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# Individual Behavioral and Neurobiological Markers Associated with a Vulnerable to Ethanol use Phenotype

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**Abstract**

Alcohol use disorder (AUD) is a chronic relapsing brain condition that is characterized by excessive alcohol consumption, continued use when faced with negative consequences, and a negative emotional state associated with withdrawals (anxiety, irritability, depression). The main challenge to treating AUD is preventing relapse. The purpose of this study was to use a prolonged-exposure model to allow rats to self-administer ethanol to determine the brain regions active during relapse events. The rats performed multiple behavioral tests such as economic demand, negative consequences, and an elevated plus maze. These tests determined how hard rats were willing to work for an ethanol reward, how much they would persist in the face of negative consequences, and the level of anxiety-like behavior during withdrawal. These data were crossed-analyzed with neurobiological data collected from *c-Fos* immunoreactivity to determine a vulnerable to ethanol use endophenotype. The data were analyzed on an individual level to highlight differences associated with AUD and relapse. The preliminary results suggested that individuals with high demand for ethanol were willing to persist more in the face of negative consequences, had higher anxiety-like behavior, and were more likely to reinstate their ethanol-seeking behaviors during reinstatement. Based on this evidence, we predicted rats with higher demand would have higher rates of neural activity in areas associated with relapse. This study will provide valuable insight into the underlying mechanisms of relapse, but further clinical studies are necessary to use these phenotype-associated markers to develop a treatment plan that prevents relapse in individuals with AUD.

***Keywords:*** ethanol self-administration; alcohol use disorder; extinction; reinstatement; addiction; relapse

## Introduction

### *Significance*

Excessive alcohol consumption is a significant global public health concern, affecting individuals of all nationalities and ages. In the United States, it remains a major contributor to preventable deaths, resulting in approximately 140,000 deaths per year, and accounts for one in five deaths for individuals ages 20 to 49 years old.[1] In addition to the adverse effects on mortality, excessive alcohol consumption has a substantial impact on the economy, costing approximately $249 billion per year, with 72% attributed to a loss in workplace productivity.[2] There are different types of excessive alcohol consumption including binge drinking and heavy drinking. Binge drinking refers to men consuming 5 or more drinks on occasion or women consuming 4 or more drinks on occasion.[3] Heavy drinking is defined as 15 or more drinks a week for men and 8 or more drinks per week for women.[3] While binge drinking and heavy drinking do not equate to alcohol dependency or addiction, they can lead to alcohol use disorder (AUD).[4]

Alcohol use disorder (AUD) is a chronic relapsing brain disease, characterized by excessive alcohol consumption, craving alcohol, continued consumption in the face of negative consequences, and withdrawal symptoms such as irritability, depression, and anxiety.[5] People suffering from this disease are often willing to endure negative consequences related to their health, social lives, and occupations.[5] Individuals with AUD often have the compulsion to find and consume alcohol and do not have the control to limit their intake.[6] Risk factors for this disease include early exposure to alcohol, family history of addiction, mental health disorders, and past trauma.[7] In 2019, there were 28.6 million adults and 894,000 adolescents aged 12 to 17 with AUD.[7] People with AUD represent 71.1% of all individuals with substance use disorders.[8]

In the U.S., there are 700,000 people seeking treatment each year, [9] which include behavioral therapy, community help groups, and medications such as acamprosate and naltrexone.[10] Despite treatment, 95% of patients with this disease relapse at least once during their recovery.[11] Understanding the neurobiological markers associated with relapse is crucial for developing more effective, long-term treatments for AUD.

***Previous Research***

Previous research has explored the neurobiology of ethanol use within humans including topics such as executive function and the dopaminergic neurotransmitter system. Executive function refers to the ability of one to inhibit impulsive thoughts, plan, pay attention to tasks, and think about the consequences of an action before performing that action.[12] If an individual lacks executive function it can lead to repetitive addictive behavior such as seeking ethanol, consuming alcohol even when paired with negative consequences, and relapsing when certain environmental cues are present.[12] Cognitive dysfunction and the model of behavioral changes suggest that behavioral therapies would be most effective when geared towards discussing motivations and goals to bring back executive function.[12]

