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Individual Behavioral and Neurobiological Markers Associated with Vulnerable to Ethanol Use Phenotype

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Abstract

Alcohol use disorder is a chronic, relapsing brain condition that affects 29.5 million Americans. The disease is characterized by loss of control over drinking, continued use of alcohol in the face of negative consequences, and the experience of withdrawal symptoms. While there are several forms of treatment available for alcohol use disorder, 95% of patients experience at least one relapse during recovery. Currently, the high tendency to relapse remains the major challenge standing in the way of successful treatment for alcohol use disorder. Research is continuing to be conducted into the behavioral and neural mechanisms underlying relapse into alcohol use, and preclinical studies are using novel methods to investigate possible new avenues of treatment. The purpose of this study is to determine a vulnerability to relapse brain phenotype for alcohol use disorder, to better understand the brain regions involved in alcohol-use relapse and aid in future treatment research. The study uses a rodent model, with a project design utilizing behavioral economics to incorporate behavioral and neurobiological components to the project. Our preliminary results show differentiation between rats with high economic demand and low economic demand for ethanol. Those with high economic demand are more likely to persist in the face of negative consequences and reinstate ethanol-seeking behavior after a period of extinction. While this data is not yet significant, we expect it to be with the addition of more subjects. The neurobiological data for this study will be collected in summer 2023.

Introduction and Background

Excessive alcohol consumption is a leading cause of preventable death in the U.S., as it claims 79,000 lives per year.[1] It causes an economic burden of approximately $249 billion per year on the U.S., comprised of lost productivity, motor vehicle accidents, criminal justice costs, and healthcare expenses.[2] 29.5 million Americans suffer from alcohol use disorder (AUD), including 16.3 million men, 12.4 million women, and 894,000 adolescents.[2] In 2020, a reported 10.2% of the U.S. population was suffering from AUD, and the disorder is most prevalent in the 18-25 age group.[3] AUD is a chronic, relapsing brain disorder that is characterized by negative emotional states associated with withdrawal, compulsions to seek and drink alcohol, and loss of control limiting the intake of alcohol.[4] AUD, like other substance addictions, has three phases associated with impulsivity and compulsivity: binging, withdrawal, and craving.[4] Risk factors for this disorder include early drinking exposure, genetics, and mental health conditions.[5] Treatments are available for AUD, comprising of medications, behavioral treatments, and mutual help groups.[5] Approximately 700,000 people in the U.S. receive treatment every day[1], however, this is less than 10% of those afflicted.[2] While treatment is available, it is still not completely successful. The tendency to relapse associated with AUD remains a major challenge for recovery, as 95% of patients will experience at least one relapse during treatment.[2]

Long-term ethanol use affects several neurotransmitter systems in the brain. One of the main systems affected is the dopaminergic neurotransmitter system. Dopamine is the most important neurotransmitter in the reward circuit, and it mediates the reinforcing effects of all addictive drugs.[6] The primary structures involved in the reward system include the extended amygdala, mesolimbic dopaminergic pathway, nucleus accumbens, prefrontal cortex, and the ventral tegmental area.[6] Ethanol consumption causes excessive firing of the dopaminergic

neurons, which over time causes imbalances and an increase in neurotransmitter tolerance in the brain regions involved in the circuit.[6] Similarly, due to its role in the stress and reward circuit, serotonin deficiency has been implicated in the development of alcohol dependency.[6] Long term ethanol use also causes an imbalance between GABA, the primary inhibitory neurotransmitter of the brain, and glutamate, the primary excitatory neurotransmitter of the brain.[6] A decrease in GABA and overstimulation of glutamate is thought to play a significant role in creating negative emotional states during withdrawal and triggering relapse events.[6] Research into adolescent ethanol consumption has also shown serious developmental impacts on the brain, and it has been linked to gray matter volume deficits, in regions such as the prefrontal cortex, hippocampus, cerebellum, and limbic system.[7] Poor white matter integrity and increased cortical thickness have also been implicated in adolescent ethanol consumption.[7]

