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Investigation of Genetic Differences In The Influence Of Ethanol On Trace Fear Conditioning Behavior In Mice

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ABSTRACT

The genetic differences between individuals are important to consider when examining the effects of drugs on learning and memory. The neural circuitry of learning and memory can be affected by a number of different drugs, including alcohol. One way to study the effects of alcohol exposure on changes in learning is to use the Pavlovian model of fear conditioning. Trace fear conditioning is a type of fear conditioning in which there is a delay between the conditioned stimulus and the unconditioned stimulus, engaging regions of the brain that can be affected by alcohol. Inbred mouse strains are a useful animal model for this type of experiment due to their lack of genetic variability within strains. Genetic differences between strains, however, are of interest in this experiment because they may lead to unique responses to the fear conditioning paradigm in the presence of alcohol. The purpose of this honors thesis is to explore genetic differences in the effects of acute ethanol intoxication on the learning response to trace fear conditioning. These differences are important to investigate when considering how genetics play a role in how alcohol causes different learning deficits between individuals. The initial hypothesis was that there would be a significant effect of both strain and drug treatment on freezing, the learning response. Male and female C57BL/6J and DBA/2J mice were treated with saline or one of two doses of ethanol and tested through a trace fear conditioning program. The results of this experiment showed that ethanol contributed to inhibited fear learning in both context and cued tests. Strain was also found to have differing effects on ethanol sensitivity between C57BL/6J and DBA/2J mice. There were no significant effects of sex. These results suggest the potential for future research and have important implications for how genetics can influence the sensitivity of learning and memory mechanisms to alcohol.

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Introduction

Learning and memory are two essential functions of the brain that allow individuals to interact with their environment. These functions are susceptible to a variety of environmental factors. Behaviors such as drug use can influence the mechanisms of learning and memory in the brain (McGaugh & Petrinovich, 1965). The processes of learning and memory have long been studied in relation to various drugs that can affect them. One such drug is alcohol, the most widely abused recreational drug (SAMHSA, 2015). The effects of alcohol on the brain and body range from desirable to aversive. Alcohol can facilitate social bonding (Sayette *et al.,* 2012), but it can also cause impairment in cognitive and executive functioning, especially in binge drinkers (Weissenborn & Duka, 2003). Cognitive deficits related to alcohol use vary in severity depending on a number of different factors. Understanding these factors is critical for treatment of alcohol-related deficits, as well as preventative measures for those who consume alcohol on a regular basis. It is already known that certain genetic factors contribute to a higher risk for alcohol dependence (Dick & Bierut, 2006). This raises the question of how else genetics contribute to the effects of alcohol in the brain.

Animal models such as inbred mouse strains serve as a useful and important tool for drug research, including alcohol research. Inbred strains exhibit a lower amount of genetic variability than outbred, or highly variable, populations of mice (Casellas, 2010). This allows researchers to perform experiments on mice within inbred strains without having to account for genetic differences between subjects. The genetic differences between inbred strains can also be studied when genetic background is meant to be considered. Strain comparisons are useful when determining the role of genetics in a subject's response to certain stimuli, as different strains exhibit different behaviors during a variety of laboratory tests (Crawley *et al*., 1997).

There are a number of paradigms that can be followed to test learning and memory in mice. A common model used to test learning in mice, specifically learned relationships between stimuli, is fear conditioning (Maren, 2001). This process follows a Pavlovian classical conditioning model in which the subjects learn to associate aversive and neutral stimuli (Pavlov, (1927), P.I. 2010). Mice are placed in a novel environment, and a neutral conditioned stimulus, an auditory cue or tone, is co-presented with an aversive unconditioned stimulus, a small foot shock. The rodents can then be put through a context test, in which they are placed in the same environment in the absence of either stimulus, or they can be put through a cued test, in which they are placed in an entirely new environment with the presence of only the conditioned stimulus. If the subjects can pair the cue to the foot shock, it is an indicator of learning. The dependent variable used to quantify learning in fear conditioning is freezing, which is the absence of any movement except for respiratory movement. Freezing is the primary defensive response to fear in rodents (Fanselow, 1994). More freezing indicates stronger learning, whereas less freezing indicates inhibited learning.