Other research has focused on the dopaminergic neurotransmitter system, specifically mesolimbic dopamine neurotransmission in humans. It was found that the activation of these neurons acts as a learning signal during times when individuals are expecting a reward.[13] The motivation to consume alcohol is often positively reinforced at the beginning of use, but over time neuroadaptive changes occur and the substance is reinforced by negative reinforcement[14] Glutamate is known to be the main excitatory neurotransmitter; however long-term alcohol consumption upregulates the glutamate systems which can trigger relapse.[14] Between the

upregulation of glutamate and the down-regulation of the GABA system, the main inhibitory neurotransmitter, it creates an imbalance and causes negative emotional states during withdrawal.[14] Long-term ethanol use can also dysregulate the stress systems, which results in a negative emotional state and potential relapse.[14] The dopamine system plays a key role in developing alcohol dependency and craving behavior. The reward system involves the extended amygdala, mesolimbic dopaminergic pathway, ventral tegmental area, ventral striatum, nucleus accumbens, and prefrontal cortex.[14] The increased release of dopamine reinforces the drug-addicted behaviors; however, hypo-dopaminergic states have been observed in individuals with chronic alcohol use and cause a negative emotional state and increase cravings, which can lead to relapse.[14]

Preclinical and clinical trials are essential components of medical research, and they are frequently utilized in the development of treatments for various diseases, including drug addiction. Clinical studies use human participants to collect data on various research topics, but ethical concerns and strict regulation limit their scope. In preclinical rat models, there are still strict regulations and extensive approval processes however, studies can be conducted that would not be possible in humans. Preclinical studies can control more variables and can employ methods that would not be ethical to conduct on human subjects. For example, it would not be ethical to force a person to become addicted to a substance and go through withdrawals to gain knowledge about the neurobiology involved. Unlike clinical models, preclinical rat models can give addictive substances to rodents, provoke withdrawal states, and not only collect behavioral data but also extract their brains for further analysis.

Preclinical rat models are a valuable tool for studying addiction since rats exhibit similarities in both neurochemical and neuroanatomical features to the human brain.[15]

Additionally, rats and humans display similar addictive behaviors, such as craving the substance, enduring negative consequences to get the substance, and withdrawal symptoms upon cessation of use.[15] Previous studies have used rodent models to study alcohol dependency and have found that many of the same neurotransmitter systems that are affected in humans are affected in rats including the regulation of dopamine, glutamate, GABA, serotonin, and norepinephrine.[16] Studying the neurobiology and behaviors of rats that consume ethanol can provide insight into AUD in humans.

Rat models studying addiction often use a self-administration model, which allows the subjects to consume as much or as little of a substance as they would like. The present study utilized an operant conditioning model, in which subjects were trained to use a nose poke on a fixed ratio (FR) schedule, and then a variable ratio (VR3) schedule during the self-administration phase. Self-administration allows researchers to explore the motivation underlying ethanol-seeking behavior. Different programs can be used to determine economic demand for ethanol and to compare behavior between extinction and reinstatement to see if ethanol-seeking behavior was reinstated.[17] One caveat to self-administration is it must be long-term, and ethanol must be consumed for intoxication and not for taste or calories.[18] It is also important that they are voluntarily consuming enough ethanol to be intoxicated to a level similar to humans.[18] To ensure the rats are consuming enough ethanol, some studies take blood samples to determine their blood alcohol content. In the current study, however, we calculated grams of ethanol consumed per kg of body weight, due to the use of male and female rats who differ in weight sometimes by 100 grams.[18] The residue volume in the sipper bottle is used as a basis for the amount of ethanol consumed and the time spent in contact with the sipper bottle can be used to verify how much was consumed. Self-administration offers a straightforward approach to determining rewards

earned and ensures voluntary consumption like humans, which makes it easy to view in the context of behavioral economics.

Preclinical rat models commonly employ behavioral economics, a field that explores the decision-making processes underlying human behavior, particularly why individuals make choices that are not the most cost-effective.[19] For instance, why would an individual continue to consume alcohol if it is not benefiting them, and instead is causing them to lose their job or have health problems? To study the behavioral economics of drug addiction, rat models are frequently used to explore why individuals make decisions that may harm them. Behavioral economics views drugs as reinforcers that have dynamic reinforcing values based on the price of the drug, other reinforcers available, and the reinforcing effects.[19] One way to study behavioral economics is called economic demand, a mathematical model that can determine how much an individual is willing to pay for a given substance.[20] In humans, this could involve assessing the amount of money an individual is willing to spend or the distance they are willing to travel to obtain the substance they are addicted to. In rat models, the cost is often measured in the number of lever presses or nose pokes required to obtain the ethanol reward. By using an escalating fixed ratio where the number of nose pokes required increases each day, researchers can determine how much each rat is willing to work for a single reward. This approach allows for a quantitative comparison of human and rat behavior.[20] Economic demand can be used to predict addictive behaviors such as relapse and ethanol-seeking, which makes it a valuable tool for alcohol use disorder research.[20]

