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The Affective Disturbance of Ethanol Withdrawal on C57BL/6J and C57BL/6NJ Mice

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THE AFFECTIVE DISTURBANCE OF ETHANOL WITHDRAWAL ON C57BL/6J AND C57BL/6NJ MICE

by

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ABSTRACT

The C57BL/6 (B6) mouse is the most commonly used inbred mouse strain in biomedical research. While the B6 mouse originated at The Jackson Laboratory, a number of separate breeding colonies are now maintained at various sites, resulting in genetic drift that has led to the emergence of both genotypic and phenotypic differences among these colonies. Two distinct substrains of B6 mice, C57BL/6J (B6J) and C57BL/6NJ (B6N), have been shown to differ on several addiction-related phenotypes, such as ethanol preference and locomotor responses to psychostimulants. Therefore, the aim of this study was to assess possible differences in depression- and anxiety-like behaviors following ethanol withdrawal between B6J and B6N mice. Male and female mice (n = 78) were exposed to a regimen of chronic-intermittent ethanol vapor or plain air and subsequently subjected to several behavioral tests at weekly intervals for four weeks. Behavioral measures included the Sucrose Preference Test, a well-established test for depression-like anhedonia; the Light-Dark box Test, a commonly used index of anxiety-like behavior; and the Forced Swim Test, a standard assessment for depression-like learned helplessness. For the Forced Swim Test, the results showed strain main effect between the J's and the N's that the J's spent more time immobile than the N's did. There was also a sex by strain by condition effect in the Sucrose Preference Test where the female N Ethanol mice consumed significantly less Sucrose water than did their control counterparts. From this we can conclude that there are significant behavioral effects associated with Ethanol withdrawal across B6J and B6N mice.

ACKNOWLEDGEMENTS

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INTRODUCTION

Alcohol Withdrawal Syndrome

Alcoholism, also known as alcohol dependence is a disease that results in an increased craving for alcohol, loss of control of when to stop drinking alcohol, and an increased tolerance to alcohol, which requires the individual to drink more to feel the same effect as previous consumptions (MedlinePlus, 2018). Alcoholism then leads into alcohol withdrawal syndrome, since an alcoholic has a physiological dependence on alcohol not consuming alcohol after excessive consumption of alcohol leads to many negative side effects (MedlinePlus, 2018). This is currently classified as Alcohol Use Disorder in the newest version of the DSM. (American Psychiatric Association, 2013). Ethanol addiction is a serious problem, and ethanol withdrawal is a near fatal condition, and there are profound differences among people, so understanding the genetics is a very important tool.

On the cellular level, ethanol withdrawal has a very significant hyper-exciting effect on the central nervous system (Sachdeva et al., 2015; Jesse et al., 2016). Acute ethanol produces mild stimulatory effects at low doses and profound depressive effects at high doses. (Sachdeva et al., 2015; Jesse et al., 2016). Normal ethanol produces euphoria and excitable behavior at lower blood concentrations because of the increase of glutamate binding to N-methyl-D-aspartate (NMDA) receptors which leads to an increase in dopamine levels (Sachdeva et al., 2015; Jesse et al., 2016). Ethanol is a NMDA antagonist and a GABA receptor agonist. (Sachdeva et al., 2015; Jesse et al., 2016). Persistent ethanol consumption leads eventually to decreased gamma-Aminobutyric-acid

(GABA) receptor responsiveness which causes an increased tolerance to ethanol exposure (Sachdeva et al., 2015; Jesse et al., 2016). NMDA receptors, which are responsible for certain forms of memory formation also suffer greatly when exposed to large amounts of ethanol, resulting in common "blackouts" and long term memory loss (Sachdeva et al., 2015; Jesse et al., 2016). Chronic ethanol consumption leads to downregulation of GABA receptor function and an upregulation of NMDA receptor function. The upregulation of NMDA receptor function is what also can cause hyper-excitability, seizures, anxiety, and circadian rhythm disruption during withdrawal. (Sachdeva et al., 2015; Jesse et al., 2016). When prolonged ethanol use is no longer taking place the body still doesn't return to normal right away. The GABA receptors are still repressed meaning that the NMDA receptors are still firing and expressing excitatory neurotransmissions which end up being the cause of seizures, delirium, and tremors (Sachdeva et al., 2015; Jesse et al., 2016). Dopamine is also still being activated without the presence of ethanol and leads to hyperarousal and hallucinations (Sachdeva et al., 2015; Jesse et al., 2016).

Alcohol withdrawal syndrome has many physiological symptoms: nausea, headaches, disorientations, and in extreme cases of withdrawal, seizures and tremors (Becker, 2008; Sachdeva et al., 2015; Jesse et al., 2016; Galbicsek, 2018). These physiological symptoms tend to last up to seventy-two hours after the last ingestion of alcohol (Philibin et al., 2010; Jesse et al., 2016; Galbicsek, 2018). The psychological effects of alcohol withdrawal syndrome, however, can last much longer than the physiological effects and may include: paranoia, anxiety, depression, irritability, and combativeness (Jesse et al., 2016; Galbicsek, 2018). It's the behavioral symptoms that had the biggest effect on most individuals, and what leads most people towards relapse

(Becker, 2008). These behavioral effects of course vary between one patient to another, but, the overall time course of withdrawal may be split into three different time frames: acute withdrawal, early abstinence, and protracted abstinence (Heilig et al., 2010).

Acute withdrawal is the stage of withdrawal that peaks at twenty-four hours after the last ingestion of ethanol and lasts approximately one week (Heilig et al., 2010). Symptoms of this stage of withdrawal include hyper excitability of the autonomic nervous system, seizures and tremors (Heilig et al., 2010; Galbicsek, 2018). Early work indicated that during this phase of withdrawal there is a much greater chance of relapse than the other two stages, however, this has recently been shown not to be the case, as relapse is most likely to occur during protracted abstinence (Heilig et al., 2010; Perry 2014). Although, this doesn't mean that the acute withdrawal phase is something to ignore. The acute withdrawal phase is the most medically dangerous phase of withdrawal because the patient is more likely to experience seizures and tremors and is often advised to spend this stage of withdrawal in a hospital or another rehab facility (Perry 2014; Galbicsek, 2018). Thankfully once this stage of withdrawal is concluded, symptoms like seizures and tremors subside, but, symptoms like anxiety and disrupted sleep and circadian rhythms may still exist (Heilig et al 2010; Perry 2014).

Early abstinence is the second stage of ethanol withdrawal that immediately follows acute withdrawal and can last upwards of eight weeks (Heilig et al 2010). This stage's symptoms are more behavioral than physiological with symptoms including anxiety, depression, and disrupted sleep and circadian rhythms (Heilig et al 2010). The increased anxiety during this stage usually subsides between three to six weeks after cessation of ethanol intake (Heilig et al 2010).

Protracted Abstinence is the third stage of ethanol withdrawal, which follows early abstinence and can last anywhere from months to years. Anxiety and depression are still very prevalent in this stage of withdrawal and minor annoyances may cause negative behavioral effects and lead to increased chance of relapse (Heilig et al., 2010).

In animal models of alcohol withdrawal, there are numerous similar behavioral effects in mice as there are in humans. Tremors, autonomic system excitability, anxiety, and agitation are all present in both humans and mice during ethanol withdrawal (Becker, 2000). The key facets of ethanol withdrawal being tested for in these mice are changes in anxiety-like and depression-like behavior. As we know about mental health disorders, there many different factors that can arise in anxiety or depression, there isn't just one single cause. Depression can arise from learned-helplessness, anhedonia, stress, etc. and anxiety can arise from trauma, stress, and alcohol abuse. It's important when considering these behavioral tests to know the facet in which we are looking to see changes in and note any differences.

J's and N's

The C57BL/6 mouse is the most well-known and widely used inbred mouse strain in biomedical research (Mekada et al., 2009; Simon et al., 2013; Jackson Laboratories). The original C57BL strain was created in 1921 by C.C. Little at the Bussey Institute for Research in Applied Biology, and the "6" substrain was the most popular of the strains that survived into modern usage. C.C Little later went on to found The Jackson Laboratory (JAX) in 1929 and the denotation of C57BL/6J ("J" for Jackson Laboratory) was then used at this facility (Jackson Laboratories). In 1951, JAX sent a number of these mice to the National Institute of Health (NIH), and those mice were used to found a new

breeding population, which has come to be known as C57BL/6N ("N" for NIH). Eventually, a breeding population of C57BL/6N mice was established at JAX; these mice are now designated as C57BL/6NJ. The mice used in this study are the C57BL/6J mice, and the C57BL/6NJ mice (referred to herein as C57BL/6N). Thus, all mice used in this study were bred and housed under standard conditions at JAX before being shipped to us at the University of Maine.