Preclinical models are commonly used to study addiction, a majority using rat and mouse subjects. While no preclinical model can perfectly mimic human behavior or neurobiology, the use of rodent subjects is less limited by ethics than the use of human subjects.[8] It is not ethical to make human subjects become dependent on ethanol for the purpose of a study, nor to cause alcohol dependency in humans under the controlled environment needed for the purpose of a preclinical study.[8] It is also not possible to extract and examine neural tissue from a human in the same way as for a rodent subject. While all rodent subject studies are held to a standard of ethics, using rodent subjects in preclinical models is a more ethical way to study addiction in a preclinical setting rather than using human subjects.[8] Rodents appear to be similarly affected at the behavioral and neurobiological level by ethanol. From prior research, the neurotransmitter systems affected by ethanol in rodents include several of the same systems affected in humans, including glutamate, GABAergic, and dopaminergic.[9] Other systems affected in rodents include

the opioidergic, nicotinic, endocannabinoid, CRF, melanocortin, and ghrelin systems.[9] Similar brain regions are implicated in being affected by ethanol in rodents, including the hypothalamus, amygdala, ventral tegmental area, prefrontal cortex, and nucleus accumbens.[9]

Behavioral economics is an emerging field of research intersecting psychology, economics, and pharmacology.[10] It is a promising method of studying substance abuse disorders in preclinical models.[10] The purpose of behavioral economics is to help scientists understand why humans and nonhuman animals make the decisions they do and to characterize systematic decision-making mechanisms.[10] One of the most relevant contributions of the field is the concept of economic demand, which uses quantitative analyses to understand the consumption of a commodity at a given cost.[10] For example, a human's economic demand for ethanol will be dependent on how much money they are willing to spend on ethanol. Using economic demand, neuroscientists can create mathematical models used for evaluating drug motivation in rodent models.[11] For example, a rat's economic demand for ethanol will be dependent on how many times it is willing to press a lever to retrieve ethanol. The use of behavioral economics in preclinical models allows for the quantitative alignment of human and rodent dependency on a given drug.[11]

In a laboratory setting, rats and mice have been shown to voluntarily consume quantities of ethanol for the purpose of strong intoxication, making them a candidate laboratory animal for addiction preclinical studies.[12] While no preclinical model is exact, the drug seeking behavior of rodents is similar enough to the drug seeking behavior of humans that it makes them a valid method of study for ethanol abuse.[12] For a preclinical rodent model to be considered a comparison to human ethanol dependency, it must meet certain criteria.[9] The rodents must voluntarily administer the ethanol to a substantial degree over a period of time in order to obtain

a pharmacologically relevant blood alcohol level comparable to humans.[9] The rodents must also be obtaining the ethanol for its intoxicating effects, and not for the purpose of taste or calorie intake.[9] Self-administration models are the most reliable method of achieving this. There are two types of self-administration models: operant and non-operant.[12] In this study, we are using an operant model of self-administration. In an operant model of self-administration, the rodent will perform a task such as nosepoking or pressing a lever on a schedule of reinforcement to earn a presentation of the drug.[12] Rodents are usually trained on a fixed ratio (FR) schedule of reinforcement, then moved to a progressive ratio (PR) or variable ratio (VR) schedule of reinforcement for maintenance.[12] Having the rodents perform a task (nosepoking or lever pressing) allows for easily quantifiable economic demand for ethanol, as these actions can be tracked and recorded.

The purpose of this study is to determine a vulnerability to relapse brain phenotype for ethanol use. We are using a rat model to investigate the behavioral and neurobiological components underlying relapse in ethanol dependency. There are three behavioral tests involved in this study, designed using the principles of behavioral economics. The purpose of these tests is to determine each rat's individual economic demand for ethanol. The rats will undergo a period of extinction before experiencing a reinstatement test. After this reinstatement test the rats will be perfused and their brains will undergo *c-Fos* antibody processing to highlight the brain regions that were active during the reinstatement event. Each rat's brain activity during reinstatement will be compared to its level of economic demand displayed during the behavioral testing portion. We are using several novel methods in this study. The rats will undergo a long-term, prolonged-exposure self-administration model to make their self-administration comparable to human ethanol use. The data from this study will also be analyzed at a group, sex-

related, and individual level, to gain insight into the individual differences in ethanol dependency rather than just at the group level.

