DIAZEPAM EFFECTS ON ANXIETY-RELATED DEFENSIVE BEHAVIOR OF HIGH AND LOW
OPEN-FIELD ACTIVITY INBRED MOUSE STRAINS

by

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Thesis directed by Associate Professor Marissa A. Ehringer

Open-field activity is a commonly used measure of anxiety-related behavior in rodents. The inbred High and Low Activity strains of mice, selected for extreme differences in open-field activity, have been used as a genetic model of anxiety-related behaviors. The goal of this study was to determine if the treatment of adult male and female High Activity (low anxiety) and Low Activity (high anxiety) mice with Diazepam, an agonist at the benzodiazepine allosteric site on the GABA_A receptor and a drug commonly prescribed to treat anxiety disorders in humans, lead to decreases in anxiety-like defensive behavioral responses as assessed in the open-field test (OFT) and elevated plus-maze (EPM). We have tested the effects of three doses of Diazepam (0, 0.5, 1.0, 3.0 mg/kg, i.p.), given 30 min before behavioral testing to one High Activity strain (H2) and two Low Activity strains (L1 and L2). The only anxiolytic response observed was in the High Activity animals, with more entries into the open arms of the elevated plus-maze, like common mouse strains. Lack of response to Diazepam suggests the Low Activity animals are not displaying classic conflict anxiety-like behavior, and instead may be displaying unconditioned fear-related behaviors, such as freezing behaviors, when exposed to novelty. Fear and anxiety are distinguishable traits, and both contribute to anxiety disorders in humans.
DEDICATION

This thesis is dedicated to my mother, Lisa Willis. I would not be where I am without your endless love and support. You taught me the importance of hard work and commitment, and I am forever grateful for your patience and encouragement.
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First, I would like to thank my advisor, Dr. Marissa Ehringer. Thank you for all your help and support these last couple of years. I am excited to continue working with you and learning from you. I am so grateful for such a wonderful mentor.

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I. INTRODUCTION

Anxiety disorders are of interest because of their impact on society, with high prevalence, considerable cost, and lack of effective treatments. Generalized anxiety disorder is one of the most common mental disorders, with up to 20% of adults affected by anxiety disorders each year (Muniv & Takov, 2020). According to the Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM–5; American Psychiatric Association, 2013) anxiety disorders consist of separation anxiety disorder, selective mutism, specific phobia, social phobia, panic disorder, agoraphobia, and generalized anxiety disorder (American Psychiatric Association, 2013). Often anxiety disorders begin early in life and negatively affect individuals for decades (Ask et al., 2021). In addition, there is evidence that anxiety disorders are significantly undertreated. A large study conducted in Europe that showed that only 21% of the respondents with an anxiety disorder sought help from a professional (Bandelow, 2020). The treatment of anxiety disorders is also costly, with an estimated cost of $48.72 billion dollars in the United States in 2013 (Shirneshan, 2013). The first line treatments for these disorders are selective serotonin or norepinephrine reuptake inhibitors (SSRIs and SNRIs). Although they are the most used treatment for anxiety disorders, successful response rates are limited to only 30 to 50 percent and generally patients must deal with multiple side effects (Munir & Takov, 2017). Much is known about the neural mechanism underlying anxiety, less is known about the genetic factors contributing to underlying risk for these disorders (Ask et al., 2021). It is also common for anxiety disorders to co-occur within individuals, and comorbidities with other psychiatric disorders, including depression, which could indicate a shared underlying genetic risk (Ask et al., 2021).

Many studies in humans have tried to uncover the genetic contribution to anxiety disorders, and other psychiatric disorders. There has been limited success because anxiety disorders are highly complex and polygenic (Ask et al., 2021; Meier & Deckert, 2019). A variety of candidate gene studies, twin studies,
genome-wide association studies, epigenome-wide association studies, rare variant studies, and others have attempted to uncover the genetic nature of anxiety disorders. While there has been some progress in human research, only a few risk loci have been identified (Meier & Deckert, 2019). However, anxiety disorders are thought to have an estimated SNP heritability from 1.7% (in childhood/adolescence) to 31% (in adulthood), which is less than the 20-60% heritability we see from twin studies (Ask et al., 2021). Common genes that have been assessed correspond to the monoaminergic neurotransmitter systems and the hypothalamic-pituitary-adrenal (HPA) axis (Meier & Deckert, 2019). Genome wide association studies have more recently been used but are still underpowered for reliably detecting causal variants for anxiety disorders (Ask et al., 2021; Meier & Deckert, 2019). As sample sizes increase these association studies will be able to uncover more variants of interest. Human studies are slowly revealing more about the genetics anxiety disorders, but with limited success thus far animal models remain invaluable.

A classical model of anxiety-related behavior tested in mice measures approach-avoidant behaviors when the animal is put in a low-threat and uncertain novel environment (La-Vu et al., 2020; Lezak et al., 2017). This assay relies on the mouse’s conflict the mouse has between a desire to explore these novel environments and avoiding danger (La-Vu et al., 2020; Lezak et al., 2017). Mice are averse to brightly lit and open spaces and tend to favor enclosed spaces that are dimly lit, to reduce the risk of predation (La-Vu et al., 2020; Lezak et al., 2017). Anxiety assays can measure these avoidance behaviors of bright open spaces and exploration. In the open-field test (OFT) the center is seen as more anxiogenic than the walls and corners of the box. Lower anxiety-related behavior is attributed to more time spent in and entries into the center and shorter latency to move to the center, and move overall distance traveled. In the elevated plus maze (EPM) the open arms are seen as more anxiogenic, meaning more time spent and entries into them are interpreted as lower anxiety levels. In addition, stretch attend posture (SAP) is a behavior associated with anxiety and is seen as a risk-assessment behavior, with increases in SAP when the mouse is placed in an open-field (Holly et al., 2016). This behavior is reduced by anxiolytic drugs (Holly et al., 2016).
Many rodent anxiety assays measure risk assessment behavior, including OFT, EPM (and variations of it), light-dark box, novel object exploration test, hole board assay, and the Vogel conflict test (La-Vu et al., 2020). It is recommended to include more than one ethological test in the study of this anxiety-related behavior (Henderson et al., 2004). The High and Low Activity strains used in this study were bred using the OFT, so it was appropriate to include this test (Figure 1A). Previously, our lab tested these strains in a behavioral battery paradigm that consisted of the OFT, light-dark box, EPM, a second OFT, a second light-dark box, and a novel object exploration test (Booher et al., 2021). To allow for better comparison across studies of these strains the other behavior test done in addition to the OFT was EPM, which was also used in previous studies on these strains (Figure 1B) (Booher et al., 2021; Flint et al., 1995; Henderson et al., 2004). In addition, many studies that have tested the effects of Diazepam on anxiety-related behavior have included OFT or EPM (Ennaceur et al., 2010; Ennaceur, 2012; Griebel et al., 2000; Salomons et al., 2012; Michalak et al., 2020; Fraser et al., 2010).