Trace fear conditioning is a specific type of fear conditioning that engages a different type of neural circuitry than delay (normal) fear conditioning. In delay conditioning, the tone and foot shock are presented simultaneously during training. However, in the trace fear conditioning paradigm, there is a delay between the end of the tone and the beginning of the foot shock. This requires the formation of a memory trace, rather than simply associating the two stimuli as a pair. Trace conditioning has been shown to involve both working and declarative memory in the hippocampus and prefrontal cortex (Connor & Gould, 2016). Study of hippocampal function, specifically the CA1 region of the hippocampus, may utilize trace fear conditioning to test learning and memory dependency upon this area of the brain (Sharma *et al.*, 2018). In contrast,

delay conditioning is not shown to be dependent upon these brain areas. Lesions of the hippocampus do not interfere with learning in cued delay conditioning (Phillips & LeDoux, 1992). It has also been argued that attention is required for trace but not for delay fear conditioning (Han *et al*, 2003). The contextual learning test, on the other hand, examines whether the subjects have formed a connection between the foot shock and the environment in which the shock took place. Like trace cued learning, contextual learning is hippocampus-dependent, and it is thought to be involved in the same mechanisms that produce declarative memory in humans (Rudy *et al.*, 2004). Contextual and trace cued learning depend upon overlapping brain regions yet utilize different types of memory. Comparing these types of learning in the presence of alcohol could provide insight into alcohol's effects on the hippocampus and prefrontal cortex, and in turn contextual and working memory. The prefrontal cortex has been shown to be more susceptible to alcohol than the hippocampus (Fowler *et al.*, 2014). It would be interesting to determine whether, as a result, these different types of memory are also differentially susceptible to alcohol.

There is limited literature on the effects of acute ethanol intoxication on the learning mechanisms involved in trace fear conditioning. It has been shown that acute ethanol withdrawal can cause differential effects on learning depending on the type of fear conditioning test. For example, withdrawal increases the cued response and decreases the contextual response in both delay and trace fear conditioning (Tipps *et al.*, 2015). Similarly, there is little research on acute ethanol exposure before the rodents are exposed to the trace fear conditioning paradigm. One study involving rats used post-training ethanol intoxication and found that this disrupted the trace conditioned fear response (Hunt *et al.*, 2009). There is little data on pre-training ethanol intoxication in mice, however. With this limited research in mind, this experiment was designed

to test the effects of acute ethanol exposure prior to training on the response to trace fear conditioning and whether these effects varied by genetic background.

The results of fear conditioning of any kind with inbred mice can often prove difficult to interpret. Significant differences in the response of different strains of inbred mice have been discovered across trace, delay, and non-associative conditioning (Tipps *et al.*, 2014). This experiment looks at males and females of two genetically distinct strains of inbred mice, C57BL/6J and DBA/2J. One recent study performed in this lab found that C57BL/6J mice exhibit higher sensitivity to ethanol than DBA/2J mice during contextual fear conditioning, but not during cued delay conditioning (Seemiller & Gould, 2021). This led to the expectation of a similar outcome in this study. The purpose of this honors thesis is to further explore genetic differences in acute ethanol-induced learning changes. This is part of a larger project which focuses on the effects of strain, age, and sex on acute ethanol-induced learning changes. The hypothesized outcome is significant effects of both strain (in which C57BL/6J mice experience more learning deficits than DBA/2J mice) and treatment (in which ethanol-treated groups experience more deficits than saline-treated groups).

Methods

This experiment used male and female C57BL/6J and DBA/2J mice (Jackson Laboratory, Bar Harbor, ME). The design of this experiment was 2 x 2 x 3 (strain x sex x drug treatment). Each mouse was assigned to either saline, 1g/kg of ethanol solution, or 1.5 g/kg of ethanol solution. The ethanol solution was 25% ethanol by volume. There were 12 treatment groups (see Table 1), with 8 mice per treatment group (n = 96). Mice were housed in pairs, keeping members of the same treatment group together.