While the C57BL/6J and C57BL/6N substrains derive from the same founder population, the two breeding lines have been separated for sixty-seven years. This separation causes genetic drifts between the two strains and leads to genotypic and phenotypic differences (Mekada et al., 2009; Simon et al., 2013; Jackson Laboratories). The C57BL/6J and C57BL/6N substrains vary more on the phenotypic end as opposed to the genotypic end, which make analysing their behavioral differences is that much easier. In fact, the C57BL/6J and C57BL/6N mice only have thirty four coding single nucleotide polymorphisms (SNPs) different between them (Mekada et al., 2009; Simon et al., 2013; Jackson Laboratories). A SNP is genetic variation in an individual's DNA, so what might be expressed as the nucleotide guanine in one individual, may be expressed as adenine in the other (National Institute of Health). These SNPs occur approximately every three hundred nucleotides in the human genome, meaning there is about ten million SNPs in the human genome (Mekada et al., 2009; Simon et al., 2013; National Institute of Health 2018; Jackson Laboratories).

Besides just using these two strains because of their genetic similarity and how widely used they are, the other main reason these mice are being tested and compared with one another is for testing alcohol preference and dependence on each strain line to

see how well they respond to ethanol testing (Mekada et al., 2009). As mentioned before, genotypically the C57BL/6J and C57BL/6N mice differ in thirty four single nucleotide polymorphisms in thirty four different coding genes. Some of these differences are in genes such as: Adamts3, Ecm1, Pdzk1, Herc2, and Zp2, all of these genes are protein coding genes, however, when expressed differently can lead to different behavioral/neurological effects (Simon et al., 2013; Mouse Genome Database). For instance, the Cytoplasmic FMR1-interacting protein 2 (Cyfip2) gene can have phenotypic effects on anxiety, startle reflex, and hyperactivity, and the Acan gene can have effects on gait (Mouse Genome Database).

Besides these genotypic differences, there are phenotypic differences between the C57BL/6J and C57BL/6N mice that are extremely relevant to this study. The C57BL/6J mice have higher alcohol preference and consumption, stronger grip strength, higher pulse startle magnitude, and higher blood sugar concentration, which over time with excessive ethanol intake can strongly affect the liver and cause glucose intolerance (Simon et al., 2013). Additionally, the C57BL/6J mice have greater contextual fear, greater alcohol deprivation effect, consume more oxygen, and produce more carbon dioxide, have a higher fat mass, and have higher prepulse inhibition (meaning they have a higher pre-response to stimuli which overall reduces their startle response) (Simon et al., 2013). These phenotypic differences between the two strains and the fact that these two strains have not been compared in an ethanol withdrawal test is the reason why this study of J's and N's is so important. Our lab has shown that they differ in running wheel activity phenotypically as well as binge eating. B6 mice in general are also known to be more resistant to the negative effects of withdrawal as opposed to other strains which also

makes them good test subjects (Jackson Laboratories). There is very little data in the area of study on the C57BL/6N mice and using this as a comparison to C57BL/6J mice could help identify areas of anxiety or depression like behavior in the genome for future research. This mice also are known to differ in ethanol preference, so it's the goal of the experiment to determine whether or not they also differ in ethanol withdrawal.

Chronic-Intermittent Ethanol Exposure

Created in 1972 by Goldstein and colleagues, the chronic-intermittent ethanol protocol is a strategy for the induction of ethanol dependence in mice through forced inhalation of ethanol vapor (Becker et al., 1997). Mice do not voluntarily consume enough to produce sustained blood ethanol levels, a prerequisite for the induction of dependence (Becker et al., 1997). A chamber is set up that is connected by a tube to a one-liter bottle of 95% ethanol which is mixed with air to flow into and out of the chamber, all of which is controlled by an air and ethanol pressure gauge on the side of the chamber (Becker et al., 1997).

The mice in the chamber are exposed to the 95% ethanol for sixteen hours of a twenty four hour day and exposed to normal air for the other eight. The reasoning behind this is called the "Kindling Effect" which states that this eight hour period of ethanol abstinence in between ethanol intake creates progressively more severe withdrawal periods (Becker et al., 1997). Therefore, once the protocol has concluded, the mice have already gone through multiple withdrawal periods.

There's many different styles of performing the CIE protocol, one is through a continuous day cycle of the sixteen hours on, eight hours off, over the course of anywhere from four to ten days. Another is a three-cycle, in which it's still the same sixteen/eight

split but it's performed four days in a row and then three days completely off ethanol and repeats three times total (Logan, 2010). Previous studies have shown that this three-cycle in studies on locomotor activity and circadian rhythms had experienced no significant change in circadian rhythms, and that there was a significant drop in locomotor activity the first seven days after the last cycle, and that locomotor activity returned to normal on the eighth day (Logan, 2010). In another study, B6 mice and C3H mice were compared using one-cycle and three-cycle CIE protocols for locomotor activity (Logan et al., 2012). Surprisingly, the one-cycle and three-cycle CIE protocols had the same results with the C3H mice experiencing thirty days of reduced locomotor activity post-CIE, and the B6 mice experiencing seven days of complete inhibition of locomotor activity and then back to normal activity (Logan et al., 2012). The overall difference between the locomotor activity between strains is most likely due to genetic differences, however, the big pull away from here would be how there is little to no difference between doing a one-cycle or three-cycle of CIE (Logan et al., 2012).

In this study, a continuous seven-day CIE protocol was utilized in the interest of saving testing time and due to previous studies within the lab using the three-cycle method. The kindling effect is still present in the continuous day CIE protocol, the main difference is that there is less time spent in minor withdrawals before a complete cessation of ethanol and the beginning of the behavioral tests.

Handling Induced Convulsions

The handling induced convulsions test is used to measure withdrawal seizure severities by scoring the mice on a scale of zero to seven comprised of the following criteria:

> "0=No activity on tail lift or after gentle 360°spin, 1=Facial grimace after 360°spin, 2=Tonic convulsion after 360°spin, 3=Tonic/clonic convulsion after 360°spin, 4=Tonic convulsion after tail lift, 5=Tonic/clonic convulsion on tail lift/delayed onset, 6=Tonic/clonic convulsion; no delay, 7=Severe tonic/clonic convulsion prior to tail lift" (INIA)

Tonic convulsions meaning stiffness of the body and clonic convulsions meaning sustained and rhythmic jerking of the body. This test was first utilized by Goldstein and Pal as a way to grade withdrawal reactions in mice (Goldstein et al., 1971). Currently, there is very little information on what neural mechanisms control the HIC response in mice, however, it is used as a dependable index of hyper excitability within the central nervous system when used in ethanol withdrawal studies (INIA). Another reason why this study includes the HIC test is due the limited data on HIC scores within C57BL/6N mice and as a manipulation check for showing that the Ethanol treatment worked.

Sucrose Preference Test

The sucrose preference test is a reward based test used to indicate the depression-like behavior of anhedonia which is the inability or lack of interest in pleasure or rewarding stimuli (Brown Institute of Science, Serchov et al., 2016). Mice have a tendency to enjoy sweet tasting foods or drinks, so testing to see if they still have that tendency after ethanol withdrawal is a good measure to determine depression like behavior (Serchov et al., 2016). The dependent variable of this test is the amount of water consumed and the amount of sucrose water consumed. The sucrose preference test

involves two bottles, one filled with plain water and the other filled with sugar water both being placed into the mouse's cage. The solution intake is measured every twenty four hours and the bottles switch sides after each test to ensure that there is no side bias in the study (Eagle et al., 2016). Mice will sometimes develop a preference for one side of their cage, therefore swapping the sides the bottles are on is important to keep accurate data (Eagle et al., 2016).