Figure 1. (A) Open-field box. (B) Elevated plus maze (La-Vu et al., 2020)
There has historically been concern and debate about the validity of these behavioral tests that rely so heavily on locomotion and general activity levels, particularly the OFT. These tests use levels of exploration to assess anxiety-related behavior but may not be able to fully disentangle changes in activity level and anxiety (Rosso et al., 2021). For example, the OFT is used to investigate both anxiety-related behavior and locomotor activity (Rosso et al., 2021). This point will be further addressed during the review of recent work done with these High and Low Activity strains.

In the 1960s John DeFries observed the inherent differences in open-field activity and defecation scores between BALB/cJ and C57BL/6J mice, with BALB/cJ mice being less active than C57BL/6J mice, with the defecation scores are inversely related (DeFries, 1966). DeFries et al. (1978) bidirectionally selected for open-field activity for 30 generations using the F3 generation as the founder population, creating two High Activity lines (H1 and H2), two Low Activity lines (L1 and L2), and two control lines (C1 and C2). Throughout selection open field activity was assessed and the most active male and female were chosen from each litter and were mated randomly within line for the High Activity lines, while the least active male and female were chosen for the Low Activity lines. The control lines used mating of randomly chosen males and females. After 30 generations there was a 30-fold difference in open-field activity (Figure 2). The defecation scores of the low activity mice were also seven times higher than the high activity. A significant negative genetic correlation exists between open-field activity and defecation scores. After selection, the six lines were randomly mated within line for 18 generations and then inbred using brother-sister mating (DeFries et al. 1978). Only the two Low Activity (L1 and L2) and one High Activity (H2) are available now.

After the creation of the inbred strains Quantitative Trait Loci (QTL) studies were done to identify regions of the genome associated with the observed behavioral differences in the strains, with both L1 x H1 and L2 x H2 F2 crosses (Flint et al., 1995, Turri et al., 2001a, Turri et al., 2001b, Henderson et al., 2004). Combined, these studies identified QTLs on mouse chromosomes 1, 4, 7, 8, 12, 14, 15, 17, 18, and
X. These QTLs in total account for between 20 and 36% of total phenotypic variation in the behavioral tests used (Henderson et al., 2004). It was determined that the QTLs that increase activity in the OFT were derived from the C57BL/6 mice, whereas QTLs from BALB/c mice where QTLs decrease activity. This is true for all loci except the one from the X chromosome (Turri et al., 2001b). The QTLs on chromosomes 1 and 15 showed the most consistent effect on anxiety-related behavior (Flint et al., 1995, Turri et al., 2001a, Henderson et al., 2004). The QTL on chromosome 1 predominantly influenced safe-area locomotor activity and autonomic reactivity (Henderson et al., 2004). The QTL on chromosome 15 had a larger effect on suppressing activity in higher anxiogenic areas. It also increased latency to move from one location to another and suppressed rearing behavior. However, the QTLs on chromosomes 4 and 8 influenced locomotor activity in novel environments and their home cages. The QTL on chromosome 7 is likely from the albino mutation and has an overall suppressive effect on activity in anxiogenic areas (Henderson et al., 2004).
More recent work on these High and Low Activity strains included confirming the behavioral phenotypes of these strains, and sequencing studies to uncover genes and pathways contributing to the behavioral differences between these strains. Booher et al. (2021) used a behavioral test battery, previously mentioned, that confirmed the extreme anxiety-related behavior differences between the High and Low Activity strains. As previously discussed, a common criticism of using mice bred for activity levels as a model for anxiety-related behavior is that the differences in activity levels in the tests are actually just differences in general locomotion (Rosso et al., 2021). To address these concerns the novel object test and home cage wheel running were assessed. The novel object test is less reliant on locomotion and the results show decreased anxiety-related behavior in High Activity mice. In addition, home cage running was assessed. The male Low Activity mice ran almost twice as much daily compared to male High Activity. However, the female High and Low Activity mice did not differ in the amount of wheel running (Booher et al., 2021). These new results provide support for the hypothesis that the behavioral differences observed between the High and Low Activity strains are not due to baseline differences in overall movement.

Thomas & Evans et al. (2021) performed whole-genome sequencing on both Low Activity strains (L1 and L2) and both High Activity strains (H1 and H2). The distribution pattern of DNA variants from both parental strains, BALB/cJ and C57BL/6J, were examined to identify and compare the variants shared between and within the High and Low Activity strains. The study found that roughly 10% of the variants are likely associated with open-field activity and further showed the polygenicity of the trait. The whole-genome sequencing was used in combination with similar genomic datasets from online databases using the GeneWeaver tool to help refine the QTLs previously identified (Thomas & Evans et al., 2021). Some genes and pathways were found that are of interest for follow up studies. These will be considered in the discussion in the context of the results of this study.

RNA sequencing was also performed using hippocampal tissue from the H2 and L2 strains (Booher, in preparation). The hippocampus was selected because pathological anxiety is associated with this brain
region, perhaps due to its critical role in contextual learning/memory (Booher, in preparation). This allowed for further prioritization of genes and pathways, which is considered in the discussion section of this study.

This study expands on previous work with the strains by using an anti-anxiety drug in the benzodiazepine class, Diazepam. Diazepam is a nonselective full agonist at the benzodiazepine allosteric site on the GABA\textsubscript{A} receptor and increases GABA potentiation (Balon & Starcevic, 2020). GABA is the main inhibitory neurotransmitter in the central nervous system, inhibiting brain activity. This system is central to the regulation of anxiety. A benzodiazepine was chosen because of its fast-acting anxiolytic effects after acute administration, whereas drugs such as SSRIs when administered acutely have anxiogenic effects (Birkett et al., 2011). Benzodiazepines have long been prescribed for anxiety disorders and are generally well tolerated but have a risk of dependence and sedative effects (Nuss, 2015). The heterogeneity of the GABA\textsubscript{A} receptors has led to an interest in receptor subtypes impact on different behavioral responses. This is seen in the nonselective GABA\textsubscript{A} receptor agonist, Diazepam, where its anxiolytic effect is seen in a\textsubscript{2} containing GABA\textsubscript{A} receptors, but not in a\textsubscript{3} or a\textsubscript{1} (Blanchard et al., 2003).