Table 1: Experimental design of strain, sex, and ethanol treatment groups

C57BL/6J						DBA/2J					
Male			Female			Male			Female		
sal	1 g/kg EtOH	1.5 g/kg EtOH	sal	1 g/kg EtOH	1.5 g/kg EtOH	sal	1 g/kg EtOH	1.5 g/kg EtOH	sal	1 g/kg EtOH	1.5 g/kg EtOH

The experiment was run in four cohorts of 24 mice (two mice per treatment group in each cohort). The mice were split into six groups of four mice (two treatment groups at a time, kept in separate cages). On training day, each group received intraperitoneal injections of the assigned drug treatment. An acclimation period of 14 minutes began after the second mouse was injected. Once acclimated, mice were placed individually into identical chambers and put through the automated trace fear conditioning training program. The program is 880 seconds and contains five tone/shock pairings with a 30 second delay in between the tone and shock. The tone (conditioned stimulus) is a 30 second long, 85-dB white noise, and the shock (unconditioned stimulus) is a 2 second long, 0.45 mA foot shock (Gould *et al.*, 2004). The mice were video recorded for the duration of this training.

On testing day (approximately 24 hours after training), each group was placed back into the same chamber as training and put through the context test (absence of tone or shock for 5 minutes and 30 seconds). Then, each group was taken to a new room with different chambers for the cued test. The cued chambers were smaller in size and contained white plastic flooring rather than grating. The walls of the chamber also differed in size and color from the training chambers. A paper towel was spotted with 0.3 milliliters of vanilla extract and placed under each chamber in the cued room. Each mouse was placed into a separate chamber and put through the cued test (absence of tone for three minutes, then presence of tone for three minutes with no shock). The mice were also video recorded for both the context and cued tests. Freezing in each mouse for every video was measured with Ethovision, an automated computer system.

Freezing was analyzed via a 3-way ANOVA (Strain, Sex, Treatment), followed by Tukey's HSD post-hoc comparisons when interactions were detected. Difference scores (representing contextual learning) were created by normalizing ethanol-induced freezing values to saline-induced freezing values, representing percent change in freezing for each ethanol treatment group [((individual ethanol-induced freezing value – saline freezing group average) / (saline freezing group average) * 100]. Difference scores were analyzed via a 3-way ANOVA (Strain, Sex, Treatment), followed by Tukey's HSD post-hoc comparisons when interactions were detected. All statistics were conducted using IBM SPSS Statistics 28.

Results

I. Strain impacts sensitivity to ethanol-induced contextual fear learning deficits

3-way ANOVA (Strain, Sex, Treatment) of freezing during the context test showed a main effect of Treatment ($F_{2,84} = 26.301$, $p < 0.001$), with ethanol-treated groups showing lower freezing than saline-treated groups. A significant Strain by Treatment interaction ($F_{2,84} = 4.177$, $p = 0.019$) was also found. Significant sex effects were not found.

To assess the Strain by Treatment interaction, Tukey's HSD post-hoc comparisons showed a significant difference between the C57BL/6J saline-treated group and the C57BL/6J 1 g/kg ethanol ($p < 0.001$), C57BL/6J 1.5 g/kg ethanol ($p < 0.001$), DBA/2J 1 g/kg ethanol ($p < 0.001$), and DBA/2J 1.5 g/kg ethanol ($p < 0.001$) groups. There was also a significant difference between the DBA/2J saline group and the C57BL/6J 1.5 g/kg ethanol ($p < 0.001$), DBA/2J 1 g/kg ethanol ($p = 0.028$), and DBA/2J 1.5 g/kg ethanol ($p = 0.044$) groups. Post-hoc comparisons showed that within each strain, contextual learning of both ethanol groups was significantly impaired when compared to the saline group. This does not explain the Strain by Treatment interaction. Additional statistical analyses were necessary to explore this interaction further.