Light/Dark Box Test

The light/dark box test is a widely used test for anxiety-like behavior in mice (Takao et al., 2006; Serchov et al., 2016). This test was originally created in 1980 by Jacqueline Crawley and Frederick Goodwin (Serchov et al., 2016). This test simulates two different areas for the mice, the first being the dark box which simulates a burrow, and the light box which simulates the outside (Takao et al., 2006; Serchov et al., 2016). The mouse is first placed in the dark box with a bottle blocking the opening, once the test has commenced, the bottle is removed (Takao et al., 2006; Serchov et al., 2016). The mouse is then able to freely move back and forth between one box to the other and a more anxious mouse is more likely to stay in the dark, while a less anxious mouse is more likely to explore the light (Takao et al., 2006; Serchov et al., 2016). This test looks for three important variables: Time until first transition, number of transitions, and time spent in light. The time until first transition and time spent in light variables are a good measure of anxiety-like behavior and the number of transitions is a measure of exploratory behavior (Takao et al., 2006; Serchov et al., 2016). Seeing if ethanol withdrawal has significant effects on time until first transition and time spent in light is the main goal of this test.

Forced Swim Test

The forced swim test is a test used to determine depression-like behavior in mice through learned-helplessness (Can et al., 2012; Yahav et al., 2015). Originally created by R. D. Porsolt in 1977 and why it is sometimes referred to as the Porsolt swim test (Can et al., 2012; Yahav et al., 2015). The test places a mouse in a chamber of water and is measured by the amount of seconds it stays immobile while in the water (Can et al., 2012; Yahav et al., 2015). Mice are natural swimmers so this test, fortunately can exclude endurance as a variable (Can et al., 2012; Yahav et al., 2015). As the test goes on, a more depression-like mouse is more likely to give up and begin to float rather than expend energy, whereas a typical mouse is more likely to spend the majority of the test actively swimming around the chamber (Can et al., 2012; Yahav et al., 2015).

All three of these tests are performed once a week for four weeks. While the sequence in which the tests are performed doesn't have any effect on the data, once the order is set, it doesn't change (I.E. it doesn't matter if SPT is the first test or the last test, as long as it is consistent). Multiple tests are also performed to show week by week changes in the data, and also display the three stages of withdrawal and see if the stages have an impact on the data. The first week of testing represents acute withdrawal, the second and third week represent early abstinence, and the fourth week borders into protracted abstinence. Finally we do two different depression tests because there are different factors that can affect anxiety-like or depression-like behavior.

MATERIALS AND METHODS

Subjects and Apparatus

Six-week old male and female C57BL/6J and C57BL/6NJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, mice were group-housed (five per cage) based on sex, strain, and assigned experimental condition. Food (ProLab RMH3000, Lab Diet, St. Louis, MO) and plain water were provided *ad libitum*. The cages were then moved into the four (60x36x60cm) vapor chambers with two cages per chamber.

Experimental Design

Ten equally male and female C57BL/6J and ten equally male and female C57BL/6N mice were exposed to seven days of the CIE vapor in their chambers, while the other twenty mice were exposed to regular air in their chambers. The mice were maintained on a 12:12 light/dark cycle throughout the entire experiment. After the last day of the CIE protocol, the mice were all moved into single-housing, where they remained throughout the four weeks of behavioral testing. Food and water were provided ad libitum and bedding was changed every week.

Chronic-Intermittent Ethanol Exposure

Seven daily cycles of 16 hours of ethanol vapor exposure, and 8 hours of regular air exposure was utilized. The ethanol vapor exposure started at the beginning of their dark cycle and finished 4 hours into their light cycle. The control mice were not exposed to any of the ethanol vapor, but, both the control and experimental mice had the same

12:12 light/dark cycle. The ethanol vapor was consistently flowing into the two CIE chambers at a rate of 10-12 L/min (Logan et al, 2009).

Before each ethanol vapor exposure cycle, mice received an injection of ethanol/pyrazole or saline/pyrazole. The solution is 136.2 mg of pyrazole added into 100 mL of 20% ethanol. The mice are then injected at 0.01mL/g, meaning that a 20g mouse would be injected with 0.2mL of the ethanol/pyrazole solution. Pyrazole is used because it helps start the intoxication process and helps stabilize the blood ethanol concentration which we measure at the end of the CIE protocol (Becker et al., 1997). The control mice were also injected in the same fashion, the only difference being that the ethanol was replaced 100 mL of 0.9% saline and still received the pyrazole. While the solution in the injection has no effect on the control mice, they are still injected to stimulate the same stress response of receiving an injection that the CIE mice receive.

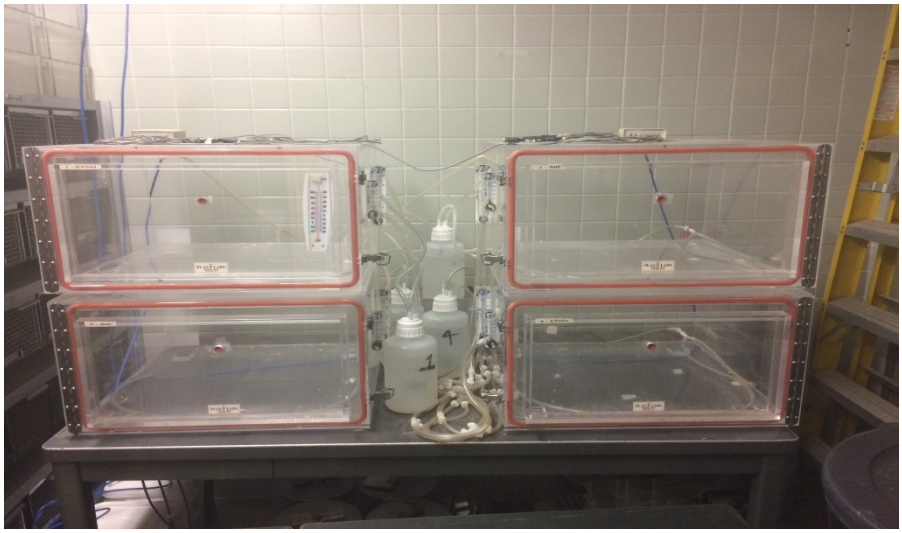

Image 1: CIE Vapor Chambers: Mice were placed into these chambers and exposed to either CIE or Air

Blood Ethanol Content and Handling Induced Convulsion

After the last sixteen hour cycle of ethanol vapor exposure, the mice are then tested for blood ethanol content and handling induced convulsions before being placed into single housing. One by one each CIE mouse is placed into an acrylic restraint to inhibit its movement. A small nick is made in the tip of the tail and a very small volume of blood is collected into a centrifuge tube, centrifuged, and then the blood ethanol content is analyzed through an Analox alcohol analyzer. The control mice also go through a similar process, except, their blood is not needed to be collected since they have not been exposed to ethanol, the small nick in the tip of the tail is done on the control mice to induce the same stress that the CIE mice experienced.

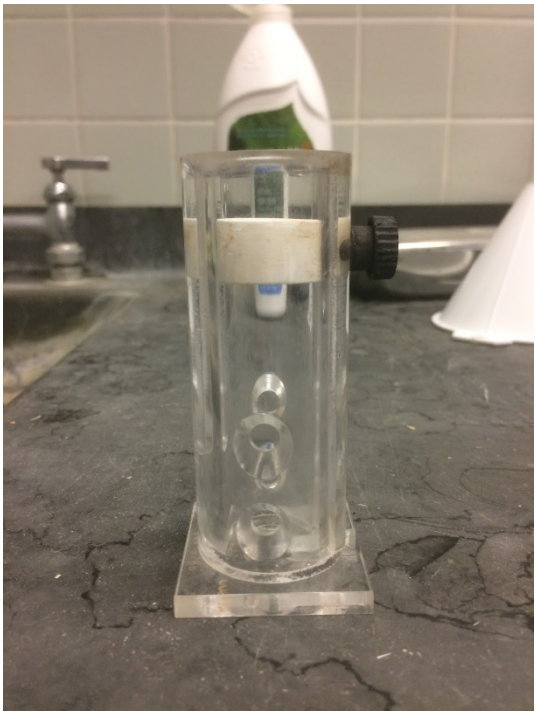

Image 2: Acrylic Restraint: This Apparatus is used to immobilize mice during BEC collection.

The handling induced convulsions test is performed approximately six hours after the blood ethanol content data is taken. Again, one by one, all forty mice are taken out of their cage, lifted up, spun gently 360° and scored zero to seven according to the handling induced convulsion scale. Once they are scored, they are placed back in their cage.