We hypothesize that rats with high economic demand will persist more in the face of negative consequences and show higher anxiety-like symptoms, compared to rats with low economic demand. We also hypothesize that rats with high economic demand will show higher levels of neural activity in brain regions associated with ethanol use relapse, compared to rats with low economic demand. Our goal is to gain a better understanding of the neural mechanisms underlying relapse into ethanol use, to eventually aid in future research that improves the treatment options available for AUD.

Methods

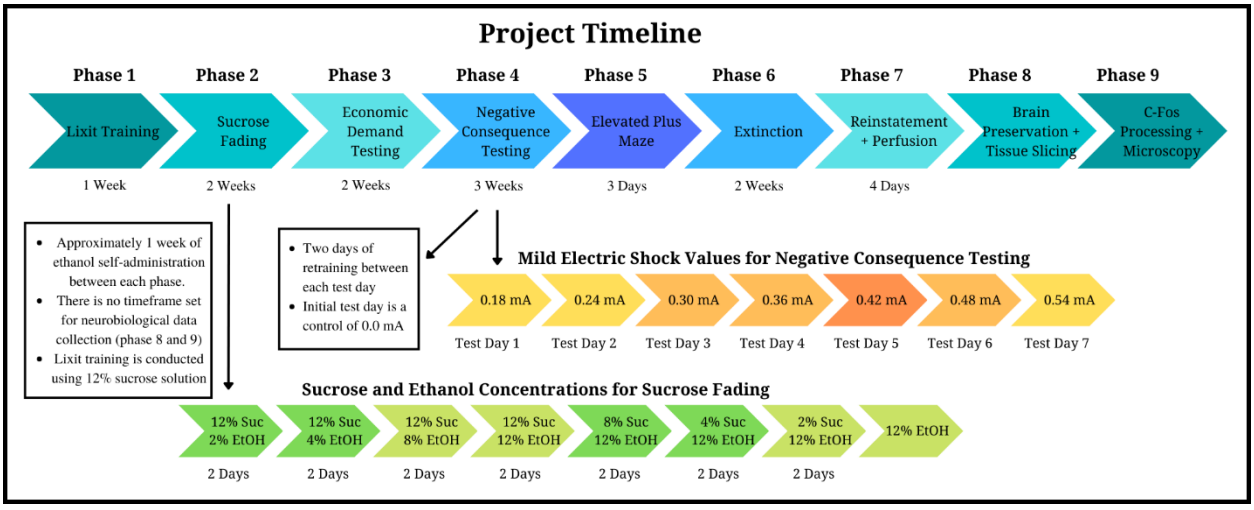

Figure 1. Visualization of the timeline of the project.

Animal Subjects

Twelve Wistar rats, six male and six female, were purchased from Envigo. The rats were housed in a temperature and humidity-controlled vivarium for the entirety of the study, with a reverse 7pm-7am light cycle. The subjects were allowed to acclimate to the vivarium for one

week, before being handled by the research team for two minutes a day for three days prior to the start of the study.

Self-Administration Procedure

Ethanol self-administration occurred for ten hours during the rats' dark cycle, from 8am-6pm. All self-administration and behavioral testing took place in Med Associates operant chambers (measuring 30.5 x 24.1 x 21.0 cm). Rats self-administered ethanol through nosepoking in the operant chambers; once the criteria for number of nosepokes was met, a sipper bottle with ethanol would extend into the chamber within access of the rat. The rats were trained to nosepoke on a fixed ratio of 1 (FR1) schedule of reinforcement, and then continued self-administration on a variable ratio of 3 (VR3) schedule of reinforcement. Extension of the sipper bottle was accompanied by activation of the stimulus lights, serving as an ethanol-associated predictive cue. Ethanol consumption was measured in g ethanol/kg rat body weight. This was achieved by measuring the volume of ethanol consumed at the end of each session.

Sucrose Fading Procedure

To acclimate the subjects to the taste of ethanol, it was introduced gradually through liquid sucrose in a sucrose fading procedure. The percentage of ethanol increased every two days, until the solution faded from 12% sucrose and 0% ethanol to 12% ethanol and 0% sucrose. Reference **Figure 1** for exact daily percentages of ethanol and sucrose. All ethanol self-administration for the study remained at VR3 schedule.