This study aimed to validate and characterize the High and Low Activity strains as a genetic model of anxiety-related behavior. It was hypothesized that we would see reduction in anxiety-related behavior predominantly in the Low Activity strains, with minimal effect on High Activity strain.
II. METHODS

Animals

Adult male and female High (H2) Activity and Low (L1 and L2) Activity were used. A total of 241 mice were tested, with 10 in each grouping of sex, strain, and dosage, except the female L2 saline group, which had 11 animals. For each strain of mice tested the animals were split into different treatment groups or the saline control group. There was consideration taken in each round of testing to include each strain, sex, and dose. The mice were aged 60-90 days old at the start of the experimental behavioral testing. The animals were bred and housed at the Biofrontiers Institute at the University of Colorado Boulder. Once the mice were weaned, they were transferred to the Institute of Behavioral Genetics at the University of Colorado Boulder. They were housed with their same-sex siblings and their ears were clipped to differentiate between individuals in group housing. The mice were housed in a colony room with a 12-hour light and 12-hour dark cycle. Testing occurred at least two hours after the start of the light cycle. Standard polycarbonate cages with 30 cm length x 13 cm width x 17 cm height dimensions were used, along with ad libitum access to water and food (Envigo Teklad 2914 irradiated rodent diet, Harlan, Madison, WI, USA). Experiments were run according to the Office of Laboratory Animal Welfare, along with protocol approval from the local Institutional Animal Care and Use Committee (IACUC).

Drugs

For each strain, H2, L1, and L2, there were four different test groups, three dosages of Diazepam and a saline control group. The three doses of Diazepam chosen were 0.5, 1, 3 mg/kg, based on previous studies using common lab strains (Griebel et al., 2000; Ohl et al., 2001; Ennaceur et al., 2010; Ennaceur, 2012; Michalak et al., 2020). The Diazepam was first dissolved in DMSO and then diluted with physiological (0.9%) saline. The DMSO concentration varied slightly by the concentration of Diazepam, but no more than 1.2% DMSO was in the final solutions.
At 9 AM (2 hours into the light cycle) all the mice to be tested were relocated to the testing room. They were left to acclimate for 30 minutes. The animals received their respective treatments via an intraperitoneal injection starting at 9:30 AM. They were then left to acclimate and allow the drugs to take effect for another 30 minutes. A 30-minute period was chosen because the half-life of Diazepam is 60 minutes (Ennaceur et al., 2010). This way the drug has enough time to have an effect but not lose potency.

**Behavioral Tests**

**Open-field Test (OFT)**

OFT was the first behavioral test completed on day 1 of the 2 days of behavioral testing. Once the mice receive their respective treatments, they are left in the testing room for 30 minutes to acclimate and allow time for the onset of drug effects. At 10 AM (3 hours into the light cycle) the OFT commenced. The floor of the center of the OFT box measured approximately 420 lux of light. A gray OFT arena was used to obtain more accurate tracking of the Low Activity mice, which are white. The gray OFT arena was also used for the High Activity mice. The OFT arena was 40 cm in length x 40 cm in width x 34.5 cm in height. The floor of the arena was divided into the outer region (40 x 40 cm), a big center area (27 x 27 cm), and a small center region (18 x 18 cm) (Figure 3). The individual mouse would be placed against the middle of the same wall facing toward the center to start each test. After release the mouse had 600 seconds, or 10 minutes, to explore their surroundings in the arena.

Between each animal trial the arena was cleaned using 70% ethanol. This behavior and movement were recorded on video and tracked using Etho-Vision® (EthoVisionXT 8.5, Noldus Information Technologies BV). The measurements examined included the total distance
traveled in the arena, the latency of initial movement, frequency, duration, and latency to enter the big center and smaller center, and frequency of stretch attend posture. Manual tracking was needed for latency measures and frequency of stretch attend posture because of issues with the tracking software.

**Elevated Plus Maze (EPM)**

EPM was the second behavioral test completed on day 2 of behavioral testing. The injections, and therefore the behavioral test, started exactly 24 hours after the OFT on day 1 (9 AM acclimation start). The floor of the center of the EPM measured approximately 620 lux of light. The EPM used was white, but black strips of a similar texture were placed on the arms to allow for more accurate tracking of the white Low Activity mice. The black strips were not used with the brown High Activity mice because the tracking was inaccurate. The EPM was elevated 30.5 cm from the table, on a table that was 75 cm tall. The maze’s center was a square of 5 cm x 5 cm. From there, four arms extend out, with the dimensions of 30.5 cm in length x 5 cm in width. Two of the arms are open, and two are enclosed by walls 17.5 cm tall. To start each test, the individual mouse was placed at the opening of the same open arm, positioned with its head toward the open arm. After release the mouse had 300 seconds, or 5 minutes, to explore their surroundings in the arena. Between each animal trial the arena was cleaned using 70% ethanol. This behavior and movement were recorded on video and tracked in real time using Etho-Vision® (EthoVisionXT 8.5, Noldus Information Technologies BV). The behaviors that were focused on were the percent of time spent in the open arms, percent of entries into open arms, and the raw number of open arm entries, as well as frequency of stretch attend posture, which was scored manually for this test as well.
III. RESULTS

Statistical Analyses

The data were split by sex because of known differences in anxiety-related phenotypes between males and females, and in the High and Low Activity strains (Booher et al., 2021; Donner & Lowry, 2013).

One-way Analysis of Variances (ANOVA) was done for each behavioral measure separated by sex and strain to compare activity at each dose of Diazepam because we were most interested in within strain effects of the Diazepam. We also noticed baseline differences in the two Low Activity strains (L1 and L2), so t-tests were done to compare their activity.