Behavioral Tests

The sucrose preference test is the first behavioral test the mice are exposed to post-CIE treatment. To create the sucrose water, 60g of sugar is dissolved into 8000 mL of water and mixed. This solution is then spread out evenly across forty bottles. Forty more bottles are then filled with just plain water. The weights of all eighty bottles are recorded and then one sucrose bottle and one water bottle is placed into each of the forty cages. While the weights of each bottle are not the same, the real values we are looking for the overall weight change in each bottle. The sucrose bottles and water bottles position within the cage is swapped every week to ensure there is no side bias in the experiment. The bottles are then left in the cage for a twenty-four hour period and then taken out and weighed to take the difference from starting value to ending value.

The second behavioral test is the light/dark box test. This test measures the light/dark box activity of each mouse for six minutes each. A mouse is first placed into the dark box with the entrance to the light box blocked by an empty bottle. Once the bottle is removed, the test begins. A camera is placed about the light box to keep track of when the mouse first transitions into the light box, the number of transitions, and the time spent in the light. Once the video is recorded, it is analyzed through the ANY-Maze computer program which automatically records and keeps track of the data measures.

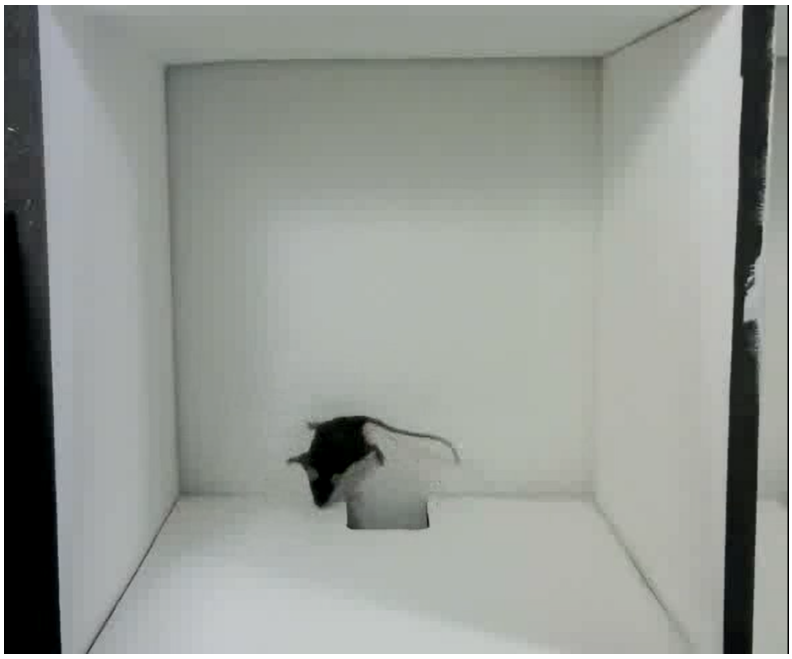

Image 3: LD Box Test Set-up

The final behavioral test is the forced swim test. This test measures the swimming activity for each mouse for six minutes each. The first two minutes of the test are not used in data collection and acts as an acclimation period, once the two minutes are over, the final four minutes are used for data collection. A vase of eight inches by twelve inches is filled approximately ¾ of the way with water keep between 23℃ and 25℃. The vase is then lined up with a camera on a kickstand to make sure it can clearly see the meniscus of the water. The mouse is then gently placed into the water to monitor for seconds of immobility. This test is also measured in the ANY-maze computer program to determine how long the mouse was immobile. Once the test is concluded, the mouse is placed into a separate cage in order to dry off before being returned to its regular cage.

Image 4: Forced Swim Test Set-up

Data Analysis

While the data itself was collected through the ANY-maze program, analysis was performed in a different program. In analyzing the data for the light/dark box test and the forced swim test a three factor ANOVA was used for the factors “male vs. female”, “ethanol vs. air”, and “J’s vs. N’s”. A repeated measures ANOVA was also utilized for independently comparing variables in both a four-week model and a three-week model. The ANOVA is used to test for main effects of ethanol exposure across different variables. In the light dark box test, individual ANOVAs were performed for time until first transition, number of transitions, and time spent in light. Whereas the forced swim test ANOVA was used for seconds of immobility. The sucrose preference test did not need an ANOVA for data analysis, it was done manually with inputs from data collected from the difference between the starting weight of the bottles and end weight of the bottles.

Ethical Considerations

This experiment was performed with approval of the University of Maine Institutional Animal Care and Use Committee.

RESULTS

Blood Ethanol Content and Handling Induced Convulsions

Figure 1 below shows the blood ethanol content by each strain and sex of the CIE mice and figure 2 below shows the handling induced convulsion scores in all of the mice. The blood ethanol content concentrations were gathered from the Analox alcohol analyzer and showed lower than expected values, as the typical range is 170 mg/dL to 200 mg/dL. However, while still a little low, it doesn't have a strong effect on the rest of the data collected.

B6 Strain	Sex	BEC (mg/dL)
J	F	155.7 ± 6.24
J	M	150.0 ± 8.74
N	F	155.7 ± 11.81
N	M	142.1 ± 8.67

Figure 1: Shows the blood ethanol content for each strain and sex of the CIE mice along with the mean error.

In terms of the handling induced convulsion scores (Figure 2), the scores did not exceed three. Meaning that there were no instances of tonic convulsions after the tail lift, or without being lifted in general. For the most part, the mice in this study scored between zero and two, meaning that they had no activity, facial grimaces, or tonic convulsions after the 360° spin.

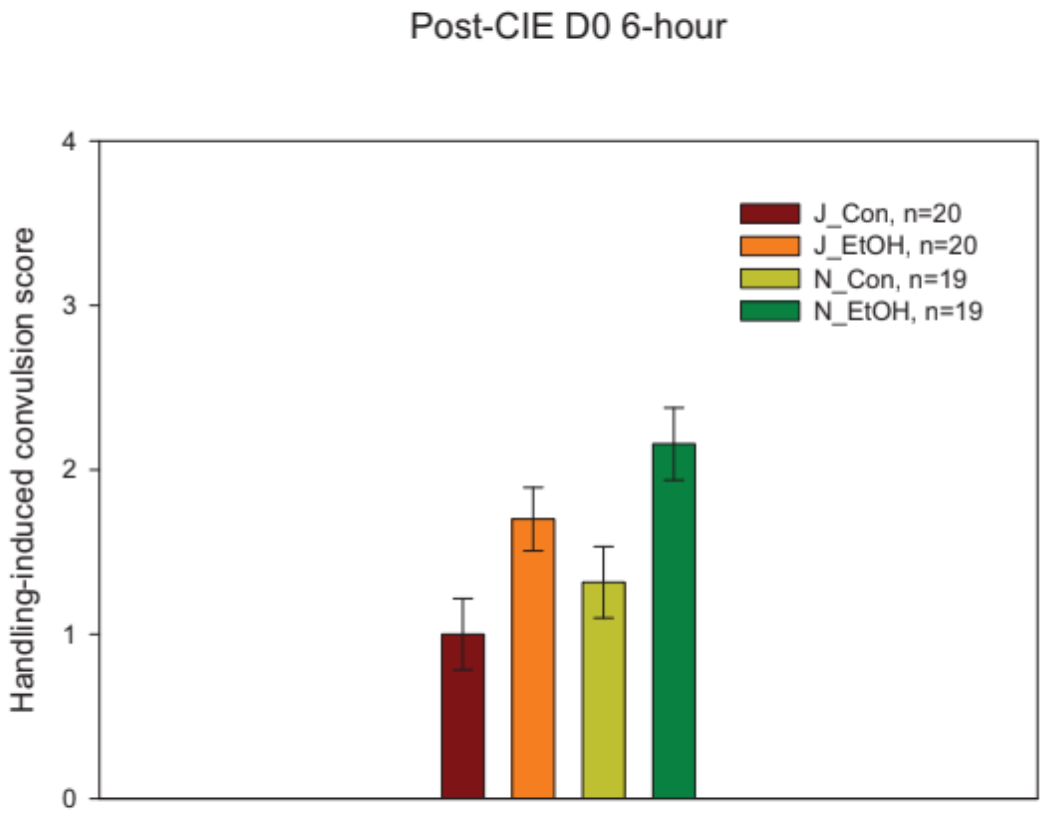

Figure 2: Shows the handling induced convulsion scores for each mouse. There is a significant difference between HIC scores in both control and ethanol treatments in N mice. However there is no significant difference between J and N mice.