### *Aims and Hypotheses*

This study's objective was to use a preclinical rat model to identify an endophenotype that makes individuals vulnerable to AUD. The project employed prolonged exposure ethanol self-administration to enable rats to consume ethanol for 10 hours per day for four months. Data were collected on the volume of ethanol consumed and the number of active and inactive nose pokes. Multiple different behavioral tests were conducted: reinforcer demand tests, responding in the face of negative consequences, and the elevated plus maze. The behavioral reinforcer demand test aimed to assess how hard the rats were willing to work for an ethanol reward. The negative consequence tests were used to determine if a mild foot shock deterred rats from seeking ethanol or if they were willing to persist in the face of negative consequences. The elevated plus maze was used to determine the level of anxiety-like behavior during withdrawal. The extinction phase extinguished ethanol-seeking behavior before a reinstatement session was conducted, which acted as a relapse event. After the relapse event, the rats were perfused, and the brains will be stained and analyzed using *c-Fos* immunostaining to highlight areas active during relapse.

This research is important because it is taking a unique approach to studying AUD by focusing on individual differences instead of grouped data. The data from the three behavioral tests and the neurobiological data will be analyzed on the group and sex level. However, the focus will be on individual differences because ethanol use in rats varies significantly, similar to how AUD varies among humans. Analyzing individual differences will prevent valuable results from being lost in the grouping process and will help find a relationship between specific rat behaviors, neurobiological markers, and relapse. Discovering these connections will be critical in developing a targeted treatment for AUD effective at preventing relapse events.

During the behavioral reinforcer demand test, we hypothesize that rewards earned will decrease as the required number of nose pokes increase. We also predict predicted that as the amplitude of mild foot shock increases the number of ethanol rewards earned will decrease. We hypothesize that individuals with high demand for ethanol would persist more when facing negative consequences, have higher anxiety-like behavior, and be more likely to reinstate their ethanol-seeking behaviors. We also expect individuals with high demand for ethanol to have more brain activity in the regions associated with relapse compared to those with low demand for ethanol, low persistence, and low anxiety-like behavior. The results from this study will inform our understanding of the neurobiological effects of ethanol use in individuals and can aid in future AUD treatment research.

## Methods

### *Animals*

A cohort of twelve Wistar rats (six male and six female) was obtained from Envigo (Indianapolis, IN, USA). They were single-housed in a vivarium with temperature and humidity controls, and a 12-hour light/dark cycle, with lights turning on at 1900. The subjects acclimated to their cages and surroundings for a week, during which ad libitum access to food and water was provided. Following the acclimation period, the rats were handled twice daily for two minutes over four consecutive days. During the last three days of handling, food was restricted to maintain the rats' body weight at 90% of their free-feeding weight. To promote normal development and growth, the weight criterion was increased by two grams each month. All procedures were approved by the University of New Hampshire Institution Animal Care and Use Committee (IACUC).

### *Apparatus*

Med Associate Conditioning Chambers were utilized for training, ethanol self-administration, negative consequence test, extinction, and reinstatement. Each chamber measured 30.5 x 24.1 x 21.0 cm (l x w x h) and was equipped with a sound and light-attenuating cubicle to isolate the rats from external stimuli. The walls of the chambers were aluminum, while the door, back wall, and top panel were made of polycarbonate. One wall featured two nose pokes, two cue lights, and an opening for a sipper bottle: the opposite wall was equipped with a house light (white 28 V, 100 mA lamp). The nose pokes were used to operate the sipper bottle during ethanol self-administration and training. When nose pokes earned the rat access to 12% ethanol, the sipper bottle was presented. Infrared beams detected movement, and metal grid floors were utilized to accurately measure the amount of time the rat was in contact with the sipper bottle. A combination of volume consumed, and time spent in contact with the sipper bottle was used to determine ethanol consumption. During negative consequence testing, Med Associate current aversion stimulation modules were used to administer mild foot shocks through the grid floors.