Before each One-way ANOVA we conducted Levene’s test for equality of variances and if the data did not meet the equality of variance assumption, then Welch’s ANOVA was done. It is noted if Welch’s ANOVA was needed for analysis. The Shapiro-Wilk test was used to check the normality of the data at every dose, and if the data did not meet the normality assumption a Kruskal-Wallis test was used. It is noted if Kruskal-Wallis was needed for analysis. In addition, studentized residuals were used to identify outliers. Only one mouse appeared as an outlier in more than one test, but it was two related measures of big center duration and frequency. Therefore, no mice were removed from analyses. If the One-way ANOVA was significant, Tukey’s HSD test was run to look at specific dose differences. If the Kruskal-Wallis test was run instead, it was followed up with the multiple comparison Dunn’s test. Student t-tests were used to analyze differences in baseline activity of the L1 and L2 strains. If the variance equality assumption was broken, a Welch’s t-test was used instead, and if the normality assumption was violated, a Wilcoxon rank-sum test was used.
Baseline differences in Low Activity strains

In the OFT there were baseline differences between L1 and L2 in every measure. A Student’s t-test found that female L2s had a significantly higher OFT distance traveled compared to L1s, t(19)= 2.83, p=.011. A Student’s t-test also found that male L2s had a significantly higher OFT distance traveled compared to L1s, t(18)= 5.30, p<.001. A Welch’s t-test found that female L2s spent significantly more time spent in the OFT big center, t(14.17)=3.21, p=.0062. A Wilcoxon rank sum test also found that male L2s spent significantly more time in the OFT big center significantly more than the female L1s, t(13.62)= 3.15, p=.0073. A Wilcoxon rank sum test also found that male L2s enter the OFT big center significantly more than the male L1s, W=6, p<.001. There were no differences between male or female L1 and L2 in any of the EPM measures.

Open-Field Test (OFT)

Distance travelled

There was no difference in the distance traveled between doses in female L1 and H2 animals (Figure 4A). However, the Kruskal-Wallis test revealed dosage difference in total distance traveled for female L2 animals, $\chi^2(3)=15.45$, $p=.0015$ (Figure 4A). Dunn’s test for multiple comparisons showed female L2s traveled significantly less distance at 3 mg/kg compared to the saline group and 0.5 mg/kg (0 and 3mg/kg $p= .019$, 0.5 and 3 mg/kg $p=.0011$). There was no difference in the distance traveled between doses in male L1s (Figure 4B). However, the One-way ANOVA revealed dosage difference in total distance traveled for male L2s, F(3, 36)=8.45, $p<.001$ (Figure 4B). Tukey’s HSD post hoc showed that the 3 mg/kg treated animals traveled significantly less distance compared to the saline group and the other two doses (0 and 3 mg/kg $p=.027$, 0.5 and 3 mg/kg $p< .001$, 1 and 3 mg/kg $p=.0011$). A One-way ANOVA also revealed dosage difference in total distance traveled for male H2s, F(3, 36)=13.33, $p<.001$ (Figure 4B). Tukey’s HSD post hoc showed that the 3 mg/kg animals traveled significantly less distance
compared to the saline group and the other two doses (0 and 3 mg/kg p< .001, 0.5 and 3 mg/kg p< .001, 1 and 3 mg/kg p< .001).

**Big center duration**

The data for the big and small center duration and entries were analyzed. Results were very similar, and the small center did not add any additional information, therefore only the big center data will be reported here. For all three strains the females did not show any significant change in time spent in the big center regardless of dose (Figure 5A). Male L1 and H2 animals did not have a significant change in time spent in the big center with any dose of Diazepam (Figure 5B). However, a One-way ANOVA for male L2s revealed dosage difference in time spent in the big center, F(3, 36)=3.07, p=.040 (Figure 5B). Tukey’s HSD post hoc showed that there was a significant decrease in time spent in the big center with 3 mg/kg compared to 0.5 mg/kg (p=.038).

**Big center entries**

There was no difference in the number of big center entries between doses in female L1 and H2 animals (Figure 6A). However, the Kruskal-Wallis test revealed dosage differences in big center entries for female L2 animals, χ²(3)=9.26, p=.026 (Figure 6A). Dunn’s test for multiple comparisons showed female L2s had significantly less distance traveled at 3 mg/kg compared to the saline group and 0.5 mg/kg (0 and 3mg/kg p= .047, 0.5 and 3 mg/kg p=.047). Male L1s did not have any significant changes in entries based on dose (Figure 6B). However, a One-way ANOVA for male L2s revealed dosage difference in entries into the big center, F(3, 36)=6.25, p=.0016 (Figure 6B). Tukey’s HSD post hoc showed that there was significant decrease in entries into the big center with 3 mg/kg compared to 0.5 mg/kg and 1 mg/kg (0.5 and 3 mg/kg p=.0012, 1 and 3 mg/kg p=.014). A One-way ANOVA for male H2s revealed dosage difference in entries into the big center, F(3, 36)=4.38, p=.010 (Figure 6B). Tukey’s HSD post hoc showed that there was significant decrease in entries into the big center with 3 mg/kg compared to 0.5 mg/kg and 1 mg/kg (0.5 and 3 mg/kg p=.011, 1 and 3 mg/kg p=.036)
Latency for first movement and latency to enter the centers needed to be tracked manually because of challenges with the tracking software. Those data were not ready in time for the preparation of this manuscript. The manual tracking of frequency of stretch attend posture was also not completed in time for inclusion.

**Elevated Plus Maze**

**Percent time spent in open arms**

Female L1 and L2s had no significant change in percentage of time spent in the open arms at any dose (Figure 7A). However, a One-way ANOVA for female H2s revealed dosage differences in percent of time spent in the open arms, $F(3, 36)=3.21, p=.035$ (Figure 7A). Tukey’s HSD post hoc showed that there was significant increase in percent of time spent in the open arms at 3 mg/kg compared to the saline group (0 and 3 mg/kg $p=.019$). Male L1 and L2s had no significant change in percentage of time spent in the open arms at any dose (Figure 7B). However, a Kruskal-Wallis test for male H2s revealed dosage difference in percent of time spent in the open arms, $\chi^2(3) =15.19, p=.0017$ (Figure 7B). Dunn’s test for multiple comparisons showed that there was significant increase in percent of time spent in the open arms at 3 mg/kg compared to the saline group (0 and 3 mg/kg $p <.001$).