Sucrose Preference Test

Figure 3 below shows the data for all four weeks of the sucrose preference test. Starting in week two and continuing until the end of the study, a main effect of condition shows a significant difference between control mice and ethanol mice with p-values of 0.001, 0.016, and <0.001 from week two to four. When running the data as a 4-week repeated measure test, there is a week by condition main effect in which for Ethanol, week 1 has the highest sucrose preference and it decreases progressively with each continuing week. For the Controls, week 1 has the lowest sucrose preference and it increases progressively with each continuing week. This main effect has a p-value of <0.001.

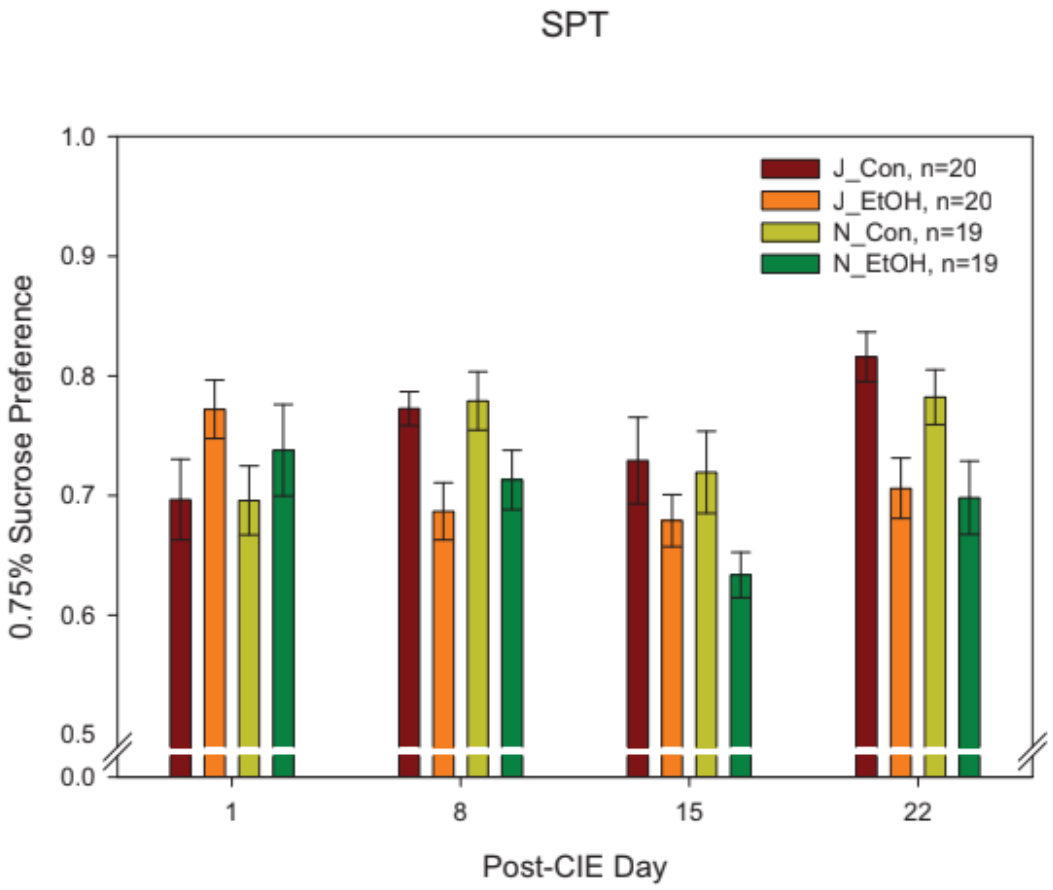

Figure 3: Shows the sucrose preference across the 4 weeks of testing.

Light/Dark Box Test

The light/dark box test has the most data by far due to three different variables being tested. First is the amount of time spent in light. For week one, two, and four, the data collected showed a main effect of strain with J mice spending more time in the light than N mice with p-values of 0.049, 0.028, and 0.002 respectively. Week one exclusively showed a main effect of condition with control mice spending more time in the light than ethanol mice with a p-value of 0.032. We also see a main effect of week when run through a 4-week repeated measures ANOVA, which shows the percent of time spent in light being significantly lower in week one than any other week with a p-value of <0.001.

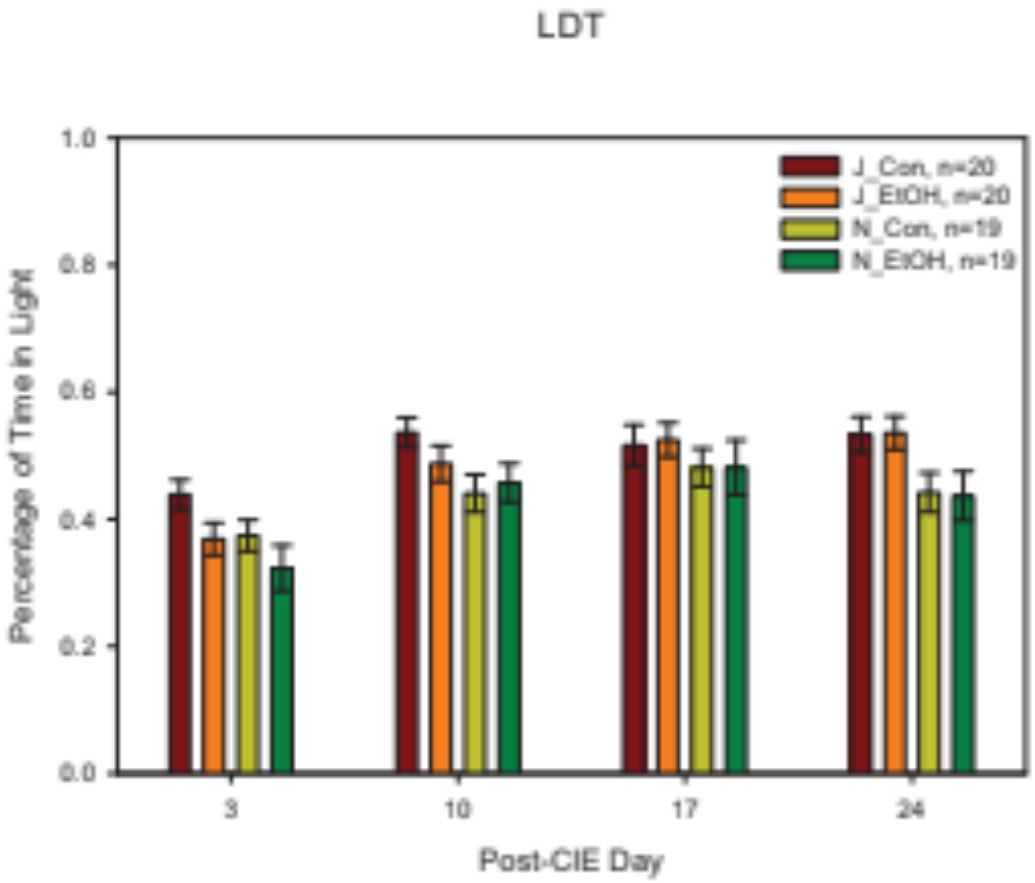

Figure 4: Shows the percent of time spent in light in the light/dark box test during the 4 weeks of testing.

Next, in testing for total amount of transitions in the light/dark box test, the following data was collected. In all four weeks, there is a main effect of strain in which J mice had more transitions than N mice did with p-values consisting of <0.001 for the first three weeks and then 0.002 for week four. There is also a main effect of week when the test is run as a 4-week repeated measure ANOVA. Week one has the lowest amount of total transitions and is significantly different from weeks two and four, and also that week two has the highest amount of total transitions and is significantly different from weeks one and three, each with a p-value of 0.004.