### *Drugs*

During the study, various solutions were used for self-administration. Solutions were made using tap water, ethanol (200 proof; Decon Labs; King of Prussia, PA, USA), and sucrose (store-bought sugar). Throughout the study, 85 ml of solution was dispensed into each sipper bottle, and the residual volume in each bottle was recorded at the end of each session.

***Phases of the Study***

The rats started by acclimating to the vivarium and human handling for one week before they started self-administration training with 12% sucrose. Then a sucrose fading procedure was administered to transition the rats to consuming 12% ethanol. After the rats learned to self-administer ethanol, they went through various behavioral tests, behavioral reinforcer demand testing, negative consequence tests, and then an elevated plus maze test. The rats went through extinction before a reinforcement session that encouraged a relapse event. The rats were then perfused, and the brains were extracted for later c-Fos staining and analysis (Figure 1).

*Ethanol Self-Administration Training*

During the rats' dark cycle, self-administration sessions were conducted for 10 hours each day, beginning at 800 and concluding at 1800. A two-week training period was conducted for the rats to acquaint themselves with the use of nose pokes and the identification of stimulus lights (FR1 program). The sipper bottles were filled with 12% sucrose solution, and stimulus lights were illuminated when rats could earn a sucrose reward. Each chamber had one active nose poke that was used to earn ethanol rewards and an inactive nose poke that even when used did not result in an ethanol reward. One active nose poke resulted in one reward, which triggered the stimulus lights to turn off, the houselights to turn on, and the sipper bottle to enter the chamber for six seconds. For training purposes, the sipper bottle was presented for 11 seconds on the first day of FR1, then 6 seconds for the remainder of the training period.

*Sucrose Fading*

After training with a 12% sucrose solution, ethanol was gradually introduced using the sucrose-ethanol fading procedure to allow the rats to become accustomed to ethanol (Figure 1). Over the course of two weeks, ethanol slowly faded in until the solution was 12% sucrose 12% ethanol, then sucrose slowly faded out. The dilution can be observed in Figure 1. Each dilution was used for three days, except 12% sucrose 12% ethanol was used for six days. By the end of the fading procedure, the solution in the sipper bottles consisted of 0% sucrose 12% ethanol, which was used for the remainder of the study for ethanol self-administration.

To make the initial 12% sucrose solution, 12 grams of sucrose was placed into a beaker and filled to a final volume of 100 ml using tap water. To make a solution containing sucrose and ethanol the sugar was measured in grams, the ethanol was measured in ml, and the final volume was adjusted to 100 ml with tap water. For instance, the 12% sucrose 4% ethanol solution was prepared by weighing 12 grams of sucrose, adding 4 ml of ethanol, and adjusting the volume with water to reach a total volume of 100 ml.

*Economic Demand testing*

During economic demand testing, the rats had to achieve a certain number of nose pokes before an ethanol reward was presented. The testing sessions were conducted for 10 hours during the rats' typical self-administration session. Each day the required number of nose pokes increased using an escalating fixed ratio in the following sequence: 1, 3, 5, 8, 12, 18, 26, 38, 58, 86, 130, 195, 292, 438, 658. (Figure 1). Once a rat failed to receive a single ethanol reward during demand testing, that rat returned to self-administration. This test measured how many ethanol rewards they received at each economic cost.

*Negative Consequences*

The rats underwent eight negative consequences tests, with one day of ethanol self-administration (VR3) to retrain the rats. During the initial two hours of the negative consequence test, 50% of the earned ethanol rewards were paired with a 0.5s mild foot shock. The foot shock did not harm the rats but was used as a potential deterrent from responding for ethanol rewards. For the last eight hours of the session, rats earned ethanol with no mild foot shocks. Over the eight sessions, the shock intensity increased from 0.18mA to 0.54mA (Figure 1). These tests measured how many ethanol rewards they earned at varying negative consequence intensities.

*Elevated Plus Maze*

During the 12% ethanol self-administration, the elevated plus maze tests were conducted 10-11 hours after the rats' last self-administration session to observe withdrawal-like symptoms. The elevated plus maze comprised four arms, two open and two enclosed. Rats spending time in the enclosed arms were correlated with anxiety-like behavior and stress, whereas rats that stayed in the open arms did not show anxiety-like behavior. ANY-maze software was used to record the 10-minute sessions and collect data that determined how much time the individual rat spent in each section. The information from the first five minutes of the test was considered an acclimation period and therefore was excluded. The measures taken from this test were time spent in open arms, time spent freezing, and total freezing episodes.