Economic Demand Behavioral Test

The purpose of economic demand testing was to determine the rats' individual economic demand for ethanol, or how much the rats were willing to work to retrieve ethanol. This was

done through measuring rewards earned while escalating the price of ethanol. Each day of the test the number of nosepokes required to earn a presentation of ethanol increased (1, 3, 5, 8, 12…685) with a fixed ratio schedule of reinforcement. Once a rat failed to retrieve a presentation of ethanol, they had completed the test and were moved back to a VR3 schedule.

Responding in the Face of Negative Consequences

The purpose of the negative consequence test was to measure how much the rats were willing to endure to retrieve ethanol, or the level that they would persist in the face of negative consequences. There were eight test days for this behavioral test, with two days of retraining between each test day. On test days, for the first two hours of the ten-hour self-administration session, rats would receive a mild foot shock on 50% of ethanol presentations. The foot shock did not harm the rat but would act as a deterrent to nosepoke for ethanol. Eight levels (amps) of shock were tested over the course of the test, see **Figure 1** for the test day schedule.

Elevated Plus Maze Behavioral Test

The elevated plus maze is a test to measure anxiety-like behavior in the rats, or to determine if the rats were exhibiting any withdrawal-like symptoms. 10-11 hours into withdrawal, they would be placed in the elevated plus maze and their movements would be tracked with a camera placed above the maze. The time the rat spent in the closed arms vs. the open arms of the maze was statistically analyzed after the test; the variables tested were total time freezing, total freezing episodes, and time spent in the open arms. The data from the last 5 minutes of the 10-minute test were used, as the first 5 minutes were for acclimation. Rats that spent more time in the closed arms of the maze were determined to have higher anxiety-like symptoms than the rats that explored the open arms of the maze. This test was repeated for three

days. After the test was completed, all rats completed their ethanol self-administration session as usual.

Extinction Phase

The purpose of the extinction phase of the study was to extinguish the rats' ethanol-seeking behavior. The rats were placed in the operant chambers for the duration of the self-administration period, 8am-6pm. The rats were allowed to nosepoke as usual, however, no ethanol was presented through the duration of the phase. There was also no activation of the stimulus lights or extension of the sipper bottle. This phase continued for two weeks.

Reinstatement Event and Perfusion

During the reinstatement event, rats were placed in the operant chambers for thirty minutes at the beginning of the usual self-administration session. The rats were presented with the activation of stimulus lights, which had been acting as an ethanol-associated predictive cue. The behavior of the rat was tracked by the operant chamber, and it was recorded if the rats began to reinstate nosepoking behavior to try to retrieve ethanol. The data from the reinstatement event is graphed in Figure 4. 60 minutes after the reinstatement event, the rats were euthanized with sodium pentobarbital and perfused using 0.9% saline and 4% paraformaldehyde. The brains were extracted and preserved in 4% paraformaldehyde for later processing. The reinstatement event took place over the course of four days, with three rats per day undergoing reinstatement and perfusion. Two rats were excluded from the reinstatement test as controls but were still perfused in the same timeline.

Brain Tissue Preservation

The rat brains were preserved in 4% paraformaldehyde for 24 hours, or until the brains had begun to float. The brains were then moved to sucrose solution for three days for further preservation. The brains were then frozen on dry ice, before being moved to a -80°C freezer. Once the third replication of the study is complete, the brains will be sectioned using a cryostat and plated onto glass slides.

c-Fos Antibody Processing

In the final phase of the project, the rat brains will undergo immunohistochemistry. The brains will be processed and stained with *c-Fos* antibodies. The processing will stain *c-Fos* proteins in the brain tissue, highlighting regions of the brain that were active during the reinstatement test. The stained brain sections will be viewed with a light microscope, and the active brain regions for each rat will be recorded. Brain regions that will undergo observation are those that are implicated in ethanol-use relapse, and include:

- Orbitofrontal cortex
- Ventral pallidum
- Ventral tegmental area
- Lateral hypothalamus
- Nucleus accumbens core and shell
- Basolateral amygdala
- Prefrontal cortex
- Hippocampus

