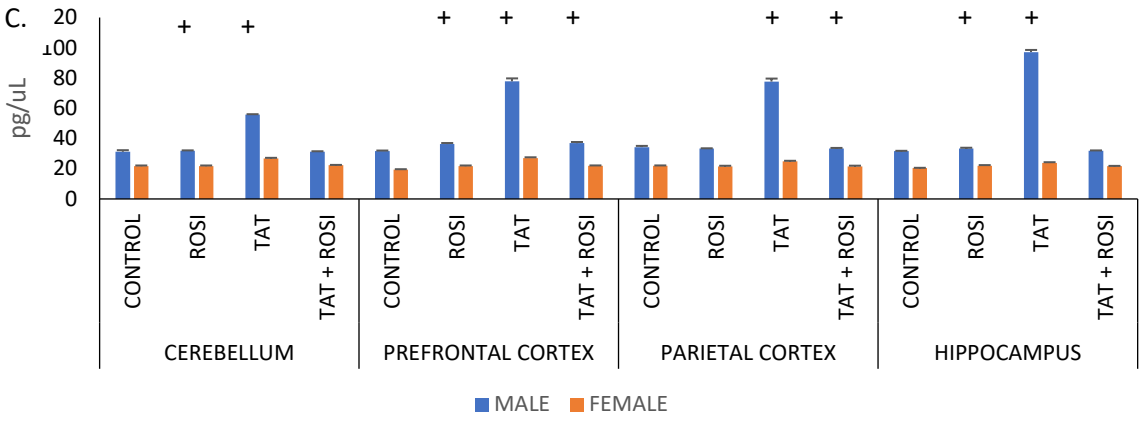
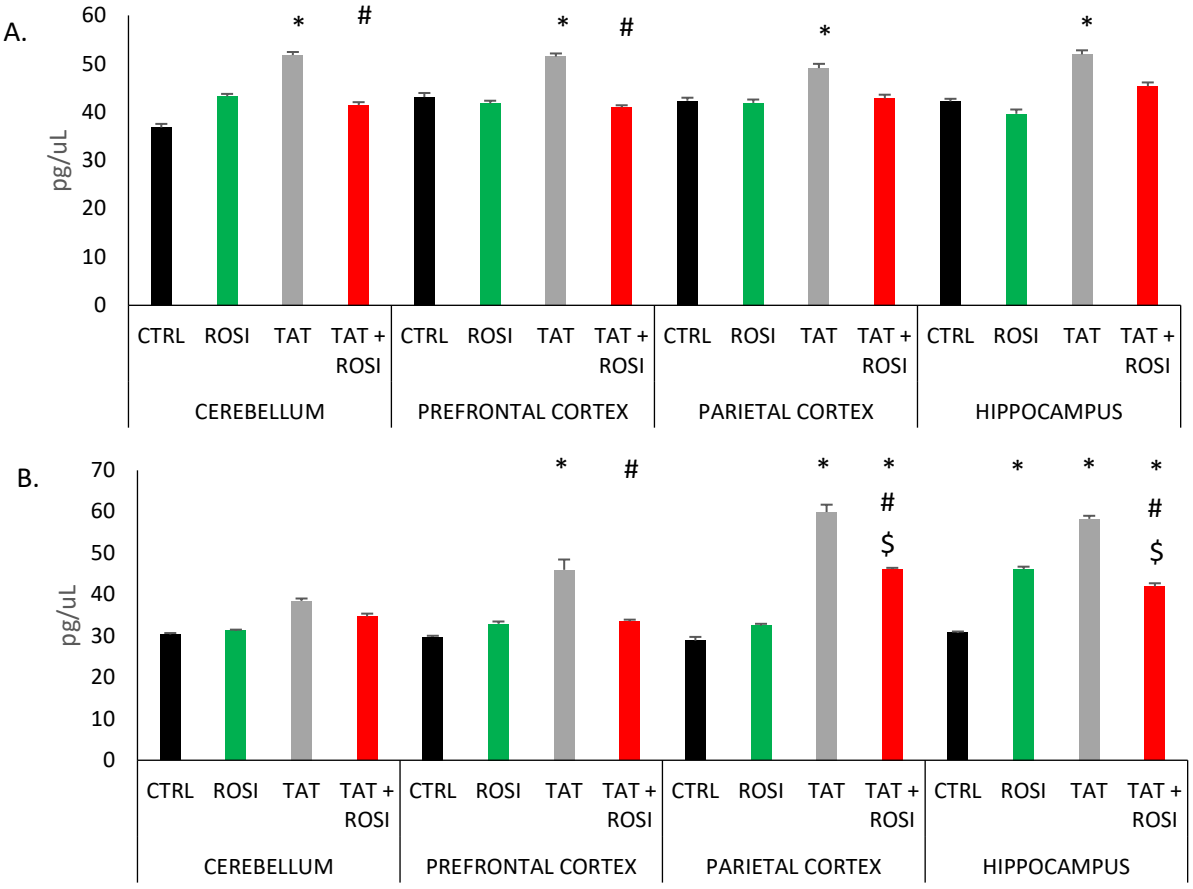


Figure 4.20 – A. Male B. Female C. Combined figures for IL-1β Levels. The levels of IL-1β show significant down regulation in all regions of the males. The levels of IL-1β show significant down regulation in two regions in the females. There significant sex difference in all regions. The * represents compared to control. The # represents compared to Tat. The + represents significant sex difference. Significance was set at $p<0.05$.