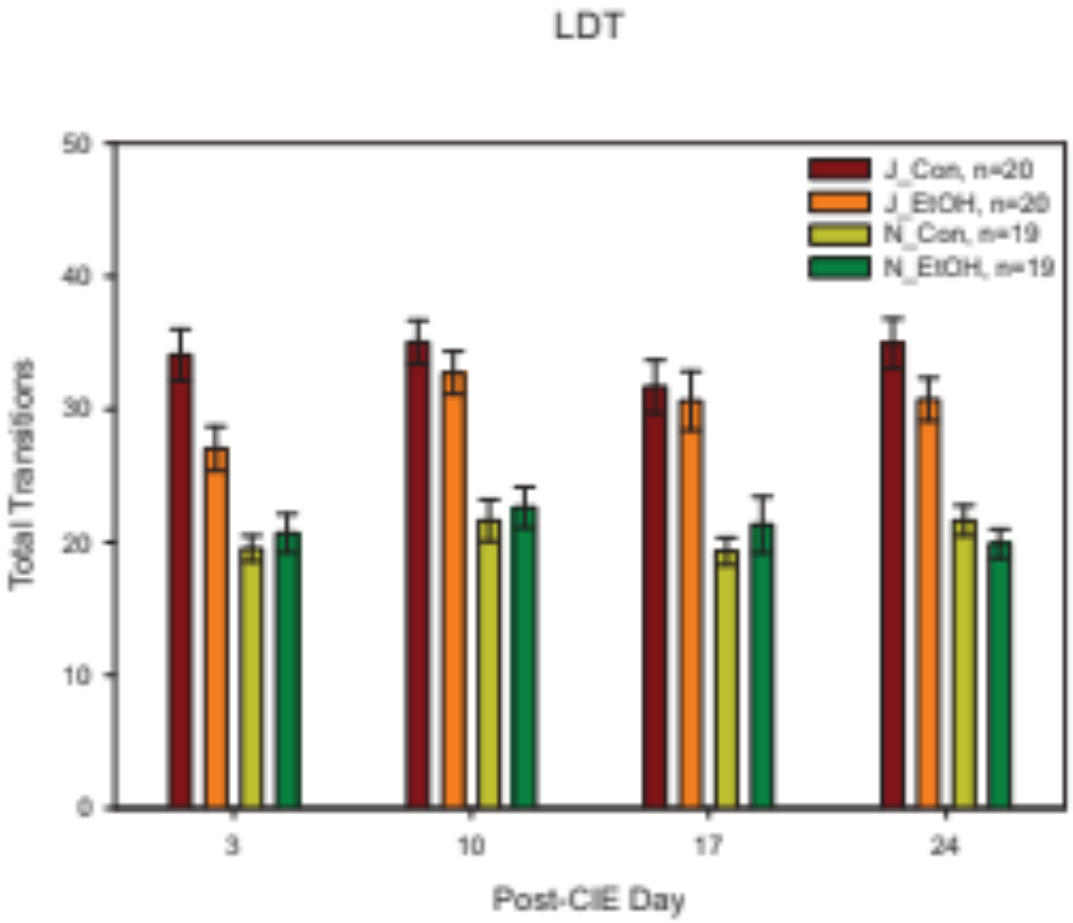

Figure 5: Shows the total transitions during the light/dark box test during the 4 weeks of testing.

Finally, the last variable being tested for is the latency until the first transition into the light. From the data collected, for weeks one and two there is a main effect of strain in which the J mice took less time to make that first transition than the N mice did with p-values of <0.001 and 0.007. For weeks three and four, there was no significant differences across any of the data. Finally, we see a main effect of week when a 4-week repeated measure ANOVA is run. Week one has the highest latency to first entry into the light and is significantly different from weeks two, three, and four with a p-value of <0.001.

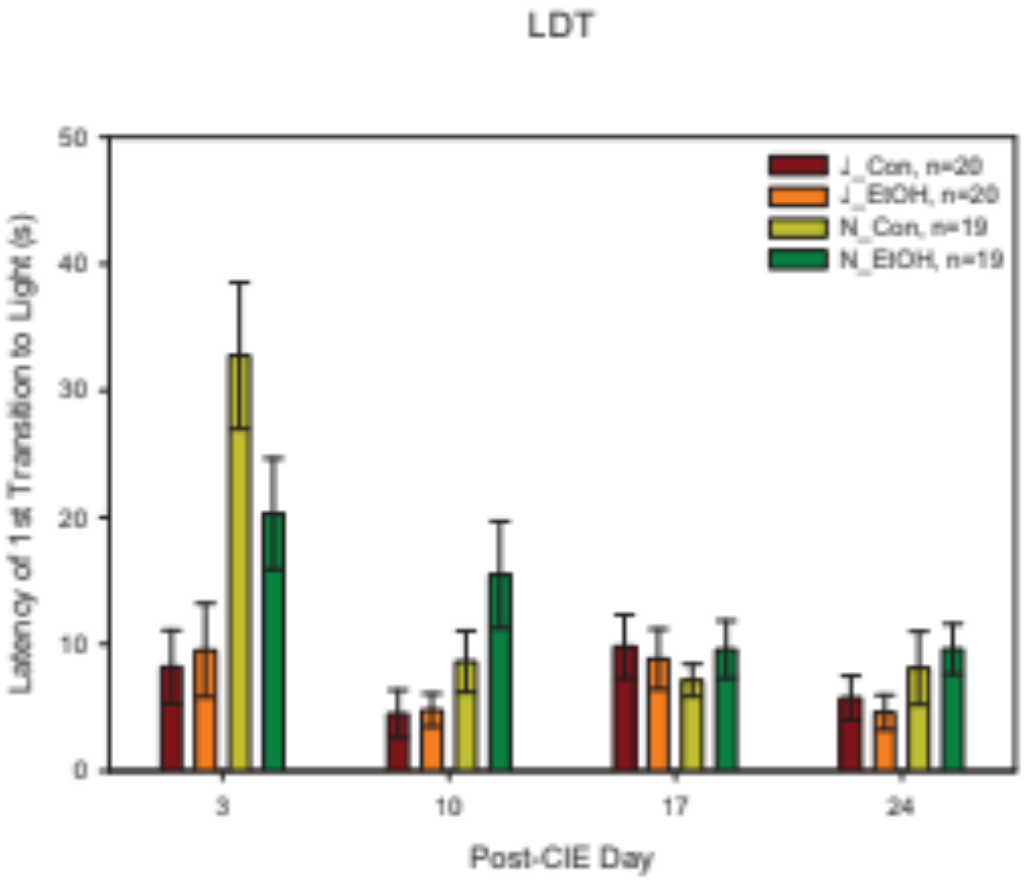

Figure 6: Shows the latency of the first transition into the light in the light/dark box test during the 4 weeks of testing.

Forced Swim Test

The final behavioral test, the forced swim test, showed the following data represented in figures 11 and 12 below. In weeks two and four, there is a main effect of strain in which J mice have more seconds of immobility than do N mice with p-values of 0.003 and 0.014. In weeks two and three, there is a main effect of condition in which ethanol mice had more seconds of immobility than did control mice with p-values of 0.17 and 0.36. Second, there is a main effect of strain by condition in which ethanol N mice had more seconds of immobility than did control N mice with a p-value of 0.005. Also, when run as a 4-week repeated measure ANOVA, there is a week main effect. Week 1 shows the lowest amount of immobility and is significantly different from all of the other weeks with a p-value of <0.001.