Preliminary Results

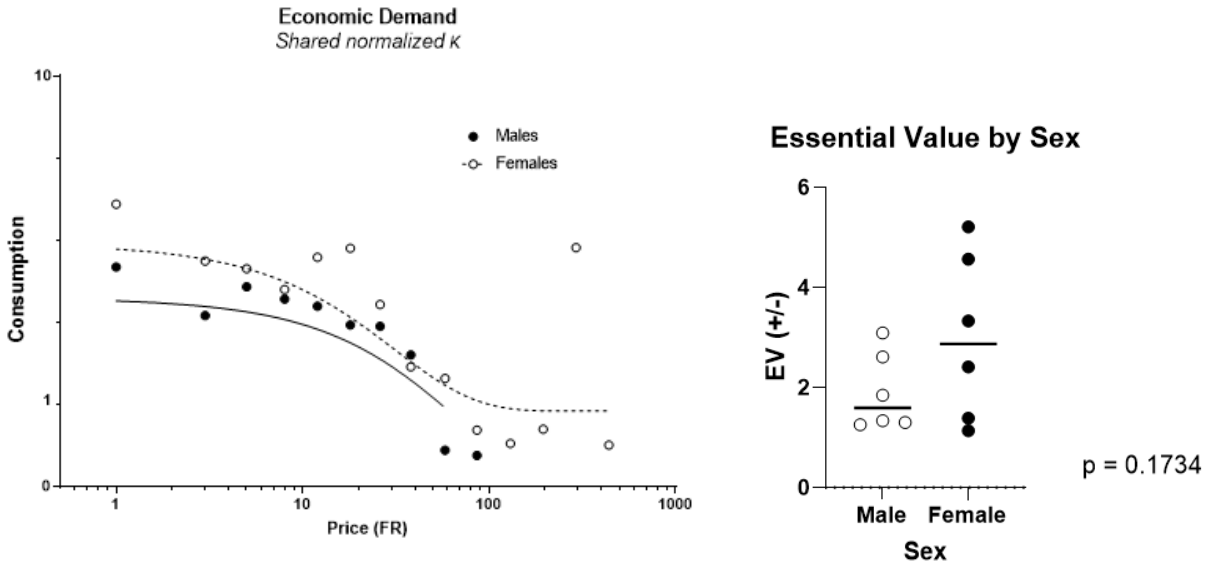

Figure 2. Graph plotting data from the economic demand test. Data (left figure) displays an inverse relationship between ethanol consumption (g ethanol/kg body weight) and price escalation (schedule of reinforcement). Differences between males and females are shown in the right figure.

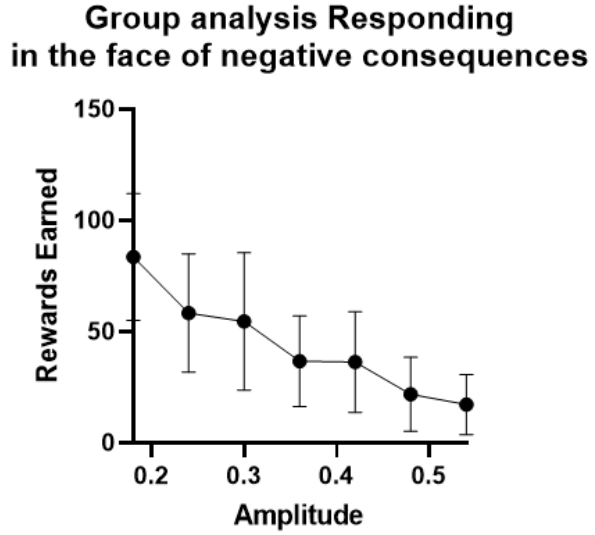

Figure 3. Graph plotting the data from the negative consequence test. Data displays an inverse relationship between rewards earned (ethanol retrievals) and mild shock value (amps). Average responses for the group are shown.