IL-1β with the presence of rosiglitazone (df_{group} 7, df_{error} 288 f=304.669 p=4.134e-129). The sexes showed specific significant interactions when comparing groups as follows: Rosi-M vs. Rosi-F p=<0.05, Tat-M vs. Tat-F p=<0.05, and Tat + Rosi-M vs. Tat + Rosi-F p=<0.05. The sexes showed significant interactions when comparing regions (df_{region} 7, df_{error} 288 f=7.725 p=5.573e-5). The sexes showed specific significant interactions when comparing the regions as follows: cerebellum vs. prefrontal cortex p=<0.05, cerebellum vs. parietal cortex p=<0.05, and cerebellum vs. hippocampus p=<0.05.

The levels of IL-12 were analyzed. The males showed significant interactions in the levels of IL-12 when in the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=39.525 p=1.07e-18). The males showed specific significant interactions as follows: Ctrl-M vs. Rosi-M p=<0.05, Ctrl-M vs. Tat-M p=<0.05, Ctrl-M vs. Tat + Rosi-M p=<0.05, and Tat-M vs. Tat + Rosi-M p=<0.05. The females

showed significant interactions in the levels of IL-12 when in the presence of rosiglitazone (df_{group} 3, df_{error} 144 f=19.420 p=1.249e-10). The females showed specific significant interactions as follows: Ctrl-F vs. Tat-F p=<0.05 and Tat-F vs. Tat + Rosi-F p=<0.05. The sexes showed significant interactions in the levels of IL-12 with the presence of rosiglitazone (df_{group} 7, df_{error} 288 f=31.116 p=5.766e-32). The sexes showed no specific significant interactions when comparing the groups. The sexes showed significant interactions when comparing regions (df_{region} 7, df_{error} 288 f=9.861 p=3.282e-6). The sexes showed specfic significant



interactions when comparing regions as follows: cerebellum vs.

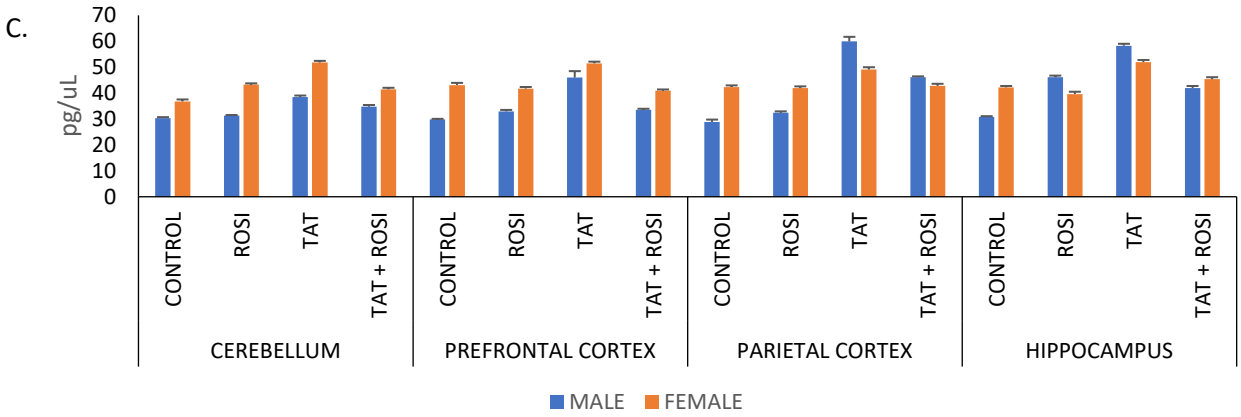


Figure 4.21 – A. Male B. Female C. Combined figures for IL-12 Levels. The levels of IL-12 show significant down regulation in two regions in the males. The levels of IL-12 show significant down regulation in three regions in the females. There was no significant sex difference detected with IL-12. The * represents compared to control. The # represents compared to Tat. The $ represents compared to Rosi. Significance was set $p<0.05$.

parietal cortex $p=<0.05$, cerebellum vs. hippocampus $p=<0.05$, and prefrontal cortex vs. hippocampus $p=<0.05$.