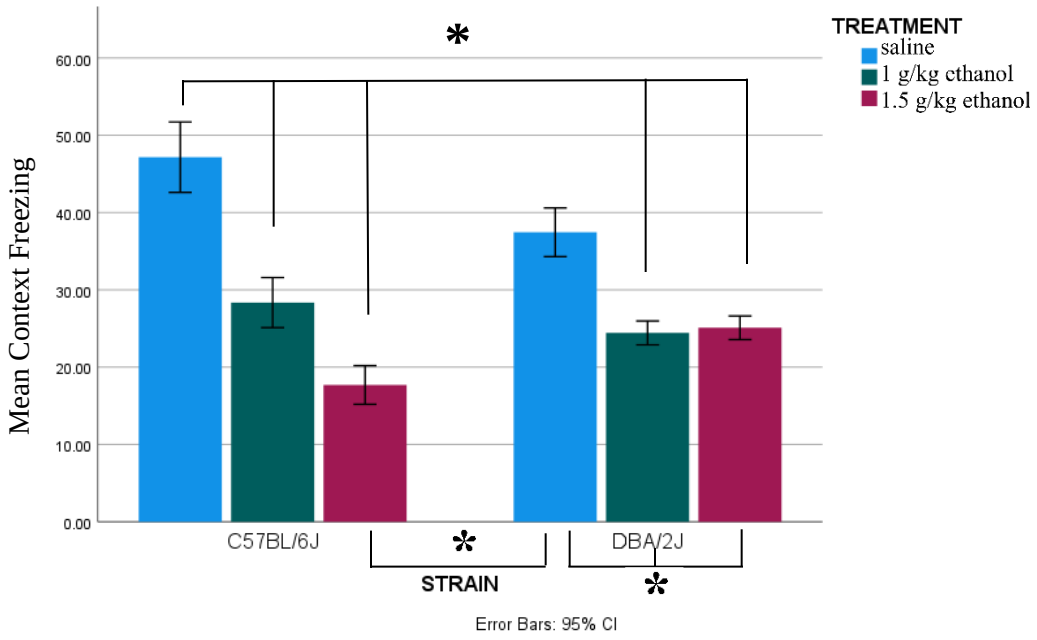

Figure 1: Effects of ethanol on contextual fear learning (collapsed across sex). n=48/strain. Data are presented as mean +/- SEM. Asterisks indicate statistical significance of values (relative to saline group) after Tukey's HSD post-hoc comparisons ($p < 0.05$).

To further examine the Strain by Treatment interaction, difference scores were calculated for ethanol-treated groups in relation to the saline-treated group within each strain. This showed the percent change in freezing from the saline group for each ethanol treatment group. Difference scores were compared by a 3-way ANOVA (Strain, Sex, Treatment). This showed a main effect of Strain ($F_{1,56} = 11.068$, $p = 0.002$), with ethanol-treated DBA/2J mice showing smaller difference scores (smaller decrease in freezing) than ethanol-treated C57BL/6J mice. A main effect of Sex ($F_{1,56} = 5.082$, $p = 0.028$) was also found, with ethanol-treated male mice showing smaller difference scores (smaller decrease in freezing) than ethanol-treated female mice. A significant Strain by Treatment interaction ($F_{1,56} = 5.126$, $p = 0.027$) was also found. Strain and sex were both shown to impact the change in contextual learning from baseline, and alcohol-induced deficits in contextual learning were again shown to differ between strains.