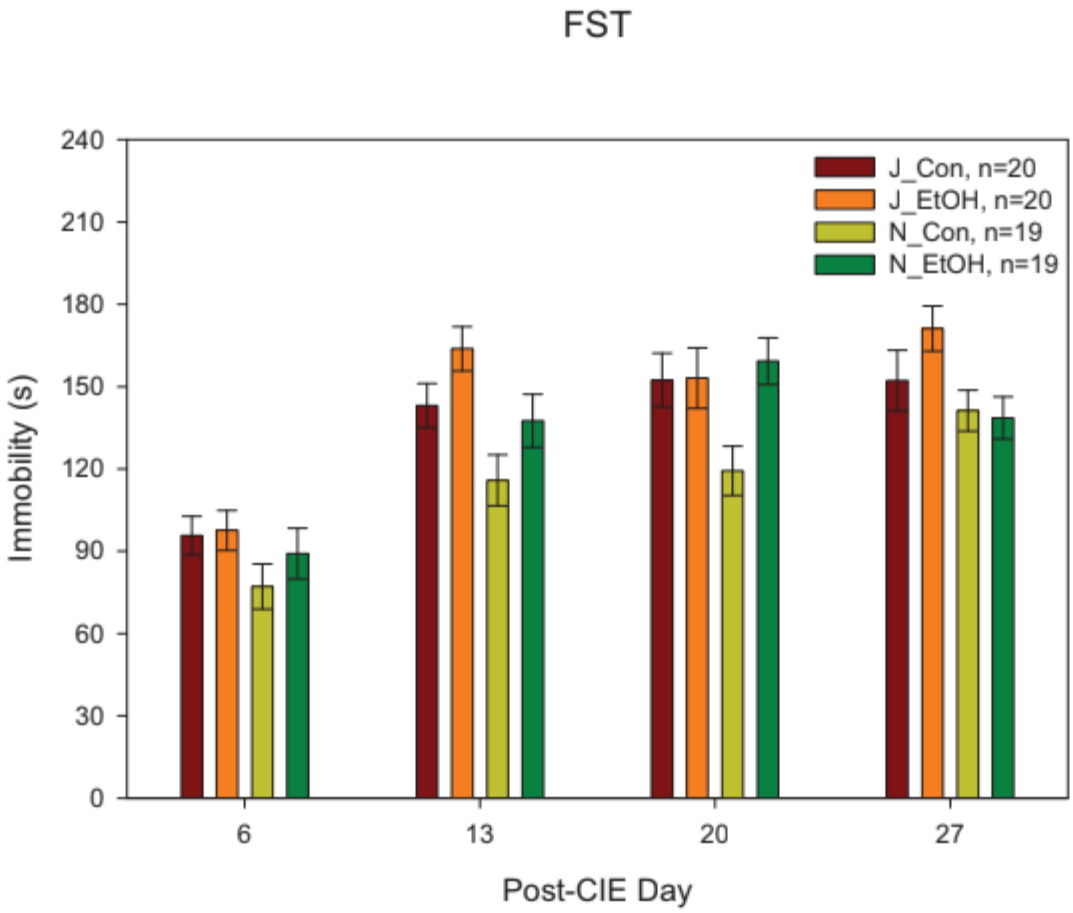

Figure 7: Shows the seconds of immobility in the forced swim test during the 4 weeks of testing.

DISCUSSION

The results above were analyzed thoroughly and originally, the graphs were split up by sex to evaluate each group as both males and females separately. However, since there were no significant sex differences in our study, the graphs were consolidated to avoid sex separation and more clearly show the results that are of significant value to the study. The sex effect in general that we saw in this study have complex interactions with each of the variables and are very difficult to interpret their meaning.

For each of the behavioral tests, the data collected gave some interesting results. For the sucrose preference test the main effect of condition was present in which the ethanol mice showed more depression-like behavior than did the control mice also during the early abstinence phase of withdrawal right up until the start of the protracted abstinence phase of withdrawal. What this means is that regardless of strain, ethanol mice in general experience depression-like behavior within the parameters of the sucrose preference test.

For the light/dark box test in terms of percent of time spent in the light in weeks one, two, and four, the J mice spend more time in the light than do the N mice signifying that J mice regardless of treatment are less anxious than N mice. Finally, in week one only, the control mice spend more time in light than the ethanol mice. What this could mean is that in the acute withdrawal phase of withdrawal ethanol mice are still more sensitive to the light than during the other phases of withdrawal.

In terms of total transitions, in all four weeks, J mice have more total transitions than do N mice, which helps further cement that regardless of treatment, J mice are less anxious than N mice within the parameters of the study.

In terms of latency until the first transition into the light, in weeks one and two there is a strain main effect in which J mice take less time to make that first transition than do N mice. What is seen by the light/dark box test in all three of the variables is that overall, regardless of treatment, J mice spend more time in the light, transition more, and take less time to make the first transition into light than do N mice. Which although doesn't utilize the ethanol treatments, is still interesting data showing a 3/3 trend of N mice having more anxious behavior than J mice. In looking at the week main effects for all three variables as well, all three show how all mice, regardless of strain or condition, spend less time in the light, transition less, and take more time to make the first transition into the light than weeks two, three, and four.

Finally in the forced swim test there is unfortunately isn't a trend within the main effects for the four weeks of this test. In weeks two and three there is a main effect of condition in which ethanol mice were more immobile the control mice, however, since this was only for weeks two and three, making the assumption that it's an effect only present during early abstinence might not be correct since it's not present during the last week of the study. Also in weeks two and four there is a strain main effect in which J mice had more seconds of immobility than N mice did. Interestingly, similar to the sucrose preference test, there was no significant effects or interactions in the first week of each test. This is interesting because it gives the assumption that depression-like behavior

does not arise during the acute withdrawal phase of withdrawal but instead during later phases of withdrawal.

So why does any of this matter? When this experiment was set out, it was common knowledge of how J's and N's differ in ethanol preference, and now after compiling all of the results and interpreting them, we can see that J's and N's do not differ in ethanol withdrawal effects due to a lack of strain by condition main effects within our results. The strain main effects help show further differences between J and N mice and the condition main effects show that the experiment was performed successfully and that ethanol withdrawal does affect the mice. However, without a strain by condition effect, it cannot be proved that ethanol has different effects on both strains. While we would have liked to see a strain by condition main effect to show a difference in ethanol withdrawal, not seeing one also opens up interesting new questions and answers about the J's and N's. For instance now we know that the gene that expresses ethanol preference is different from the gene that expresses ethanol withdrawal and is different between both J's and N's.

Future Directions

This is such an open ended study that there are some many different ways this study could go from here. In the future, it might be beneficial to do five or six weeks of behavioral testing to further solidify current data and add new data on protracted abstinence effects as well. Doing more repeats of this test would also be helpful to help confirm certain aspects of the study and to see if what is currently qualified as a trend is actually a trend or not. Finally, possibly repeating this study again, only with the addition

of C3H mice, or other non C57BL mouse to compare the differences between not only J mice and N mice, but J and N mice with an entirely different strain.

Conclusion

From this study, there are quite a few interesting things that we can conclude about the ethanol withdrawal effects in C57BL/6J and C57BL/6N mice. First, it appears that while C57BL/6J and C57BL/6N mice differ in ethanol preference, in the parameters of this study, they do *not* differ in ethanol withdrawal. Second, it can be concluded that J mice experience less anxiety-like behavior regardless of treatment than N mice during the light/dark box test study. Third, in the anxiety-like behavior testing of the light/dark box testing, all three variables showed anxiety-like behavior in week one only and then starting to phase out during the progression of the study. This is an interesting result and seems to conclude that anxiety-like behavior in these two strains of mice may only be prominent during the first phase of ethanol withdrawal and fades out during the latter stages. Finally, within the parameters of the study, it can be concluded that during the acute withdrawal phase of ethanol withdrawal, that C57BL/6J and C57BL/6N mice do not experience depression-like behaviors. According to the results of the sucrose preference test and the forced swim test, we see that there is high to progressively low amounts of sucrose preference, and low to high amounts of immobility in forced swim. Meaning that depression-like behavior isn't present near the start of withdrawal, but, arises during the later phases of withdrawal.

REFERENCES

A tool for telling apart C57BL/6J versus C57BL/6N substrains. (Jackson Laboratories). (2013, April). Retrieved February 14, 2018, from https://www.jax.org/news-and-insights/2013/april/ a-tool-for-telling-apart-c57bl-6j-versus-c57bl-6n-substrains

Alcohol Withdrawal Treatment, Symptoms and Timeline with Visuals. (n.d.). Retrieved January 13, 2018, from https://americanaddictioncenters.org/withdrawal-timelines-treatments/alcohol/

Alcoholism and Alcohol Abuse: MedlinePlus. (2017, November 17). Retrieved January 13, 2018, from https://medlineplus.gov/alcoholismandalcoholabuse.html

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (5th Ed) (5th ed., Diagnostic and statistical manual of mental disorders). American Psychiatric Association.