*Extinction*

Extinction was 14 days long and occurred during the 10-hour sessions within the Med Associate chambers. During this phase, all environment cues that had been associated with

ethanol self-administration were removed including house light cues, stimulus lights, sipper bottles, and the scent of ethanol. Environmental cues and ethanol were not present during the extinction phase to extinguish the ethanol-seeking behaviors.

*Reinstatement*

Following the extinction phase, each rat went through one 30-minute reinstatement session before being perfused. During reinstatement, the house light turned off and the stimulus lights flashed on and off 5 times every five minutes. The use of active and inactive nose pokes was recorded to determine if the ethanol-seeking behavior was reinstated. Reinstatement occurred over 4 days, with three rats going through reinstatement each day. The first rat started reinstatement at 0730, the second rat at 0800, and the third rat at 0830. Two rats did not go through reinstatement to act as controls for the *cFos* processing.

*Perfusions and Brain Tissue Preservation*

Perfusions started one hour after reinstatement, to ensure peak *c-Fos* expression in regions of the brain associated with reinstatement in rats and relapse in humans. Rats were euthanized with sodium pentobarbital and transcranial perfused (150 ml 0.9% saline, 150 ml 4% paraformaldehyde). The brains were then extracted and placed in 4% paraformaldehyde at 4° C for 24 hours. The brains were cryoprotected using a 30% sucrose solution that was diluted with 0.02 M PBS at 4° C for 3 days. The brains were stored at -80° C for future sectioning and analysis.

*c-Fos Antibody Processing*

The last phase of the study consists of processing the preserved brains and collecting neurobiological data. The rat brains will be stained using *c-Fos* antibodies, which stain *c-Fos* proteins within the brain tissue to show the brain regions active at the time of death. The brains will be mounted and stained for viewing in the confocal microscope to measure the intensity of c-*Fos* expression in various regions. The regions that will be observed include the orbitofrontal cortex[21], ventral pallidum[22], ventral tegmental area[23], lateral hypothalamus[24], nucleus accumbens core and shell[24], basolateral amygdala[24], prefrontal cortex[25], and the hippocampus. [25]

*Statistical Analyses*

We will analyze the data collected from the behavioral tests and *c-Fos* staining on three levels: individual, grouped by gender, and cohort. By analyzing the data on the individual level, we hope to highlight the individual differences in addiction, while also showing sex differences and general trends for grouped data. Multivariate analysis will be used to determine if there are any connections between economic demand, response in the face of negative consequences, level of anxiety-like behavior, and neural activity during reinstatement. In addition to individual data analysis, we will analyze the behavioral data via conducting repeated measures ANOVA tests to compare sex and group differences.