4.4 Discussion

We have completed a study that exhibits significant differences in different parameters, markers, and sex differences. These changes are reflected especially in the Barnes maze, water maze, flow cytometry, and ELISA assay. We have seen other studies that reflect these differences in behavior and the in vitro assays.

We, in this study, looked at how the component of HIV-1, Tat, causes issues with neurodeficit. In regards to Tat, we have shown that it causes decrease in motor coordination and gait, and both working memory and spatial learning and memory. Tat has also been known to cause neurodeficit with destruction of cells releasing cofactors and other debris that are known to be toxic and can cause cell death (Mattson, Haughey, and Nath 2005; Kim, Yoon, and Kim 2013; Pu et al. 2003; Rice 2017; Lu et al. 2011; Ajasin and Eugenin 2020; Perry et al. 2005;

Wallace 2022; Jadhav and Nema 2021; Pocernich, et al. 2005; Saro, et al. 2021; Nookala and Kumar 2014; Bagashev and Sawaya 2013; Nath, et al. 1999). Tat has been well documented in the literature and is known to cause receptor and ligand modifications. Increases in a variety of expression of cytokines (e.g. IL-6) have been known to show varying levels (Pu, et al. 2003; Rice 2017; Ajasin and Eugenin 2020; Nookala and Kumar 2014).

We have looked for literature that describes the drug effects. It is important to understand how drugs work together to ascertain their ability to cause good effect, no effect, or detrimental effect. In the in vitro testing, we see that this is true and can be see at a variety of level of intensity or can cause detrimental effect depending on the parameters being measured.

Our behavioral assays are selected and are scaled based to reflect methods performed in literature. The roto-rod (motor coordination and gait), novel object recognition (NORT) (working memory), Barnes maze (spatial learning and memory), and Morris water maze (spatial learning and memory) are all designed to and show significant measurements of various brain regions and functions. This modification comes in three separate ways. The first is changes in motor coordination and gait. The second a return or increase of ability to recognize objects and showing decrease anxiety to new surroundings. The third and last behavioral modification is changes in the ability to learn spatially and changes of how systematic an individual is about learning. The search strategies returning to normal or showing similar track patterns compared to control is evidence that the rosiglitazone is lessening the burden that is caused by presence of HIV-1 Tat

proteins (Huang, et al. 2015). Our assays show that neurodeficit is reversed, returns to normal function, or back to basal levels. The effect that is seen demonstrates the efficacy and how this treatment could be beneficial to multiple populations (Labandeira, et al. 2022; Omeragic, et al. 2017; Sagheddu, et al. 2021; Morsy, et al. 2022).

The receptors being up regulated and the cytokines are down regulated which would tend toward a shift toward a non-toxic environment e.g. the synergistic effect of Tat + Rosi when analyzing the CD25 level showing up regulation. This shift is because the fact that rosiglitazone has the property that most TZD drugs have, they have anti-inflammatory properties and have ability to increase synaptic plasticity. This effect is anticipated because of the pharmacology and toxicology of the drug. We anticipated the effects to be uniform across the sexes. The fact that the receptors and cytokines did not show the anticipated effect was suprising considering that this would be considered a general property of TZD drugs. This would speak to efficacy how these drugs have been used to explain the effects, stabilization, and reversal of some of the effects of usage of the rosiglitazone in treating Alzheimer's disease.

Overall, we have performed a study to show that how rosiglitazone or other PPAR agonsit can cause reversal of neuro deficit or lessen the effects of the neuro deficit. The first is that we have shown that the addition of rosiglitazone return or ameriolates the levels or changes in the receptor constitution. Changing the expression of receptors on cells decreases the ability of the cellular response to toxicants and other agents that would cause further manipulation of this

environment. Secondly, the presence of PPAR γ agonist decreases the levels of cytokines which would decrease the chance and available agents to cause an increase in productive neurodefict. The resulting changes seen in our studies would cause down stream changes that will decrease the ability to cause and subsequent release of cell debris, free radicals, and other materials that are chemoattaractant substances in the neural environment causing inflammation or inflammatory processes (Ramesh, MacLean, and Philipp 2013; Ogura and Yamaguchi 2022; Behl, et al. 2021; Porter and Sutliff 2012; Zamanian, et al. 2022; Sola, et al. 2022; Potula, et al. 2008; Omeragic, et al. 2019; Huang, et al. 2014; Vázquez-Carrera and Wahli 2022; Huang, et al. 2008; Sundararajan, et al. 2006; Villapol 2018; Huang, et al. 2015; Shrestha, et al. 2022; Barnstable, Zhang, and Tombran-Tink 2022). This evident by the resulting drug effect that is present with the GFAP marker, which was antagonistic, in the flow cytometry assay. This

modification that is apparent in our study shows to be antagonistic for some markers or addive in effect.