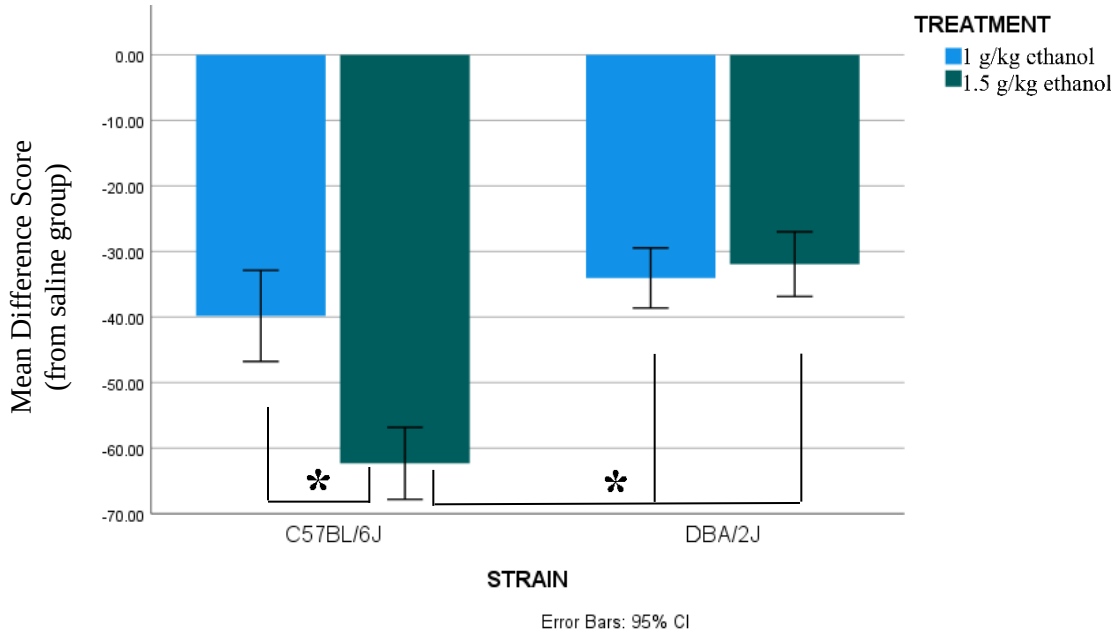

Figure 2: Difference scores of ethanol-treated groups in relation to saline-treated group (collapsed across sex). n=16/treatment/strain. Data are presented as mean +/- SEM. Asterisks indicate statistical significance of values (relative to C57BL/6J 1.5 g/kg ethanol group) after Tukey's HSD post-hoc comparisons ($p < 0.05$).

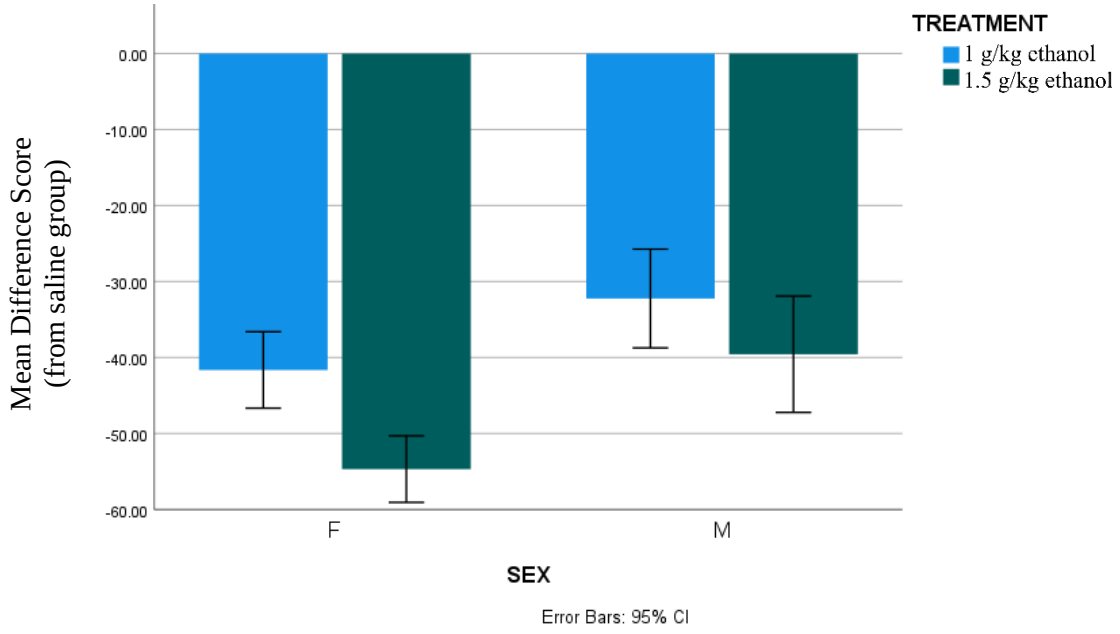

Figure 3: Difference scores of ethanol-treated groups in relation to saline-treated group (collapsed across strain). n=16/treatment/sex. Data are presented as mean +/- SEM.