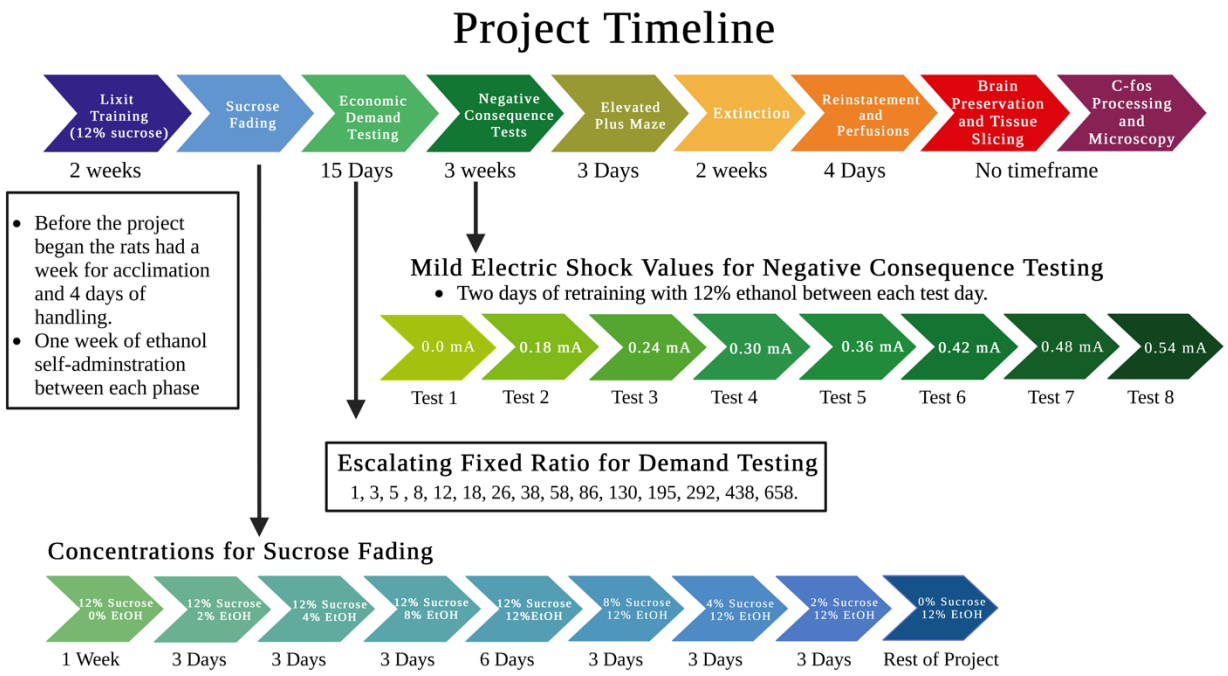


**Figure 1:** The project timeline above shows the order and duration of each phase of the study. Two iterations of the study will be conducted.

## Results

The first iteration of the study provided valuable behavioral data that showed general group trends. However, neurobiological data and elevated plus maze data will not be analyzed until the end of the second iteration. Grouped economic demand testing data showed a decrease in ethanol consumption (g/kg) as economic cost increased (Figure 2). Some rats consumed more than others at higher economic costs, but all rats stopped consuming ethanol by the time the cost reached 658 nose pokes. Although the individual responses have not been analyzed yet, there is economic demand variation within the subjects, which can be observed in Figure 2. Essential value is a parameter gathered from the behavioral economic demand model, and it is the value that each individual assigns to ethanol as a reinforcer. The essential value will be analyzed based

on the ethanol consumed (g/kg) using R studio with the "beezdemand" package. Although the mean essential value for female rats was higher than that of male rats these differences were not significant ($p = 0.1734$). (Figure 3). Grouped negative consequence data showed that as the amplitude of the mild foot shock increased the number of ethanol rewards earned decreased (Figure 4). Nose poke data from extinction and reinstatement were compared to determine if ethanol-seeking behavior was reinstated during the reinstatement session. There were nearly significant differences in active nose poke responses from extinction to reinstatement tests, with there being more active nose pokes during the reinstatement session than the average nose pokes during extinction ($p = 0.0514$; Figure 5).

The data from the first iteration suggests that individuals with higher economic demand also persist more in the face of negative consequences and have higher anxiety-like behavior. On the other hand, individuals with low demand often do not persist in the face of negative consequences and have lower anxiety-like behavior, which is likely due to low consumption. Rats with higher demand, higher persistence, and higher anxiety-like behavior are more likely to reinstate their ethanol-seeking behavior during reinstatement. The data trends observed in the first iteration are also being observed within the second iteration, although full statistical analysis will not be completed until the end of the second iteration in the Summer of 2023. The final multivariate analysis will determine the significance of these relationships.

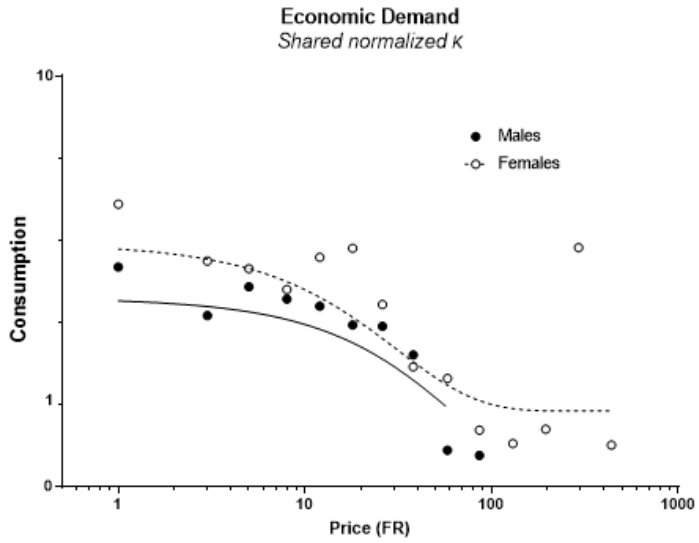


**Figure 2:** Grouped economic demand data by sex showing the relationship between ethanol consumption (g/kg weight) and increasing economic cost (FR).