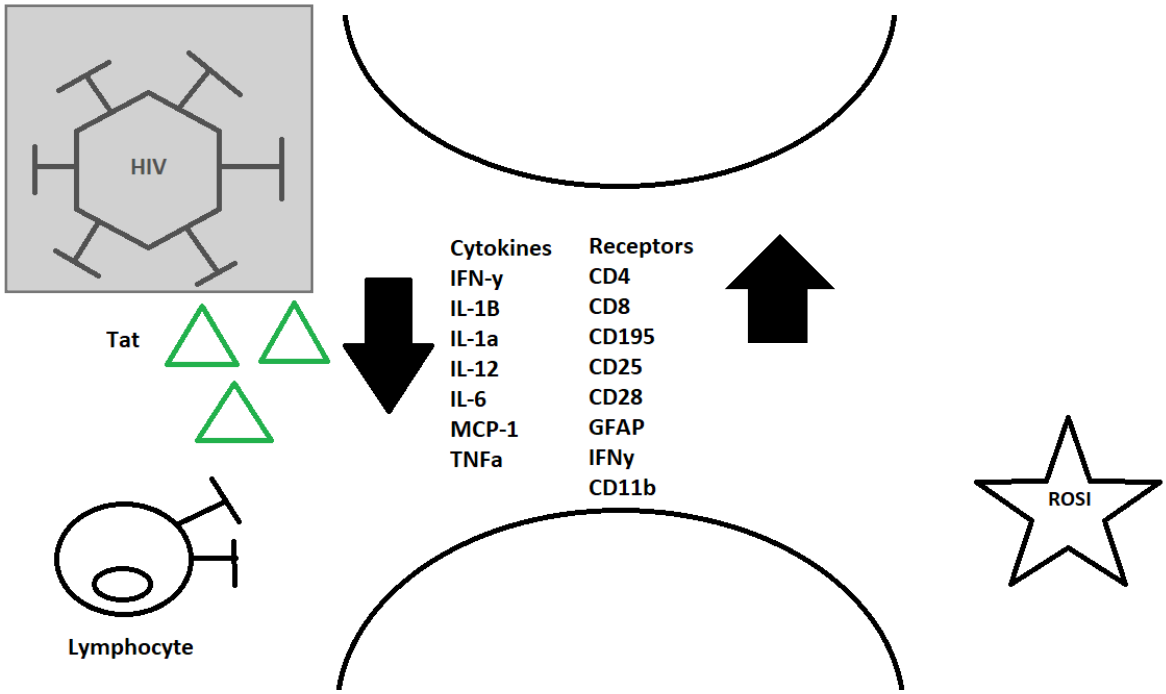


Figure 4.22 – Summary conclusion schematic. With addition of Rosiglitazone, the behavioral data shows recovery of function with respect to gait and memory testing. The receptors show up regulation in the presence of Rosiglitazone. The cytokines down regulate in the presence of Rosiglitazone.

In conclusion, our study demonstrates that addition of rosiglitazone causes an antagonistic drug effect by performing certain behavioral assays and achieved desired behavioral outcomes and decrease significant neurodeficit. The addition of rosiglitazone works to cause antagonistic behavior which decreases significant changes in receptor and ligand constitution to decrease neurodeficit from resulting exposure to HIV-1 Tat.

CHAPTER 5

SUMMATION OF RESEARCH OF ACTIVITIES AND FUTURE DIRECTIONS

5.1 Conclusion of Findings

5.1.1 Specific Aim 1

In specific aim one, we have asked the following questions:

1. 1 How long must exposure to alcohol persist for it to cause a significant change in spatial learning and memory and motor coordination changes in an animal system?
2. How long must exposure to alcohol persist for significant synaptic plasticity changes to occur?

The following hypothesis was made to answer the following questions:

1. We hypothesize that with prolonged duration of exposure to alcohol, there will be changes in motor coordination and spatial learning and memory as well as changes in synaptic plasticity.

Our findings for this specific aim show with prolonged exposure to alcohol causes alterations of motor coordination, executive function as measured by the novel object test, and spatial learning and memory as measured by the Morris water maze. These changes were noted to have existing sex differences. The effect was more pronounced in females than in males. This effect is believed to be weight and dose dependent lining up with typical effects of prolonged alcohol exposure.

The changes that are observed in the behavior show the same effect in the synaptic plasticity analysis. The fact that at 12 weeks of exposure to alcohol, there

is global significant knock down of synaptic plasticity shows that the brain is highly susceptible to toxic insults, whether that be from alcohol or some other drug. The brain expression of BDNF demonstrates that BDNF acts as an apparent protective mechanism for toxic insult or other traumatic insults to the brain. This effect is seen in both males and females.