To better understand the Strain by Treatment interaction, Tukey's HSD post-hoc comparisons were performed on the difference scores for each strain-treatment pair. A significant difference was found between the 1.5 g/kg ethanol-treated C57BL/6J mice and the 1 g/kg ethanol-treated C57BL/6J ($p = 0.029$), the 1 g/kg ethanol-treated DBA/2J ($p = 0.004$), and the 1.5 g/kg DBA/2J ($p = 0.002$) mice. There was no significant difference between ethanol-treated DBA/2J mice, indicating that the Strain by Treatment interaction exists because ethanol-treated C57BL/6J mice freeze less in response to higher doses of ethanol. In contrast, ethanol-treated DBA/2J mice do not. C57BL/6J mice experience greater contextual learning deficits associated with higher amounts of alcohol, whereas DBA/2J mice experience similar contextual learning deficits between different amounts.

II. Ethanol weakens trace cued fear learning in C57BL/6J and DBA/2J mice

3-way ANOVA (Strain, Sex, Treatment) of freezing during the cued test also revealed a main effect of Treatment ($F_{2,84} = 3.302$, $p = 0.042$), with ethanol-treated groups expressing less freezing than saline-treated groups. There were no significant strain or sex effects, and no interactions were found, suggesting that genetics and sex do not play a role in the sensitivity of trace cued fear learning to alcohol.

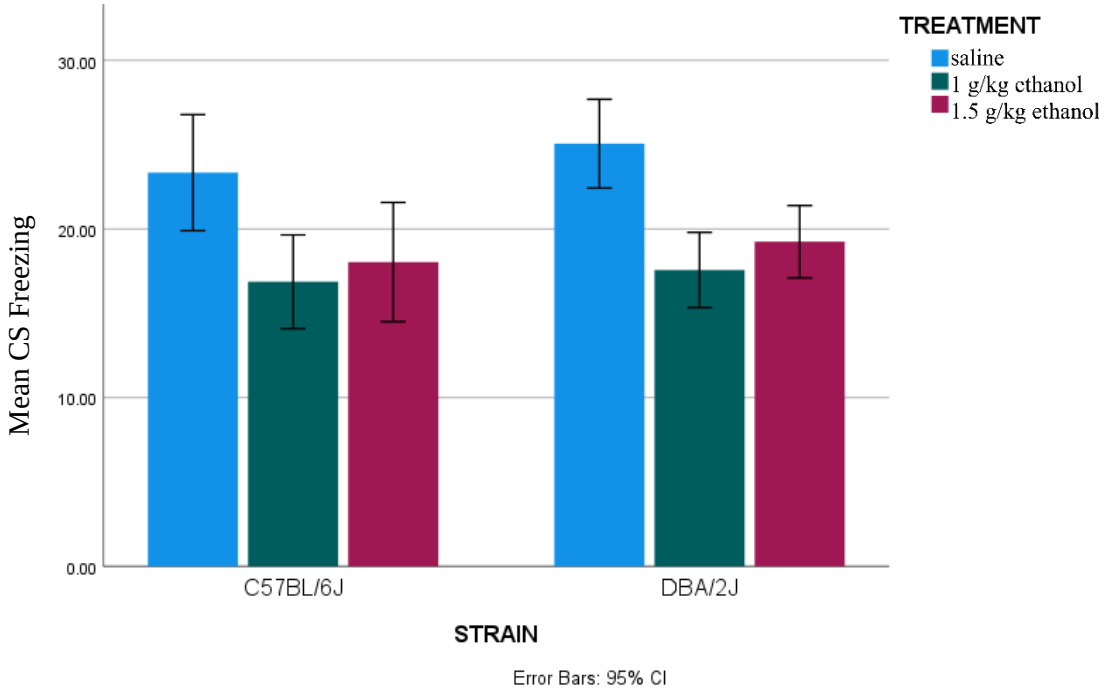

Figure 4: Effects of ethanol on trace cued fear learning (collapsed across sex). n=48/strain. Data are presented as mean +/- SEM.