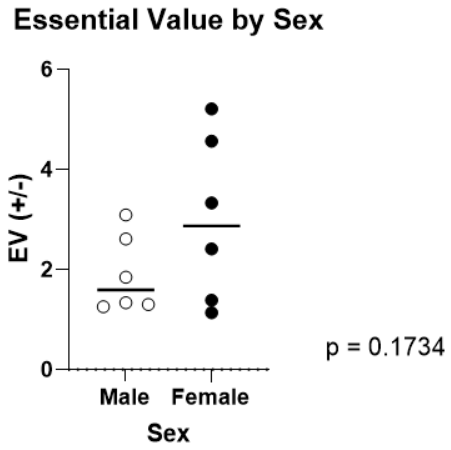


**Figure 3:** The grouped sex essential values during economic demand testing. The horizontal bars represent the mean essential value for each sex.

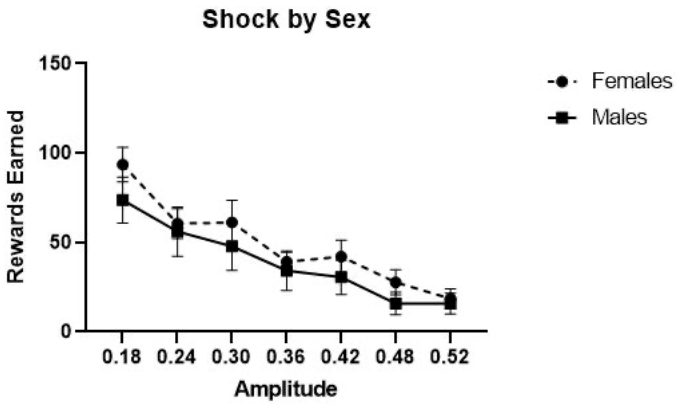


**Figure 4:** Average grouped rewards earned for each negative consequence test as the amplitude of the mild foot shock increased.

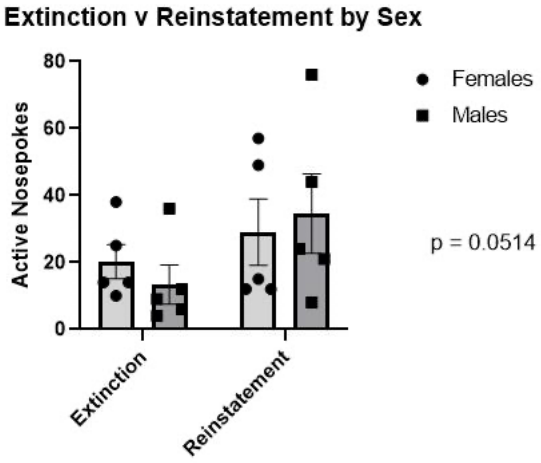


**Figure 5:** Group data for the average number of active nose pokes during extinction versus during reinstatement.

## Discussion

After analyzing the data collected during the first iteration of the study, our hypotheses are supported. The preliminary grouped data from economic demand tests showed an inverse relationship between the cost of the reward and the number of rewards earned. This suggests that as the cost of the reward increases, individuals are less likely to earn rewards due to the high

economic cost involved. The essential value (EV) is an important measure because it quantifies the value that each individual assigns to ethanol as a reinforcer. It is expected that individuals with a higher EV will be more likely to continue use as the cost of ethanol increases, be more persistent in the face of negative consequences, and be more likely to relapse. Previous studies have shown this relationship in rats consuming ethanol[26] and heroin.[27] There was no statistically significant difference between males and females during the economic demand testing. However, it is expected that with more data, there will be significant differences in behavior between the sexes. One study found that there were sex differences in ethanol-withdrawal behavior, which was attributed to different hormone levels and differences in brain structure.[28]

In the negative consequence testing, the grouped data showed an inverse relationship between the rewards earned and the amplitude of mild shock. The rats were willing to endure negative consequences, but as the severity of the negative consequences increased, their ethanol-seeking behavior decreased. Extinction and reinstatement nose poke data showed an increase in active nose pokes during reinstatement. This suggests the ethanol-seeking behavior was being reinstated. Our findings align with a prior study that found after ethanol-seeking behavior is suppressed through extinction the behavior can be reinstated with environmental cues, this is called context-induced relapse. [29]