Baca, G. J. (2006, July). Kindling by repeated alcohol dependence and withdrawal experiences: effects on handling induced convulsions and voluntary alcohol drinking in ethanol withdrawal seizure -prone and - resistant mouse selectedlines. Retrieved February 21, 2018, from https://digitalcommons.ohsu.edu/cgi/viewcontent.cgi?referer=https://www.google.com/&httpsredir=1&article=1817&context=etd

Becker, H. C., Diaz-Granados, J. L., & Weathersby, R. T. (1997, August). Repeated ethanol withdrawal experience increases the severity and duration of subsequent withdrawal seizures in mice. Retrieved February 20, 2018, from https://www-sciencedirect-com.prxy4.ursus.maine.edu/science/article/pii/S0741832997879499?_rdoc=1&_fmt=high&_origin=gateway&_docanchor=&md5=b8429449ccfc9c30159a5f9aeaa92ffb&ccp=y

Becker, H. C. (2000). Animal Models of Alcohol Withdrawal. Retrieved February 6, 2018, from https://pubs.niaaa.nih.gov/publications/arh24-2/105-114.pdf

Becker, H. C. (2008). Alcohol Dependence, Withdrawal, and Relapse. Retrieved January 13, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3860472/

Brown Institute for Brain Science. (n.d.). Sucrose Preference Test. Retrieved February 23, 2018, from http://rndb.clps.brown.edu/task/sucrose-preference-test/

C57BL/6J. (Jackson Laboratories). Retrieved February 14, 2018, from https://www.jax.org/strain/000664

C57BL/6N. (Jackson Laboratories). Retrieved February 14, 2018, from https://www.jax.org/strain/005304

Can, A., Dao, D. T., Arad, M., Terrillion, C. E., Piantadosi, S. C., & Gould, T. D. (2012, January 29). The Mouse Forced Swim Test. Retrieved March 1, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3353513/

Costa, A. P., Vieira, C., Bohner, L. O., Silva, C. F., Santos, E. C., & DeOliveria, C. L. (2013, May 09). A proposal for refining the forced swim test in Swiss mice. Retrieved March 15, 2018, from https://www.sciencedirect.com/science/article/pii/S0278584613000882

Eagle, A. L., Mazei-Robison, M., & Robison, A. J. (2016, June 5). Sucrose Preference Test to Measure Stress-induced Anhedonia. Retrieved February 23, 2018, from https://bio-protocol.org/e1822

Farook, J. M., Krazem, A., Lewis, B., Morrell, D. J., Littleton, J. M., & Barron, S. (2008, September 3). Acamprosate attenuates the handling induced convulsions during alcohol withdrawal in Swiss Webster mice. Retrieved February 21, 2018, from https://www-sciencedirect-com.prxy4.ursus.maine.edu/science/article/pii/S003193840800190X?_rdoc=1&_fmt=high&_origin=gateway&_docanchor=&md5=b8429449ccfc9c30159a5f9aeaa92ffb

Galbicsek, C. (2018, January 10). Alcohol Withdrawal - Learn the Symptoms and Signs. Retrieved January 13, 2018, from https://www.alcoholrehabguide.org/alcohol/withdrawal/

Goldstein, D. B., & Pal, N. (1971, April 16). Alcohol Dependence Produced in Mice by Inhalation of Ethanol: Grading the Withdrawal Reaction. Retrieved February 26, 2018, from http://www.jstor.org.prxy4.ursus.maine.edu/stable/pdf/1731059.pdf?refreqid=excelsior%3Aa5b37c598476729a701b832af5abe77f

Heilig, M., Egli, M., Crabbe, J. C., & Becker, H. C. (2010, April 1). Acute withdrawal, protracted abstinence and negative affect in alcoholism: are they linked? Retrieved January 13, 2018, from https://web-b-ebscohost-com.prxy4.ursus.maine.edu/ehost/pdfviewer/pdfviewer?vid=1&sid=c7a17622-7cb3-4966-ba64-71a5edd19817%40sessionmgr102

INIA. (n.d.). Acute EtOH withdrawal and handling-induced convulsions. Retrieved February 21, 2018, from http://iniastress.org/acuteetoh

INIA. (n.d.). Dependence Testing: Handling-Induced Convulsions (HIC). Retrieved February 21, 2018, from http://iniastress.org/hic

INIA. (2013, March). Chronic Intermittent Ethanol (CIE) Exposure Model. Retrieved February 20, 2018, from http://iniastress.org/cie

Jesse, S., Bråthen, G., Ferrara, M., Keindl, M., Ben-Menachem, E., Tanasescu, R., . . Ludolph, A. (2016, September 01). Alcohol withdrawal syndrome: mechanisms, manifestations, and management. Retrieved January 13, 2018, from http://onlinelibrary.wiley.com/doi/10.1111/ane.12671/full

Logan, R. W. (2010, December). Circadian Wheel-Running Activity During Withdrawal from Chronic Intermittent Ethanol Exposure in Mice. Retrieved February 20,2018, from https://digitalcommons.library.umaine.edu/cgi/viewcontent.cgi?article=1839&context=etd

Logan, R. W., McCulley, W. D., III, Seggio, J. A., & Rosenwasser, A. M. (2012), October 20). Effects of Withdrawal from Chronic Intermittent Ethanol Vapor on the Level and Circadian Periodicity of Running-Wheel Activity in C57BL/6J and C3H/HeJ Mice. Retrieved February 20, 2018, from http://onlinelibrary.wiley.com.prxy4.ursus.maine.edu/doi/10.1111/j.1530-0277.2011.01634.x/full

Luessen, D. J., Sun, H., McGinnis, M. M., McCool, B. A., & Chen, R. (2017, July 21). Chronic intermittent ethanol exposure selectively alters the expression of Gα subunit isoforms and RGS subtypes in rat prefrontal cortex. Retrieved February 20, 2018, from https://www.sciencedirect.com/science/article/pii/S000689931730313X?via%3Dihub

Mekada, K., Abe, K., Murakami, A., Nakamura, S., Nakata, H., Moriwaki, K., . . . Yoshiki, A. (2009, April). Genetic differences among C57BL/6 substrains. Retrieved February 14, 2018, from https://www.ncbi.nlm.nih.gov/pubmed/19448337

Miller, S. M., Piasecki, C. C., & Lonstein, J. S. (2011, November 7). Use of the light-dark box to compare the anxiety-related behavior of virgin and postpartum female rats. Retrieved February 24, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3183346/

Mouse Genome Database. (2018, March 06). Retrieved March 15, 2018, from http://www.informatics.jax.org/genes.shtml

Perry, E. C. (2014, May 1). Inpatient Management of Acute Alcohol Withdrawal Syndrome. Retrieved January 16, 2018, from https://web-a-ebscohost-com.prxy4.ursus.maine.edu/ehost/pdfviewer/pdfviewer?vid=2&sid=be62f22d-13fc-43f6-abff-13ef90ed9d06%40sessionmgr4008

Philibin, S. D., Cameron, A. J., Schlumbohm, J. P., Metten, P., & Crabbe, J. C. (2011, September 24). Ethanol Withdrawal-Induced Motor Impairment in Mice. Retrieved January 14, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3939695/

Sachdeva, A., Choudhary, M., & Chandra, M. (2015, September 1). Alcohol Withdrawal Syndrome: Benzodiazepines and Beyond. Retrieved January 13, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4606320/

Serchov, T., Calker, D. V., & Biber, K. (2016, October 5).Sucrose Preference Test to Measure Anhedonic Behaviour in Mice. Retrieved February 23, 2018, from https://bio-protocol.org/e1958

Serchov, T., Calker, D. V., & Biber, K. (2016, October 5). Light/Dark Transition Test to Assess Anxiety-like Behavior in Mice. Retrieved February 24, 2018, from https://bio-protocol.org/e1957

Simon, M. M., Greenaway, S., White, J. K., Fuchs, H., Gailus-Durner, V., Wells, S., Brown, S. D. (2013, July 31). A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. Retrieved February 16, 2018, from https://www.ncbi.nlm.nih.gov/pubmed/23902802

Takao, K., & Miyakawa, T. (2006, November 13). Light/dark Transition Test for Mice. Retrieved March 15, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2504462/

University of Kentucky. (n.d.). Commonly Used Mouse Strains. Retrieved February 14, 2018, from https://www.research.uky.edu/dlar/documents/CommonMouseResearchModels.pdf

What are single nucleotide polymorphisms (SNPs)? National Institute of Health – Genetics Home Reference. (2018, March 13). Retrieved March 14, 2018, from https://ghr.nlm.nih.gov/primer/genomicresearch/snp

Yankelevitch-Yahav, R., Franko, M., Huly, A., & Doron, R. (2015, March 2). The Forced Swim Test as a Model of Depressive-like Behavior. Retrieved March 1, 2018, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4401172/

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After Graduation, Eric will be taking two gap years to work within the biology field and study for the MCAT before applying to medical school.