THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

INVESTIGATING A POTENTIAL EFFECT OF AN IMMUNE SYSTEM CHALLENGE ON TEMPERAMENT

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SPRING 2013

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with honors in Biology

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ABSTRACT

Because both the immune and neural systems are known to require large amounts of energy and other resources (e.g. nutrients and time), it seems likely that trade-offs may occur between these two systems. In order to test whether such a trade-off does indeed occur, C57BL/6 mice were introduced to either a combination of *Bordetella pertussis* and saline or just saline when young (4 weeks old). At the same time they were introduced to cage enrichment, which is linked with neural stimulation. All of the mice were kept under a control feed ration in order to limit their available energy. To determine whether simultaneous immune and neural challenges led to a decreased investment in cognition, adult mice (12 weeks old) were tested with a learning and memory assay, the Barnes maze. As immune challenges have been shown to heighten expressed anxiety and other related temperaments (such as activity, exploration, and boldness), these temperaments may contribute to performance on the Barnes maze as opposed to solely their cognitive abilities. Thus temperament assays (the Elevated Plus Maze, the Open Field test, and a Novel Object Test) were given before the Barnes Maze. The results from the four treatment groups (vaccinated/enriched, vaccinated/not enriched, not vaccinated/enriched, not vaccinated/not enriched) were compared. Temperament behaviors were changed both by exposure to a vaccine and through exposure to enrichment. Exposure to enrichment increased general activity patterns in the Elevated Plus Maze and the Open Field Test.

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ACKNOWLEDGEMENTS

I would like to thank my parents, Dr. Lonnie Golden and Deborah Meyer. They have been supportive and encouraging throughout all of my decisions and have sent me many a neuroscience article. They have engrained female empowerment into my head enough to let me believe I can do anything, which has only been a positive thus far.

I would like to thank Dr. Victoria Braithwaite for providing me with all the necessary grants, techniques, materials, insights, and post docs to work on a wonderful project that truly complimented my research interests. I thoroughly enjoyed working with her and Adrianne Vasey on this project. Adrianne not only taught me about the exciting graduate school road ahead, but also the unconventional ways to enjoy life (whether it be dancing in the air or running a Half Ironman). After working with them I am that much more excited to get into the world of academia and run many more of my own research projects in the future.

Finally, I would like to thank Dr. Bernhard Luscher for reviewing this honors thesis and advising me on my academic career path.

INTRODUCTION

Background

As resources are often limited there is a tendency for animals to experience trade-offs when two or more factors simultaneously need investment, particularly if the systems concerned are energetically expensive. Two such high-resource demanding systems that may naturally require simultaneous investment are the immune system and the brain. For example, animals with challenged immune systems may potentially exhibit trade-offs with processes such as neural development. Changes to investment in neural development may have direct consequences for processes such as cognitive ability. To determine whether a simultaneous immune and neural challenge leads to a decreased investment in the brain, a comparison was made of the cognitive ability of adult mice (12 weeks age) that had been treated with either saline (control) or a vaccine (treatment) and then either experienced cage enrichment or not (in a 2x2 design). Cognition was assessed using a learning and memory task called the Barnes maze. However, because a change in neural development might also influence affective state, such as an individual's tendency for anxiety or boldness, and since these changes could affect performance in the Barnes maze, temperament should be tested alongside learning and memory to determine whether it is a confounding variable (Harrison et al, 2008). The research described in this thesis used assays of temperament (the elevated plus maze, the open field test, and the novel object test) to compare affective state in the mice being tested as part of a larger study that tested for the potential trade-off between immune system and neural development and its long-term effects on learning and memory.

Anxiety

Anxiety can be defined as an affective, or emotional, state induced by a threat that involves impaired homeostasis and can be observed in the form of various changes to behavior (Belzung and Griebel, 2001). It is also a potential by-product of stress (Tsigos et al, 2002). Thus observable anxiety comes in two forms: state and trait, where "state anxiety" is fear in response to a stimulus and "trait anxiety" is an enduring feature of an individual that is often coded in their genes (Belzung and Griebel, 2001). The research in this thesis will be testing "state anxiety," since the elevated plus maze and the open field test involve forced entry into a novel arena and therefore are associated with a stress response, in order to explore whether introducing an immune challenge and cage enrichment to young mice has long lasting effects on adult behaviors. In addition to the measures of anxiety, other temperament traits were assessed using a novel object test. Here the activity and willingness to explore a novel environment were quantified (Driscoll & Battig, 1982; Drent et al., 2003).

Behavioral Measures of Temperament

Three assays will be used to measure temperament: the elevated plus maze, the open field test, and the novel object test. Each measure will test exploration in and reaction to a novel environment, which is a critical feature of the holeboard, Barnes maze, test that will later be used to assess learning and memory.