Discussion

The purpose of this honors thesis was to examine genetic differences in ethanol-induced learning deficits using the trace fear conditioning paradigm. The results showed main effects of alcohol treatment on both contextual and trace cued learning, with the drug-treated groups freezing less than the saline-treated groups one day after training. This indicates that in both cases, pre-training ethanol contributed to deficits in fear learning. Existing research has also shown alcohol-induced impairment of contextual and trace cued fear learning, arguing that these deficits are due to the effects of alcohol on hippocampal function (Weitemier & Ryabinin, 2003). Therefore, the current finding that alcohol impairs fear learning in both contextual and trace cued fear conditioning supports previous findings that the hippocampus is particularly sensitive to the effects of alcohol. It also suggests that the prefrontal cortex is not disproportionately affected because greater deficits for trace cued learning, which additionally engages the prefrontal cortex (Raybuck & Gould, 2010), were not observed.

A Strain by Treatment interaction indicated that there was a genetic difference in contextual freezing in response to ethanol treatment. Post-hoc comparisons of strain-treatment pairs showed significant differences in freezing between the C57BL/6J saline group and all of the ethanol groups, as well as between the DBA/2J saline group and both DBA/2J ethanol groups in addition to the C57BL/6J 1.5 g/kg ethanol group. This does not provide any explanation for genetic differences in ethanol sensitivity because within each strain, both ethanol groups were significantly different from the saline group.

Difference scores were calculated for each strain to represent the percent change in freezing from the saline group to each ethanol group. Analysis of difference scores showed main effects of both strain (with DBA/2J mice showing smaller learning deficits than C57BL/6J mice)

and sex (with male mice showing smaller learning deficits than female mice). A Strain by Treatment interaction was found again. Post-hoc comparisons of difference scores in strain-treatment pairs showed significant differences between the C57BL/6J 1.5 g/kg ethanol group and every other strain-treatment pair. This shows that the C57BL/6J ethanol groups significantly differed from one another in the percent change in freezing from baseline, whereas the DBA/2J ethanol groups did not. This means that in contextual fear learning, C57BL/6J mice were more susceptible to higher doses of alcohol than DBA/2J mice, providing an explanation for the Strain by Treatment interaction. A similar interaction was found in a related study examining the effects of strain, age, sex, and two different doses of ethanol on fear conditioning. Analysis of contextual learning showed that C57BL/6J mice were susceptible to alcohol-induced contextual learning deficits with both doses, and the DBA/2J mice were only susceptible to these deficits with the higher dose (Seemiller & Gould, 2021). Thus, the findings in this study were consistent with the existing notion that different genetic backgrounds contribute to different sensitivities to alcohol. Studies comparing hippocampal activation and gene expression in C57BL/6J and DBA/2J mice during ethanol withdrawal suggest that the hippocampus is differentially affected by withdrawal in these strains (Chen *et al*., 2009; Daniels & Buck, 2002). It is possible that similar inconsistencies in hippocampus activity could contribute to the strain differences observed in this study.

The genetic difference in alcohol sensitivity as shown in contextual learning was not observed in trace cued learning. Contextual fear conditioning depends on hippocampal function (Rudy *et al.*, 2002), whereas trace cued fear conditioning depends on function of both the hippocampus and prefrontal cortex (Raybuck & Gould, 2010). Because different brain regions are involved in contextual versus trace cued fear conditioning, this could mean that, between

strains, certain regions of the brain are more differentially sensitive to ethanol than others. A lack of genetic difference in freezing between treatment groups with trace cued learning could mean that the prefrontal cortex is more similar between strains with regard to ethanol sensitivity.

Analysis of freezing in both tests showed no effects of sex on fear learning. Sex differences in contextual fear learning have been reported in the past, with females exhibiting higher freezing than males (Gresack *et al*., 2009). It is interesting that this difference was not observed in the context test. However, analysis of the difference scores in contextual learning did show a main effect of sex in which ethanol-treated male mice exhibited a smaller deficit in learning than ethanol-treated female mice. This relates more to the way in which male and female mice may be differentially sensitive to alcohol-induced contextual learning deficits. It is possible that a main effect of sex was seen in difference scores but not in contextual freezing due to a power issue. A larger sample size may have lead to results that are more consistent with previous findings.

Based on these results, an interesting comparison involving the genetic discrepancy in the context test would be to compare the results of post-training ethanol on contextual learning after both delay training and trace training. Regardless of whether the context test is performed during delay or trace conditioning, the results should in theory be the same because the association is between the shock and the context. However, because the training programs are different between the two types of fear conditioning, it would be interesting to compare the two in case the training module itself has any effect on learning.