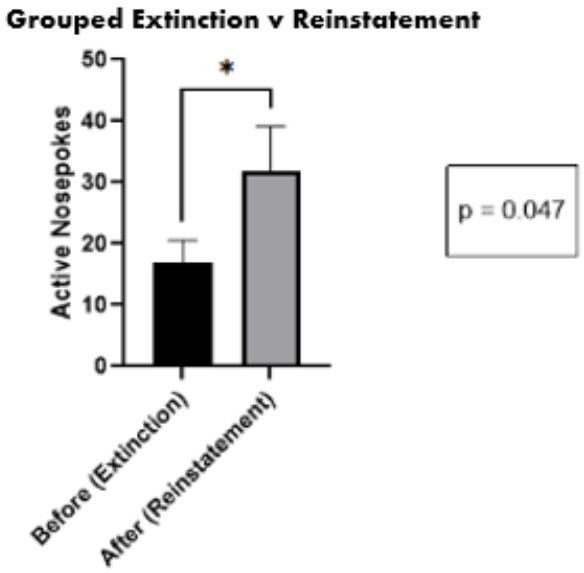

Figure 4. Graph plotting the data from the extinction and reinstatement phase of the study. Average responses for the group are shown for extinction vs. reinstatement.

Preliminary analysis of the behavioral data from this replication of the study has been performed. In the economic demand test, the data shows an inverse relationship between consumption (ethanol g/kg body weight) and price escalation (**Figure 2**). Similarly, in the negative consequence test, there is an inverse relationship between rewards earned and mild shock value escalation (**Figure 3**). The results from the reinstatement event show a statistically significant increase (p-value=0.047) in nosepoke responses during reinstatement event vs. during the extinction phase of the study (**Figure 4**).

The data trends from this replication are aligning with predictions made for the study. So far, there is a clear differentiation between rats with high economic demand and low economic demand. The rats with higher economic demand are persisting more in the face of negative consequences and are showing higher anxiety-like symptoms in the elevated plus maze compared to the rats with lower economic demand. The rats with higher economic demand are also showing to be more likely to reinstate ethanol-seeking behavior during the reinstatement event compared to rats with lower economic demand. This data is not statistically significant yet, but it may be with more subjects.

Discussion

The preliminary results from this study support our predictions. Rats with higher economic demand are persisting more in the face of negative consequences and showing higher anxiety-like symptoms, compared to those with lower economic demand. The rats with higher economic demand are also more likely to reinstate ethanol-seeking behavior during the reinstatement event, compared to those with lower economic demand. While we have not completed the neurobiological component of this study yet, this is an important finding. There is a clear differentiation between rats that have high economic demand and low economic demand, supporting the significance of analyzing this data at the individual level. Analyzing this data at the group level would be inappropriate as rats do not all consume ethanol in the same way, as not all humans consume ethanol in the same way. An important takeaway from this study will be the validity of using this method of data analysis, and our findings so far are promising.

Compared to previous literature, this study used a long-term, prolonged-exposure self-administration model. Many prior studies have utilized the operant self-administration model to study ethanol addiction; however, most have been short-term projects that only allow the rats

limited ethanol exposure during the day. As humans have access to ethanol all day for months to years, we decided that this model was more accurately mimicking human ethanol consumption. Our goal from this study is to produce a better understanding of the exact neural mechanisms underlying relapse into ethanol use, and to do this, we used the most accurate methodology possible. Gaining a better understanding of how the brain operates during relapse is crucial to improving the treatment options available to those suffering from AUD. Identifying the specific brain circuitry involved in relapse opens doors to future pharmacological or deep brain stimulation treatments for AUD, creating more successful outcomes for AUD recovery.

A second replication of this study is currently being conducted to add more subjects for publishable data. Once the second replication is completed at the end of spring 2023, neurobiological data collection for both replications will occur in summer 2023. After the collection of the neurobiological data, a full statistical analysis will be run on both the behavioral and neurobiological data for publication. Future directions for this project include further preclinical studies. One potential project involves using lesioning or DREADDs technology to inhibit regions of the brain that were implicated in relapse, to test the effect it has on the rats' behavior during the reinstatement test. Eventual clinical trials will measure the effect inhibitory pharmacological drugs have on the brain regions involved in relapse, to determine if they improve treatment outcome.

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