**Percent of entries into open arms**

Female L1s had no significant change in percent of entries into the open arms (Figure 8A). However, a Kruskal-Wallis test for female L2s revealed dosage difference in percent of open arm entries, $\chi^2(3) =8.59, p=.035$ (Figure 8A). Dunn’s test for multiple comparisons showed that there was significant increase in percent of open arm entries at 3 mg/kg compared to the saline group (0 and 3 mg/kg $p=.027$). A One-way ANOVA for female H2s revealed dosage difference in percent of open arm entries, $F(3, 36)=7.54, p <.001$ (Figure 8A). Tukey’s HSD post hoc showed that there was significant increase in percent of open arm entries at every dose compared to the saline group (0 and 0.5 $p=.011$, 0 and 1
p=.031, 0 and 3 mg/kg p<.001). Male L1 and L2s had no significant differences in percent of open arm entries at any dose (Figure 8B). However, a Kruskal-Wallis test for male H2s revealed dosage difference in percent of open arm entries, χ²(3) =17.77, p <.001 (Figure 8B). Dunn’s test for multiple comparisons showed that there was significant increase in percent of open arm entries at 3 mg/kg compared to the saline group (0 and 3 mg/kg p <.001).

**Number of open arm entries**

In all three strains the females had no significant differences in number of open arm entries at any dose (Figure 9A). Male L1 and L2s had no significant differences in number of open arm entries at any dose (Figure 9B). However, a One-way ANOVA for male H2s revealed dosage difference in total number of open arm entries, F(3, 36)= 4.29, p=.011 (Figure 9B). Tukey’s HSD post hoc showed that there was significant increase in total number of open arm entries at 0.5 mg/kg compared to the saline group (0 and 0.5 mg/kg p=.0069).

Latency to enter arms was analyzed and no differences were found. However, we did not expect these results to be useful because of the starting placement of the animals. They were placed at the base of the opening of one of the open arms, which would confound these latency measures.
Figure 4. Total distance traveled in the open-field during the 10 minute session, measured in centimeters. (A) Females. n=10 for each box plot, except female L2 saline n=11. (B) Males. n=10 for each box plot.
‘***’ p <.001, ‘**’p <.01, ‘*’ p <.05
Figure 5. Total time spent in the big center of the open-field during the 10 minute session, measured in seconds. (A) Females. n=10 for each box plot, except female L2 saline n=11. (B) Males. n=10 for each box plot.

*** p < .001, ** p < .01, * p < .05
Figure 6. Total number of entries into the big center of the open-field during the 10 minute session. (A) Females. n=10 for each box plot, except female L2 saline n=11. (B) Males. n=10 for each box plot. ‘***’ p <.001, ‘**’ p <.01, ‘*’ p <.05
Figure 7. Percent of time spent in the open arms of the EPM during the 5 minute session. (A) Females. n=10 for each box plot, except female L2 saline n=11. (B) Males. n=10 for each box plot.

*** p < .001, ** p < .01, * p < .05
Figure 8. Percent of EPM open arm entries during the 5 minute session. (A) Females. n=10 for each box plot, except female L2 saline n=11. (B) Males. n=10 for each box plot.

*** p <.001, ** p <.01, * p <.05
Figure 9. Total number of entries into the EPM open arms during the 5 minute session. (A) Females. n=10 for each box plot, except female L2 saline n=11. (B) Males. n=10 for each box plot.

‘****’ p < .001, ‘***’ p < .01, ‘**’ p < .05
IV. DISCUSSION

The prominent differences in baseline activity between the High and Low Activity strains remain consistent with previous testing of these animals (Booher et al., 2021, DeFries et al., 1978). However, we did see differences in the baseline activity of the L1 and L2 strains in the OFT, but not the EPM. The L1 had significantly less activity than the L2 in the OFT total distance traveled and duration and entries in the big center. These strains are not genetically identical, which could explain the slight differences observed (Thomas & Evans et al., 2021, Turri et al., 2001b). There are no large QTLs that were found that are different between the strains, but there may be smaller QTLs that differ between the two replicate strains (Turri et al., 2001b). The L1s have not been tested in many years, so this phenotypic difference could be interesting to study further.

The highest dose of 3 mg/kg appears to be sedative, because we see decreased activity in most measures at this dose. In the OFT we see the 3 mg/kg decrease total distance traveled in female L2s and male L2 and H2s compared to baseline. Female L2s also showed a significant decrease in OFT big center entries at 3 mg/kg, compared to baseline. In the EPM the female and male H2s with a longer time spent in the open arms only in the 3 mg/kg compared to baseline. The mice would be placed at the opening of the open arm and would partially enter the open arm before remaining still for the majority of the trial. This accounts for why many of the animals had 100 percent of time spent in the open arms at higher doses, which caused a wider range of values and explains the wide boxplots seen in percentage measures (Figures 7 and 8). For this reason, raw values of open arm entries were analyzed as well, including an analysis of raw values using n-1, to make sure that first entry was not confounding results. The n-1 results are not presented here because the results did not differ from the analyses using the total number of entries. The impairment in locomotion was likely due to the sedative effect of high doses of Diazepam, which has been shown in other studies from doses of 2 mg/kg to 5 mg/kg (Pádua-Reis et al., 2021, Lepicard et al., 2000, Ohl et al., 2001, Salomons et al., 2012).
The only non-sedative Diazepam effect was observed with lower doses in High Activity mice in the EPM. For the male H2s there was a significant increase in open arm entries at 0.5 mg/kg. For the female H2s all the doses caused an increase in percentage of open arm entries compared to the saline group. These increases in open arm entries in the H2 strain show there is at least some decrease in anxiety-like behavior in this strain, as assessed by EPM. These results are similar to other studies that have used Diazepam with a variety of common mouse strains. Michalak et al. (2020) studied male swiss mice and found an increase in percent of open arm entries with 1 mg/kg of Diazepam. Lepicard et al. (2000) assessed male BALB/cByJ and C57BL/6J mice and found a dose dependent (0.5, 1, and 2 mg/kg) increase in the number of open arm entries, percentage of open arm entries and time spent in the open arms. Lastly, Griebel et al. (2000) tested nine common lab strains and found that most had increases in percent time spent in open arms and percent of entries into open arms (Griebel et al., 2000). These included BALB/c, Swiss, C57BL/6, DBA/2, NMRI, and NZB. The effective dosage depended on strain but was between 1 and 3 mg/kg. In some of the strains decrease in activity was observed at 3 mg/kg, which could be related to drowsiness such in this study. However, the effects of Diazepam detected in the H2 mice in the EPM were not observed in the OFT, as some other studies have seen (Salomons et al., 2012, Michalak et al., 2020). There are many reasons this could be the case. One meta-analysis of anxiety related defensive behavior tests found discordance in results across studies when comparing the OFT and EPM (Mohammad et al., 2016). It has also been hypothesized that there is a higher anxiety load of the EPM as compared to the OFT (Heinz et al., 2021).