We found that 12 weeks of exposure to alcohol caused the more severe neurodeficit for this time point, unlike the 4- and 8-week time points. Specific aim 1 was figured to be a chronic life-long low dose alcohol study that has mimicked social drinking of an individual with monitored alcohol intake. Further studies that were completed in this research program used this treatment paradigm and worked well to test other agents.

5.1.2 Specific Aim 2

In specific aim 2, we asked the following question

> Do HIV-1 Tat and alcohol work to cause alterations in spatial learning and memory, motor coordination, receptor constitution changes, and change in cytokine levels?

The following hypothesis was made to answer the question:

> We hypothesize that with prolonged exposure HIV-1 Tat and alcohol will work to create a alterations and significant changes in spatial learning and memory, motor coordination, receptor constitution, and cytokines.

We found that the prolonged exposure to a combination of HIV-1Tat and alcohol exhibit a synergistic drug effect. The treatment time frame followed the results that were laid out in specific aim 1, as described above. This drug effect causes alterations in all behavioral testing including, motor coordination, executive function as measured by the novel object test, and spatial learning and memory as measured by both the Barnes maze and the Morris water maze. These changes were also noted to exhibit sex difference with more severe changes being noted in the females. Drug effects were measured and shown to be synergistic in nature, meaning HIV-1 Tat and alcohol work together to cause neurodeficit in the treated animals.

With the prolonged exposure to both HIV-1 Tat and alcohol, we found apparent changes in both receptor constitution and cytokine composition in the brain of treated animals. These changes in the brain were noted to experience sex difference that was noted from the behavior testing. The changes receptor and cytokines allow for a more toxic and inflammatory environment to potentiate neurodeficit and loss of function. The males and females exhibited synergistic drug effects with respect to receptors and cytokines. This drug effect means that both HIV-1 Tat and alcohol work together to cause an environment that would potentiate possibly neurodeficit in the treated animals and possibly humans.

The conclusion can be made that with prolonged exposure to a combination of both HIV-1 Tat and alcohol, that neurodeficit for the affected individual is potentiated and made worse.

5.1.3 Specific Aim 3

In specific aim 3, we asked the following question:

> Does the addition of Rosiglitazone to HIV-1 Tat exposed animals cause circumvention of neurodeficit with respect to spatial learning and memory, alterations in motor coordination, receptor constitution changes, and change in cytokine levels?

To answer this question, we posed the following hypothesis:

> We hypothesize that the addition of rosiglitazone to HIV-1 Tat exposed animals will circumvent the neurodeficit and associated changes in spatial learning and memory, motor coordination, receptor constitution, and cytokines.

We found with the addition of rosiglitazone to HIV-1 Tat treated animals showed recovery of neurocognitive function. This recovery was evidenced in the behavioral testing using tests of motor coordination, executive function as measured by the novel object test, and spatial learning and memory as measured by both Barnes maze and the Morris water maze. These changes were noted to experience sex differences with the males and females being able to regain function at a constant rate. In the behavioral assays, the drug effect that was exhibited was antagonism and this was noted in both males and females in all behavioral assays.

We next analyzed the effect of rosiglitazone on receptor constitution inside the brain. The most significant finding was that the amount of GFAP receptor being

expressed. This is a marker of astrocyte health in the brain. To refresh, this cell is a supporting cell inside the brain with functions to aid blood brain barrier integrity and help with neuron function. This receptor expression was elevated in the combination group meaning that with addition of rosiglitazone to individuals that are infected HIV-1 have been shown to have some recovery of neurocognitive function. This is important because the first place the virus goes in the beginning stages of infection is the central nervous system and acts as a viral reservoir throughout the disease and in later stages, with the formation of HAND. The drug effect that was elucidated from the work was a drug effect of antagonism, which was seen in both males and females.

We lastly analyzed how the cytokine constitution was affected. The testing of the system for cytokines shows with treatment of rosiglitazone to HIV-1Tat animals' levels of cytokines returning to basal levels or lower. This is in line with published data and facts about the drug Rosi, with this drug having action on the PPARγ receptor superfamily. The conclusion can be made with prolonged treatment of Rosi to HIV-1 Tat animals, that the drug acts by an anti-inflammatory mechanism.

The fact that there is upregulation of the receptors is observed and the downregulation of the proinflammatory cytokine demonstrates that the environment has shifted away from a toxic environment that would be observed with prolonged exposure to Tat without intervention by pharmacologic aid. The increase in receptors means that there is an increase survival of cells in the neural environment and increases synaptic plasticity. This documented results of

administration of PPARγ drugs. We anticipated that these effects would uniform across the sexes, but these effects did not occur that way. They more sporadically across the receptors and cytokines. The effect generally gave the effect as discussed above.