Because the goal of this research was to examine genetic differences in ethanol sensitivity, a limitation of this study was that only two inbred mouse strains were used as a comparison. Since there was not a significant effect of sex in the context or trace cued tests, an

alternative experimental design could include only one sex and more strains. Another limitation is that the only type of learning studied was fear learning. Had the subjects been taken through other learning paradigms involving other neural circuitry, the effects of alcohol may have been different. For example, the Morris water maze is another type of learning paradigm designed to test spatial learning, utilizing brain regions such as the basal forebrain, striatum, and cerebellum in addition to the hippocampus (D'Hooge & De Deyn, 2001). Using a test such as this could lead to highly different results regarding alcohol and its effects on learning. Within fear learning, the dependent variable is typically freezing because it has been shown to directly relate to conditioned fear (Fanselow, 1980), which is why freezing was used to quantify learning in this experiment. However, it is possible that certain strains may show alternative fear behaviors in addition to or instead of freezing (Seemiller *et al*, 2021). In the future, it may be worthwhile to examine how alternative fear behaviors may play a role in fear learning across strains.

This research provides important implications for the role that genetics play in sensitivity to alcohol. In humans, alcohol is known to cause a variety of deficits in cognition, including impairment of spatial awareness and overall reduction in activity of cortical and subcortical brain regions (Jacob & Wang, 2020). Understanding how different genetic backgrounds lead to different responses to alcohol is important for knowing how to treat and prevent these cognitive deficits. The finding that subjects of one genetic background may experience more cognitive susceptibility to different amounts of alcohol than subjects of a different genetic background relates in part to the concept of tolerance. Knowing the genetic factors that contribute to tolerance also allow for a better understanding of factors that influence dependence and addiction.

In this study, it was found that alcohol induced learning deficits in both context and cued tests of trace fear conditioning in C57BL/6J and DBA/2J mice. In addition, the C57BL/6J mice were more susceptible to higher doses of ethanol than the DBA/2J mice during contextual fear learning. These results will serve to supplement the results of a larger ethanol study involving strain, age, and sex. This research could provide worthwhile implications for the importance of genetic background with regard to alcohol and its effects on learning and memory.

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ACADEMIC VITA

Emily Henry

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Objective

To be accepted into a four year MD program

Education

The Pennsylvania State University — University Park, PA
Eberly College of Science — B.S. Biology Candidate
Schreyer Honors College — Psychology, Neuroscience minors
Expected Graduation: May 2022

Related Coursework

Organic Chemistry I, II	Organic Chem Lab	Honors Biochemistry I, II
Clinical Neuropsychology	Biostatistics	Behavioral Neuroscience

Relevant Experience

Undergraduate Research Assistant — 9/2019-present
Gould Lab, University Park, PA

- Assist graduate students and postdoctoral scholars in conducting research
- Work both in lab and virtually on projects with mice
- Current project: Honors Thesis on Genetic Differences in the Influence of Ethanol on Trace Fear Conditioning Behavior

Learning Assistant — 1/2021-12/2021
Organic Chemistry Laboratory Course, University Park, PA

- Hosted office hours and attended online workshops or in-person labs weekly
- Reviewed course content and answered students' questions
- Communicated with instructors and teaching assistants

Honors and Organizations

Volé Penn State Dance Company — 8/2018-present

- Current positions: Treasurer, member of performance company, showcase choreographer
- Previous positions: THON Family Relations Chair, weekly technique teacher

Alpha Epsilon Delta

- National Health Preprofessional Honor Society

Academic Honors

- The President's Freshman Award, Spring 2019
- The President Sparks Award, Spring 2020
- The Evan Pugh Scholar Senior Award, Spring 2021

Other Experience

Brand Ambassador — 8/2017-1/2021 (seasonal)
American Eagle Outfitters, Hershey, PA

- Communicated with and assisted customers
- Handled money and transactions at the register
- Unpacked shipment and performed tasks in the stockroom

Delivery Driver — 5/2021-present
DoorDash, Hershey, PA

- Complete orders on Dasher app
- Pick up orders and deliver to customers in a timely fashion