The anxiolytic effects of Diazepam were only seen in the H2 strain, not in the L1 or L2 strains. It was expected that the Low Activity strains would experience a larger anxiolytic effect because generally mice with higher anxiety-like phenotypes are more sensitive to Diazepam. This can be seen in both animals that are selectively bred for high and low anxiety-related behavior, as well as with common lab strains that have inherent differences in anxiety-related behavior. Liebsch et al. (1998) used male rats selectively bred for high-anxiety–related behavior (HAB) and low-anxiety–related behavior (LAB) using the EPM.
These strains initially differ in their time spent and entries into the open arms, with the HAB spending less time and entering the open arms less. With 1 mg/kg of Diazepam they saw a 20-fold increase for the HAB in the percentage time spent on the open arms, while the LAB animals had only a 2.5-fold increase. Similarly, there was a 5.6-fold increase for the HAB rats in the percentage of open-arm entries, while the LAB animals had a 1.2-fold change (Liebsch et al., 1998). It has been observed that BALB/c animals have higher levels of anxiety-related behavior compared to other strains, such as C57BL/6, and are more sensitive to Diazepam (Singewald, 2007, Ennaceur et al., 2010). The lack of effect of the Diazepam, even at lower non-sedative doses, suggests the inbred Low Activity strains may not be displaying the classical approach-avoidant conflict, but rather fear-related behavior. The anxiety-related behaviors assessed using the EPM and OFT are Diazepam sensitive, as previously discussed, which provides support for an alternative behavioral phenotype, such as fear. These two behaviors are often considered together, however there is a distinction between fear and anxiety-related behaviors.

Historically, the baseline differences in activity of the High and Low Activity strains have been classified as differences in approach and avoid conflict and risk assessment, which is challenged by this study’s findings with Diazepam. It is possible that the tendency of the Low Activity strains to freeze is confounding the results of the OFT and EPM. This can be seen with a previous study reported by Booher et al. (2021), where there was a difference in OFT total difference traveled but not in time spent in the center and outer section. In that study, the Low Activity mice were placed in the middle of the open-field box and freeze, with some displays of stretch attend posture. This starting placement was used because of the tendency of the Low Activity animals to freeze but was still an issue when they were started in the center. For the current study, the animals were placed against the wall to try and avoid the center freezing. Using this different starting place, differences between the High and Low Activity strains were observed, which could be due to freezing in the outer region opposed to the center. Other studies have found a decrease in center time in their higher anxiety lab strains. Stead et al. (2006) used male and female Sprague- Dawley rats selectively bred for exploratory locomotion in a novel environment as high
responders (HR) or low responders (LR). The LR display a higher anxiety-related phenotype. In the OFT the LR animals spent significantly less time in the center (Stead et al., 2006). In another study male BALB/c animals were tested against 129P3 in an OFT. As previously mentioned BALB/c animals have been shown to have higher levels of anxiety-related behavior, which was seen in this study with less center entries for the BALBs compared to the 129P3 (Salomons et al., 2012).

Freezing behavior can be classified under the fear-related behaviors (Daniel-Watanabe & Fletcher, 2021; Blanchard et al., 1997; Blanchard et al., 2003). Generally, animals will attempt to flee first, but if that is not possible then they will freeze (Blanchard et al., 2003). Given the limited space in the open-field box and EPM this freezing makes sense. The flight and freezing behaviors associated with fear are more powerful than any general anxiety of the mouse (Blanchard et al., 2003). In addition, Diazepam does not decrease freezing, or flight, whereas panicolytic drugs do (Blanchard et al., 2003). This freezing behavior is usually induced with an imminent threat, such as a predator like a cat (Blanchard et al., 1997; Blanchard et al., 2003). These selected strains could be displaying an innate fear phenotype, with the experimenter as the potential predator.

A lot of studies do not differentiate between anxiety and fear, and they are often grouped together. Distinctions can be made between anxiety and fear, but these can be difficult and there are some inconsistencies that need further research (Daniel-Watanabe & Fletcher, 2021). Fear is defined as phasic whereas anxiety is sustained fear (Daniel-Watanabe & Fletcher, 2021; Davis et al., 2010). The phasic fear is more dependent on the presence, or imminent presence, of an aversive stimulus, generally a predator, that causes fleeing, or hiding or freezing if flight is not an option (Daniel-Watanabe & Fletcher, 2021; Blanchard et al., 2003). This phasic fear has been linked to the central nucleus of the amygdala (Daniel-Watanabe & Fletcher, 2021, Davis et al., 2010). The sustained fear attributed to anxiety is caused by a sustained expectation that an aversive event, such as the potential of a predator is likely (Daniel-Watanabe & Fletcher, 2021; Blanchard et al., 2003). This is seen with more ambiguous stimuli and risk assessment.
behaviors (Daniel-Watanabe & Fletcher, 2021; Blanchard et al., 2003). The sustained fear has been linked to the bed nucleus of the stria terminalis (BNST) (Daniel-Watanabe & Fletcher, 2021; Davis et al., 2010).

The central extended amygdala plays a crucial role in both anxiety and fear, with the two major subdivisions being the central nucleus of the amygdala and the BNST (Shackman & Fox, 2016). It has long been believed that these structures were distinct and separate in fear and anxiety (Davis, 2010). More recent work done by Shackman and Fox has shown that these structures are interconnected and overlapping with regard to their role in both anxiety and fear (Shackman & Fox, 2016; Fox & Shackman, 2019). Both structures have similarities in components such as connectivity as well as cellular composition and gene expression (Fox & Shackman, 2019). However, within these structures there are different types of neuronal cell populations. Some of these neurons are response-specific, while others are threat-specific (Fox & Shackman, 2019). The periaqueductal gray projecting neurons in the medial division central nucleus of the amygdala are one example, as these are involved in the trigger of freezing behavior, but in the same region medulla-projecting neurons instead trigger changes in cardiovascular activity (Fox & Shackman, 2019). There are other brain structures involved in anxiety and fear, including the hippocampus, ventromedial hypothalamus, periaqueductal gray, several brain stem nuclei, thalamic nuclei, insular cortex, and some prefrontal regions (Shin & Liberzon, 2010).