This statement of the efficacy of the long-term treatment of HIV-1 infected individuals that are suffering from variant of HAND might benefit from long-term treatment with Rosi.

5.2 Personalized Precision Medicine

Given our findings, we will now discuss the premise of individualized treatment strategies using precision medicine. The use of precision medicine to treat conditions is a practice that gained approval over about 15 years and is increasingly being employed for many diseases and other conditions (Zhang 2015; Kohler 2018). The use of precision medicine has allowed practitioners to design treatment strategies around patient's conditions and comorbidities that the patient may have or may develop over the life span of the patient ("Precision Medicine: From Science to Value" 2018; Barker 2017; Crews et al. 2012; Zhang 2015). These comorbidities may involve the status of multiple comorbidities such as cardiovascular, development of diabetes, and substance use and abuse just to name a few.

Use of precision medicine allows the practitioner to offer strategies of treatment that will be more specialized for the patient. The use of pharmacogenetic profiles have been employed to consider a patient's genetic

profile with respect to drug metabolization status, such as being a fast metabolizer or a slow metabolizer of a HIV drug or combination of therapies that may hinder the ability of a drug to work (Chen and Snyder 2013; Crews, et al. 2012; Dlamini et al. 2021; El-Sadr, Rabkin, and DeCock 2016; Kumbale and Voit 2021; Mu et al. 2018). This can be used to anticipate and avert toxicities or toxic side effects (Cook et al. 2018). The practitioner taking this information into account allows the patient to have better therapeutic outcomes.

The fact that some patients have multiple diseases and may be taking multiple medications allows for the practitioner to use the pharmacogenomic profile to anticipate how a patient will respond to therapy ("Precision Medicine: From Science to Value" 2018; Chen and Snyder 2013; Crews, et al. 2012; Ma and Lu 2011; Whirl-Carrillo et al. 2021). The use of the pharmacogenomic profile lessens the amount of drug drug interactions that can occur. This a major concern that is present in the field of pharmacovigilance, which relates to the collection of information related to adverse effects of drug therapy (Eaton 2008).

For our study, that the patient would be infected with HIV-1 and drinking alcohol would cause issues with drug therapies the patient might be taking during the treatment. The patient is already infected by a virus that has cofactors (Tat, gp120, etc.) that are toxic by themselves in the neural environment. The drugs that these patients take to suppress the virus and viral replication are toxic in their own respects (Shah, et al. 2016). It would be well to know the patient's genotype for drug metabolizing systems. This would offer two good benefits. First, this will allow the practitioner to better manage the patient on their medication regimen.

Second, the practitioner would be able to predict drug interactions (Cook, et al. 2018; Ma and Lu 2011; Dlamini, et al. 2021; El-Sadr, Rabkin, and DeCock 2016; Kumbale and Voit 2021; Mu, et al. 2018; Marais et al. 2019).

The fact these patients are infected with HIV and drink alcohol will possibly amount to the patient having compromised hepatic system. The principal organ of the hepatic system is the liver. This is important for several reasons when considering drug metabolism and possible therapies for these patients. First, the patient having a compromised liver will allow parent drugs and metabolites to build to toxic levels. Second, the presence of parent drug and metabolites will cause prolonged toxicity for the patient causing undesired effects and possible biochemical irregularities. Third, the patient experiencing the biochemical irregularities will cause dysregulation of other body systems that will cause undesired effects for the patient.

There have also been recent advances and initiatives to undertake using precision personalized medicine in the US. President Obama worked towards increased funding for personalized medicine, and society as a whole is moving more toward an age of precision medicine in part to make treatments more efficacious and health care more efficient (Jaffe 2015). There are also some medical facilities that give their patients pharmacogenetic profiles at time of admission to help lessen the likelihood that they have drug interactions or unwanted side effects from drug therapy during the patient's time in that facility.

In conclusion, personalized precision medicine allows for the practitioner to give more reasonably targeted therapy to patients instead of a one size fits all

therapy (Kohler 2018). Knowing patient's genotype prior to prescribing drugs allows the practitioner to know what possible toxic side effects that may experience by the patient. The use of precision medicine, especially with the evidence provided in the three specific aims, demonstrates that each patient should be treated separately and individually and not as a collective to assign a particular treatment. Sex differences, alcohol use or abuse, and a myriad of other factors greatly complicate HIV treatment and should be taken into account in developing a treatment profile for patients. The present work advances our understanding of these factors and should help advance precision therapy for HIV patients experiencing HAND. The potential usage of rosiglitazone as a repurposed therapeutic for HAND patients was demonstrated here.

5.3 Future Directions

Based upon our findings, future investigators should perform these studies on two different fronts. Those fronts are using animals and coculture of human cells to verify synaptic plasticity, cell death, oxidative stress, and neurotransmitter analysis and ultimately perform clinical trials using rosiglitazone. First, we will discuss the animal front of this study.