The preliminary data suggest a relationship between the subject's economic demand, willingness to persist in the face of negative consequences, and their likelihood of reinstatement. Individuals with higher economic demand for ethanol tend to persist more in the face of negative consequences and are more likely to reinstate ethanol-seeking behavior during reinstatement. Conversely, individuals with low economic demand tend to persist less in the face of negative consequences and are less likely to reinstate ethanol-seeking behavior. Once both iterations are

complete, it is expected rats with high demand and high persistence in the face of negative consequences will have high anxiety-like behavior and that rats with low demand and low persistence will have low anxiety-like behavior. The implications of these findings suggest that individuals with AUD who consume higher amounts of ethanol, work harder for ethanol, and continue consuming ethanol even when faced with negative consequences are more likely to relapse. These results provide valuable insight into the complex relationship between economic demand, negative consequences, and anxiety-like behavior in the development and maintenance of AUDs.

Preliminary data were analyzed at the group level, but the final statistical analyses will focus on individual and sex differences. Throughout the data collection process, variability was observed among the subjects in their ethanol consumption, willingness to work for rewards, and resilience to negative consequences. Variations in ethanol self-administration are similar in human substance users and therefore it is important to look at the connections between these behavioral tests and neurobiological data on an individual level, to represent the human population most accurately. It is important as well to look at sex-specific differences in order to capture all variability that could help influence tailored treatment in the future. Our goal is to look at the group for general trends and to look at individuals and sex to determine specific relationships between alcohol consumption, economic demand, response to negative consequences, anxiety-like behavior, and neural activity.

Prior studies on AUD have primarily focused on grouped data and employed short-term exposure protocols in animal models. In such studies, rats self-administer ethanol for only two hours every other day.[29] However, people with AUD often drink throughout the entire day, every day. Therefore, in this study, a long-term exposure method was adopted, which allowed the rats

to self-administer ethanol for 12 hours per day, seven days a week, over 4 months. Our goal was to mimic human ethanol consumption as closely as possible to produce more consistent and representative data. Data from this method will better contribute to our understanding of the underlying mechanisms involved in AUD and relapse events.

The next step of the study involves the completion of the second iteration, which is currently underway with 12 rats. The current data and general trends align with the predicted results; however, the data and trends are not significant due to the small sample size. Finishing the second iteration will increase our sample size and statistical power, further strengthening our results and supporting our hypotheses. During the summer of 2023, the collected data will be analyzed, and the brains will be processed using *c-Fos* antibody staining and confocal imaging. The *c-Fos* staining technique stains regions that are active at the time of perfusion by staining *c-Fos* proteins. In this case, areas of the brain active during the reinstatement session, relapse event, will be highlighted. It is expected that areas such as the nucleus accumbens core and shell, basolateral amygdala, prefrontal cortex, hippocampus, lateral hypothalamus, orbitofrontal cortex, ventral pallidum, and the ventral tegmental area will be highlighted.

Since AUD is a chronic relapsing brain disease, the main challenge with treatment is preventing relapse. This study aims to identify specific brain regions associated with relapse; however, it will not determine the pathways nor present treatments for AUD. Further preclinical studies are necessary to explore inhibiting the pathways involved with relapse. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs), for instance, could be utilized to selectively inhibit specific pathways to investigate if they are involved in relapse within rat models. Preclinical rat models can be used to simulate human addiction as rats exhibit similar alcohol-seeking behaviors and brain structures. However, AUD is a complex human condition

that requires extensive investigation at multiple levels to develop effective treatments. Clinical studies need to be conducted to further study the identified brain regions associated with relapse. Pharmaceutical studies could then use these data to tailor treatment methods to the specific brain regions identified to improve AUD patient care and prevent relapse events.

In conclusion, the preliminary findings show that as the cost of the ethanol reward increases the number of rewards decreases, and as the amplitude of the mild foot shock increases the number of rewards also decreases. Upon multivariate analysis, we expect to see individuals with higher economic demand, higher persistence in the face of negative consequences, and higher levels of anxiety-like behavior to be more likely to reinstate ethanol-seeking behavior. Further analysis of brain regions will be necessary to determine the specific areas active during a relapse event. This study takes a unique approach to ethanol self-administration by using a prolonged exposure model and analyzing the data individually and grouped. The present study will provide valuable data about individual differences associated with AUD and the brain regions active during relapse. With this information, preclinical studies can determine specific pathways involved with relapse. Further clinical research will provide additional insights into the underlying mechanisms that contribute to relapse and inform the development of targeted interventions to treat AUD.

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