Distinguishing fear and anxiety in rodents rely on dissociating predictable and unpredictable threat. The Mouse Defense Test Battery (MDTB) was developed for measuring both fear and anxiety-related behaviors (Blanchard et al., 1997, Blanchard et al., 2003). The fear/defenses aspect includes a test with an oval runway that utilizes an anesthetized rat as the threat stimulus, where the mice will flee. When the runway is changed to a straight alley that is now closed off the mice freeze. The anxiety/defenses aspect includes a visible burrow system where a cat is briefly placed on the apparatus. The mice will flee and then freeze, but then will begin risk assessment behaviors. Another test that more specifically looks at fear, opposed to risk assessment is the beetle mania test. A robo-beetle is used as a threat stimulus and it

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was found that the behaviors were fear-related and distinct from exploration and anxiety using factor analysis (Heinz et al., 2021).

Blanchard et al. (2003) did factor analysis on the multiple measurements taken with the MDTB done on the mice. They found four factors that loaded on flight, freezing, defense threat and attack, and risk assessment. These findings show that there are separate phenotypes being assessed in the battery. These factors also agree with Gray’s three neuronal networks that distinguish between behaviors (Bijttebier et al., 2009). There is a dimension that accounts for exploratory drive and approach behavior that is called the Behavioral Activation System (BAS). Next is the avoidance behavior that is called the Fight Flight Freeze System (FFS). Lastly, the conflict solving that is called the Behavioral Inhibition System (BIS). These dimensions that make up Gray’s Reinforcement Sensitivity Theory apply animal learning research to human. It is based on the differences in sensitivity of individuals’ brain systems that are related to responding to stimuli that could be hurtful or rewarding. These differences appear to be related to human personality dimensions of anxiety (Bijttebier et al., 2009). The distinct dimensions may give insight into the Low Activity mice and their apparent freezing behavior. It may be that the Flight Freeze system is activated. And it was shown that if the threat outweighs the potential reward, then the FFS will be activated further by the BIS and the BAS will be inhibited (Bijttebier et al., 2009).

These distinctions are supported using different pharmacological agents during defensive behavior tests. Specific defensive behaviors in MDTB could differentiate drug effects on generalized anxiety and panic (Blanchard et al., 1997). Blanchard et al. (2003) used Diazepam in both the anxiety/defenses test and fear/defense test. They saw more effects of Diazepam in the anxiety/defense than in fear/defenses, including effects on risk assessment. The nonselective GABA<sub>\lambda</sub> – benzodiazepine receptor full agonists, such as Diazepam, were less effective in reducing flight, which agrees with clinical data of the limited efficacy of these drugs on panic disorder. Chronic administration of imipramine and fluoxetine, both 5-HT re-uptake inhibitors, reduced flight (Blanchard et al., 2003). The use of these drugs for the treatment
of panic disorder is well established (Blanchard et al., 2003). In addition, cholecystokinin-2 receptor antagonists, PD 135,158 and LY 288513, also reduced flight, but had no impact on risk assessment (Blanchard et al., 2003). Miles et al. (2011) also found that phasic and sustained fear are pharmacologically dissociable, using rats. Using fear conditioning they found that acute buspirone administered reduced phasic fear, but not sustained startle increases (Miles et al., 2011). Whereas chlordiazepoxide blocked sustained startle increases, but not phasic fear (Miles et al., 2011).

In addition to looking at the behavioral phenotypes and drug effects on these strains, we wanted to see how genes of interest we have previously identified came into play in fear-related behavior. With similarities and overlap in brain structures and circuitry between anxiety and fear the results we see with genes of interest continue to be of interest even if attention is shifted to fear-related behavior.

The previous whole-genome sequencing on the High and Low Activity strains used pathway analysis and found an over representation of genes involved in glutamate signaling (Thomas & Evans et al., 2021). Glutamatergic signaling has shown to play a role in anxiety and stress-related disorders (Peterlik et al., 2016). Specifically, the metabotropic receptor subtypes of this glutamatergic system have been associated with acute stress and fear. First, using an allosteric antagonist called MPEP (2-methyl-6-(2-phenylethynyl)pyridine) on mGlu5 revealed an anxiolytic affect in rodent models. This anxiolytic activity of MPEP can be seen in different behavioral tests, such as EPM, Vogel-conflict test, marble burying, and fear-potentiated startle paradigm (Peterlik et al., 2016). In addition to mGlu5 we also see a role of mGlu7. There have been mGlu7-KO mice that have shown decreased aversion, conditioned fear, and innate anxiety that is dependent on the amygdala. In addition, these knock out mice have upregulated suppression of the HPA axis that is based on glucocorticoid receptor feedback (Peterlik et al., 2016).

Two genes that showed up in the overlap of studies that also appeared in the High and Low Activity mice. These were Vnn1r1 and Ctsc. Ctsc codes for cathepsin C seems important in neuroinflammation, which has been linked to anxiety and stress disorders (Won & Kim, 2020). Of more interest, given the
results of this study, is $Vmn1r1$. $Vmn1r1$ codes for a vomeronasal receptor essential for pheromone and semiochemical detection, which helps animals of the same species communicate with each other (Jaio et al., 2019). Smell and pheromone detection is related to anxiety-related behaviors in rodents (Chen et al., 2019). This gene is of interest because of the fear-related tests involving predator odor and its influence on rodent fear behavior. In addition, olfactory bulbs project to the medial amygdala, which, as previously discussed, is important in acute anxiety and fear reactions (Davis, 1997).

Hippocampal RNA-sequencing of the High and Low Activity mice found that out of the top 264 candidate genes, 46 of them encode parts of the mitochondrial oxidative phosphorylation. In female L2 mice 39 of those 46 genes were upregulated. This highlights the importance of oxidative phosphorylation in anxiety-related behavior. There is growing evidence that shows a link between mitochondrial dysfunction and anxiety disorders (Filiou & Sandi, 2019). There appears to be a bidirectional relationship between mitochondrial function in the brain and anxiety (Filiou & Sandi, 2019). Disruption to mitochondrial function has been shown to increase anxiety. In humans, patients who reported higher symptoms, including phobias and panic attacks with agoraphobia, had an adenine-to-guanine mutation on mitochondrial transfer RNA (Filiou & Sandi, 2019).