5.3.1 Animal

Future researchers should use the same treatment strategies that are described in specific aims 1,2, and 3. They should perform the behavioral testing as described above. Once the treatment and the behavioral testing are completed, the samples should be collected and they should focus their efforts performing a

metabolomic study looking at synaptic plasticity changes and other concerning items, such as cell death and oxidative stress. They should also consider performing mass spectral imaging using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) to visualize the neurotransmitter relative abundance in key areas of the brain that are known to be associated with memory functions (Caprioli, Farmer, and Gile 1997; Chen et al. 2009; Chen and Li 2010; Esteve et al. 2016; Seeley and Caprioli 2012). These are key in understanding how the two toxicants (HIV-1 Tat and alcohol) work together to cause the toxicity and alter brain chemistry and cause brain environment alterations. It is also important to understand how the neurotransmitters are affected in the presence of these two toxicants.

Performing the same analysis with rosiglitazone is important. This will allow us to know how the presence of Rosi to decrease inflammatory environment and promote increased synaptic plasticity. This would verify the findings in specific aims 1,2, and 3. To continue to vet Rosi as a possible therapy for recovery of neurocognitive function, the researchers propose using the same methods and reasoning for performing a neurotransmitter analysis.

The performance of the animal front of this study will give more information in several ways. First, the information gathered from this study will allow to elucidate further the mechanism of how Rosi performs the effect of recovery of neurodeficit from the standpoint of decreasing neurocognitive function with the co-exposure to HIV-1 Tat and alcohol. Secondly, using mass spectral imaging will give information about how neurotransmitters and other metabolic products are

available in the brain for usage. Third, the study recommended will provide additional information of how rosiglitazone acts as a neuroprotective agent from the standpoint of use as an anti-inflammatory agent. The results gained from these studies will be used for the purposes of correlation in future human studies.

5.3.2 Human

For the human studies, future investigators use a co-culture system that involves human astrocytes, neurons, and other glial cells that will simulate the neural environment. The goal of using the co-culture method is to ascertain changes in the nervous system and blood brain barrier (Janigro et al. 1998; Nakagawa, Castro, and Toborek 2012; Strazza et al. 2016; Wilhelm, Fazakas, and Krizbai 2011). The co-culture will focus specifically on the brain looking for changes in synaptic plasticity, cytokine expression, metabolomic analysis, pharmacogenomic determinations, and finally visualization of receptors. These are optimal methods to ascertain how Rosi performs for circumvention of neurodeficit in the presence of alcohol and HIV-1 Tat. These results will help further our goal of using Rosi as a neuroprotective agent and will allow for further studies, such as clinical trial of Rosi for the purposes of demonstrating circumvention of HIV-1 Tat and alcohol induced neurodeficit.

Future investigators should also consider clinical trials to demonstrate the efficacy of Rosi as a neuroprotective agent that is able to circumvent both exposure of HIV-1 Tat and alcohol. This would allow them to perform three possible clinical trials. During these clinical trials, the researchers will collect peripheral blood to ascertain several items that will aid in the efficacy of the treatment and allow for a

robust analysis of how Rosi is being received by trial participants. These tests will involve cytokine determinations, proteomic and metabolomic determinations, and receptor evaluation. The other information that will be ascertained is the pharmacogenomic profile. This will give the information about how the trial participant will metabolize Rosi and will give a measure of variability to the study.

To perform the studies, the future investigators must have participants. For the first clinical trial, they should test whether how Rosi circumvents alcohol induced neurodeficit. The participants should be people with a history of or current alcohol users. These people should be free of cardiac history, which is based upon the pharmacology and toxicology of the drug.

The clinical trial will follow a particular sequence of events. These events are aimed at providing the researchers with the most information to gather on the usage of Rosi to have possible usage as a neuroprotective agent. First, the participants will have blood collected to ascertain a biochemical profile, and other testing that is mentioned above. Secondly, the participants will take part in a pre-test of cognitive function prior to taking a dose of Rosi. Throughout the clinical trial, the patients will provide blood samples throughout the study to monitor biochemical, receptor, and cytokine changes. At the end of test, the patients will take a post-test of cognitive function. The scores of both tests will be compared by the researchers.

The second clinical trial will be completed with the purpose of using Rosi to help ameliorate the neurodeficit that is induced by the exposure to HIV-1. The participants should be known infected with HIV and should be known to already be

demonstrating or diagnosis of a form HAND. Again, the participants should be free of any cardiac involvement or history. The participants should also be free of substance use, such as alcohol. The clinical trial should take place using the same framework as described above, with peripheral blood being collected and testing of neurocognitive function.

The third clinical trial will be completed with the purpose of using Rosi to help ameliorate the neurodeficit that is induced from co-exposure to HIV-1 and alcohol. These participants should show some form of neurocognitive involvement, such as diagnosis or current patient with HAND. These participants should be either current or have a history of alcohol usage, but free of any cardiac involvement or history. The clinical trial should take place using the same framework as described above, with peripheral blood being collected and testing of neurocognitive function.

The goal of the three clinical trials is to show how in multiple ways that Rosi will increase neurocognitive function. First, the researchers will want to show how the cytokine expression changes from the beginning of the clinical trial, duration, and end of the clinical trial. Secondly, the researchers will gain a better biochemical understanding of how Rosi changes the metabolic process to decrease neurodeficit induced by alcohol, HIV-1, and a combination of alcohol and HIV-1. Third, the researchers will be able to monitor the receptor constitution of the participant that will further information on how Rosi performs anti-inflammatory action. Lastly, the participant will receive a pharmacogenomic profile that will allow researchers to understand and account for variability on how participants

metabolize Rosi. All of this benefit will allow the formation of possible new therapy to be available for these cohorts of patients that are in need of additional therapies that would give relief from symptoms and outcomes from alcohol and HIV-1 Tat exposure.

5.4 Conclusion

In conclusion, we have completed a study that has allowed the following conclusions to be drawn:

1. We performed a study that allowed for the setup of a controlled system for alcohol study. We found that at 12 weeks of exposure there is considerable alterations in behavioral testing, Morris water maze, and down-regulation of synaptic plasticity factors.
2. We performed a study that allowed for the exploration of interaction between two toxicants that have known synergy. We showed that HIV-1 Tat and alcohol exposure cause alterations in motor coordination, executive function, and spatial learning and memory. We also showed that there shift in receptor constitution and cytokine expression with co-exposure to both toxic agents. The fact that the receptors down regulate and the cytokines increase show shift toward toxic environment which would potentiate the gained neurodeficit from prolonged exposure to Tat and alcohol.
3. We performed a study that allowed for the exploration of new possible therapy to circumvent neurodeficit induced by two toxic agents. The exposure to HIV-1 Tat and Rosi decreased induced neurodeficit and

caused increase in GFAP expression and decreased the production of pro-inflammatory cytokines. This increase in receptor up regulation and proinflammatory cytokines down regulation shows shift to a non-toxic environment. This is because of the drug properties of TZD and PPAR γ drugs. These drugs act as anti-inflammatory agents which further reinforces that a non-toxic environment is being formed.

With the conclusions from this study, we have offered a further study option that will further this work on two fronts. The first front is a continuation of this work by metabolomics and neurotransmitter analysis in animals. The second has two objectives to establish a co-culture setup to ascertain the effect on Rosi in the brain environment and performance of three possible clinical trials as described above. The completion of these studies also allows for the production of pharmacogenetic profiles. These profiles will allow the researcher to account for adverse reactions in clinical trial. It will also give necessary information to the researcher about prospective participants and information about other metabolic states that would influence the proposed clinical trials. The information gained will help give power and help with experimental design. These works will provide additional information to the field and will provide a possible new therapy to patients who need relief from symptoms they experience with exposure to HIV-1 and alcohol.

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VITA

Daniel Christopher Schwartz was born on January 21, 1985 in Garden City, Kansas to Frank and Nancy Schwartz. Daniel graduated from Liberal High School in the year 2004. He completed two undergraduate degrees from Wichita State University in Wichita, Kansas. He earned two separate but related degrees in medical technology and forensic science, where he graduated with the position of "Rite" with both degrees. Daniel proceeded to take and pass the registry exam from the American Society for Clinical Pathology, for which he currently holds licensure and membership. After completing the registry exam, Daniel started working as a staff medical laboratory scientist for Southwest Medical Center in Liberal, Kansas for four years. During this time, Daniel started and completed a master's degree in Clinical Toxicology from the University of Florida in Gainesville, Florida. Daniel now works for Prime Health Systems of the MOKAN region as a staff Clinical Laboratory Scientist.

Mr. Schwartz began his Ph.D. in pharmacology and pharmaceutical science at the University of Missouri-Kansas City (UMKC) in the Fall of 2015. While pursuing his Ph.D., he has presented abstracts at conferences, such as the BioKansas Innovation Festival and the Health Sciences Student Research Summit. He has also presented posters at these events. He has helped publish a paper in *Brain, Behavior, and Immunity*, which is a peer-reviewed journal.

With completion of this degree, Daniel will pursue research interests in clinical and research sciences. He will also continue to work to understand how infectious disease and toxic substances can alter drug response and response of

individuals to those substances. He will continue to work understand the need for documenting these responses, and how responses to substances should interpreted on an individual basis for patients requiring personalized, targeted medical care.