Many mouse models have found similar associations between mitochondrial function and anxiety. A study by Misiewicz et al. (2019) used BNST tissue and blood from two inbred strains, C57BL/6NCrl and DBA/2NCrl, after chronic social defeat stress. They also analyzed blood from panic disorder patients. With pathway analysis they found that oxidative phosphorylation genes were differently expressed in the mice and human patients (Misiewicz et al., 2019). Another study that utilized high (HAB) and low (LAB) anxiety-related behavior mouse models found similar results (Filiou et al., 2011). They used quantitative proteomics and metabolomic in the cingulate cortex for analysis. These strains were originally bred from CD-1 mice using EPM. They found changes in pathways related glycolysis, neurotransmission, and mitochondrial function. Some oxidative phosphorylation-related genes were upregulated in the HAB over the LAB animals (Filiou et al., 2011).
To further investigate the behavioral phenotype displayed by the Low Activity strains, more testing is needed to distinguish between fear and anxiety. Many tests that could be used to examine the potential heightened fear response of the Low Activity mice. One possibility is to investigate innate fear responses in the animals, which is ‘unlearned’ fear. The MDTB could be used to characterize the behaviors of the Low Activity mice, as well as compared to the High Activity mice. Different panicolytic drugs could also be used with the assay, such as chronic administration of imipramine or fluoxetine and PD 135,158 and LY 288513. In addition, other predator/prey interaction experiments using predator odors such as cat urine and fur or fox feces could be done (Ganella & Kim, 2014; Rampin et al., 2018). Social interaction tests could also be done, looking at “social fear,” which refers to decreases in the investigation and fleeing or freezing, risk assessment, defensive burying, and/or alarm cries (Toth & Neumann, 2013). Food restriction before behavioral testing can be used to increase rodents’ exploration. In tests such as OFT, EPM, and light dark box food restriction has increased general exploration (Heinz et al., 2021). However, in a fear-related test, the beetle mania task, the food restriction did not decrease fear-related behavior and further enhanced avoidance behavior (Heinz et al., 2021). The beetle mania test could also be used without food restriction. In addition, conditioned or learned fear could also be of interest, because of the applications to studying phasic fear. Fear-potentiated startle and acoustic startle can be used to look at both phasic and sustained fear (Davis et al., 2010). A short and discrete cue that is imminent, such as an air blast or shock, is predictably paired with an aversive stimulus and allows measurement of phasic fear (Davis et al., 2010). Other tests could investigate other anxiety-related behaviors besides the innate approach-avoidant conflict. The Vogel conflict test uses learned approach-avoidant conflict, which differs from the inherent conflict aspect of OFT and EPM (La-Vu et al., 2020). It would be interesting to assess behavior in the OFT and EPM tests using a different anxiolytic, such as long-term injections of an SSRI, to see if behavior outcomes differ from Diazepam effects.

There are limitations to the current study. First, only a moderate number of behavioral measures were analyzed thus far. Next, behavioral testing is very sensitive to confounders, such as previous exposure to
handling, lighting conditions, noise, sex of handlers, smell, and other mice. The animals were not habitually exposed to human handling before testing to decrease fear of experimenter. This likely increased the freezing response seen in the Low Activity animals. The different color arm color between strains also limits our ability to accurately compare behavior in the EPM between the High and Low Activity strains. However, the results of this study still provide support for further testing into distinguishing the behavioral phenotype of the Low Activity strains.

Although animal models are useful in studying psychiatric disorders, there are clear and important differences between rodents and humans. These models cannot capture the human condition, for example emotional expression. However, these models are still beneficial because of conserved pathways, structures, and genes of interest between humans and rodents. In this study the fear behavior is a relevant aspect of anxiety-related behavior that parallels human behavior. Defense reactions of mice exposed to a threat stimulus may relate to different emotional states and perhaps model different aspects of human anxiety (Blanchard et al., 2003). Making these distinctions could be very useful in understating the underlying causes of the broad range of anxiety disorders, such as panic disorder, agoraphobia, social phobia, separation anxiety disorder, selective mutism, specific phobia, and generalized anxiety disorder (American Psychiatric Association, 2013).


<table>
<thead>
<tr>
<th></th>
<th>Low Activity (L1)</th>
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<th>Low Activity (L2)</th>
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<th>High Activity (H2)</th>
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<tr>
<td></td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
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<tr>
<td>Open Field</td>
<td>Mean (standard error)</td>
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<td>Mean (standard error)</td>
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<tr>
<td>Total distance traveled (cm)</td>
<td>2536.76 (398.64)</td>
<td>2660.89 (469.48)</td>
<td>2998.88 (721.05)</td>
<td>1632.46 (371.00)</td>
<td>2206.45 (340.55)</td>
<td>2255.40 (384.98)</td>
</tr>
<tr>
<td>Big center duration (s)</td>
<td>23.44 (6.61)</td>
<td>19.40 (7.67)</td>
<td>19.36 (6.32)</td>
<td>11.64 (3.06)</td>
<td>23.85 (8.54)</td>
<td>22.73 (9.82)</td>
</tr>
<tr>
<td>Big center entries</td>
<td>16.69 (4.91)</td>
<td>16.80 (6.16)</td>
<td>20.90 (6.87)</td>
<td>7.80 (2.62)</td>
<td>16.00 (5.56)</td>
<td>12.10 (4.75)</td>
</tr>
<tr>
<td>Elevated Plus Maze</td>
<td>Percent time in open arms (%)</td>
<td>27.22 (9.52)</td>
<td>39.43 (12.84)</td>
<td>53.36 (12.81)</td>
<td>44.14 (12.76)</td>
<td>19.44 (5.30)</td>
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<tr>
<td>Percent open arm entries (%)</td>
<td>31.86 (11.27)</td>
<td>39.51 (9.12)</td>
<td>53.17 (13.29)</td>
<td>50.56 (10.12)</td>
<td>23.50 (5.34)</td>
<td>50.54 (8.10)</td>
</tr>
<tr>
<td>Total open arm entries</td>
<td>20.30 (16.65)</td>
<td>24.60 (18.74)</td>
<td>3.00 (0.83)</td>
<td>5.80 (2.19)</td>
<td>7.00 (2.74)</td>
<td>7.10 (1.39)</td>
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Table 1. Mean and standard error for open-field and elevated plus maze measurements. (A) Female and male L1 animals. (B) Female and male L2 animals. (C) Female and male H2 animals.