The elevated plus maze, a raised plus-sign with perpendicular arms that alternate between walled and open, measures anxiety since anxiogenic drugs have been shown to decrease open-arm entries and time spent in the open arm in rodents (Carobrez and Bertoglio, 2005). Aversion to such open spaces could be related to thigmotaxis, an animal's preference for vertical surfaces as a natural defensive strategy which results in the animals tending to stay in the enclosed arms and avoid entering the open arms (Carobrez and Bertoglio, 2005). Head dips, stretch-attends, and

rears are risk assessment behaviors mice exhibit when they are anxious (Carobrez and Bertoglio, 2005). These ethological measures should be counted in conjunction with open and closed arm entries in order to validate that the mice were anxious when not moving, and not simply lethargic.

The open field test, an empty arena with marked concentric rings, measures activity, exploration, boldness, and neophobia (File, 2001). The test was designed around the concept that mice have a natural aversion to exposed fields, thus the activity in the central area was recorded and compared to the periphery of the box (Carola, et al, 2002). The open field therefore tests the mouse's thigmotaxis response, but it highlights the added measure of response to larger open fields where the expectation is that more anxious individuals will stay close the edge, but less anxious animals will move in an out of the exposed, open central area more frequently. Also much like the elevated plus maze, behaviors indicative of anxiety are recorded in the open field test, such as number of rears, stretch-attends, and groomings (Carola, et al, 2002) so as to avoid simply testing locomotion (File, 2001).

The novel object test uses the same apparatus as the open field task, but includes an object that the mouse has never interacted with before. Therefore the same behaviors and activities (such as number of entries into the center and the total time spent in the center) are reevaluated in this test (Dulawa et al, 1999), with the added measure of response to a novel stimulus (Zhuang, et al., 2001). It therefore tests forced exploration in a novel space.

Neural Mechanisms of Anxiety

Multiple neural mechanisms of anxiety have been proposed and studied. For example, one biological correlate of anxiety is heightened endogenous serotonin (5-HT) neurotransmission (Clement and Chapouthier, 1998). Pharmacological studies show that the ligands of three of serotonin's 14 receptor subtypes, 5-HT_{1A}, 5-HT_2, and 5-HT_3, cause anxiogenic and anxiolytic effects (Clement and Chapouthier, 1998). Other systems associated with anxiety disorders, or trait

anxiety, include cholecystokinin, $GABA_A$-benzodiazepine, NMDA, dopamine, corticotropin-releasing factor (CRF), glutamate, acid sensing ion channels, and neuropeptide Y (Clement and Chapouthier, 1998). Excitation of cholecystokinin's receptor, CCK-B, has anxiogenic effects in male hooded Lister rats (Singh et al, 1991). Knockout mice deficient in neuropeptide Y, and $GABA_A$ exhibit heightened anxiety in the elevated plus maze and the open field test (Crestani et al 1999; Bannon et al 2000).

Neophobia, measured as a longer latency to approach a novel object, has been associated with a heightened adrenal response. This seems to be driven by an increase in the release of corticotropin-releasing hormone in the brain, which is controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Cavigelli, 2005). Fearful rhesus monkeys were shown to have increased levels of corticotropin-releasing hormone in their brains (Kalin, 2000). Corticotropin-releasing hormone and arginine-8-vasopressin, found in the parvocellular neurons of the hypothalamic paraventricular nucleus, cause the anterior pituitary to release adrenocorticotropic hormone (ACTH) (Plotsky, 1991). ACTH stimulates the secretion of corticosterone, the primary glucocorticoid in rodents (cortisol in humans) (Varman et al, 2011). Rats that demonstrate low exploration in response to novel situations have also been shown to have elevated levels of glucocorticoids (Kabbaj et al, 2000). Glucocorticoids up-regulate the corticotropin-releasing hormone, thus completing the negative feedback loop that regulates the HPA axis (Plotsky, 1991). Since regulation of the HPA axis can adjust rapidly in response to the animal's environment (Plotsky, 1991) it could be a biological mechanism of both trait and state anxiety.

Stress and the Immune System

Both chronic and short-term stressors that activate an organism's HPA axis have been shown to impair an organism's immune function, which might be why stressed individuals have weaker immune systems (Gurfein et al, 2012). For example, people who indicated more

psychological stress were found to be more susceptible to the common cold (Cohen, 1991). Stress hormones, including ACTH, can modulate the immune system in two ways: 1) directly, by binding to immune cells and 2) indirectly, by causing a decrease in the production of cytokines (such as interferon-γ, interleukin-1, 2, and 6, and tumor-necrosis factor) (Glaser, 2005). Both paths ultimately weaken the cell-mediated immune response (Padget and Glaser, 2003).

It is also possible that the immune system could influence the HPA axis, and thus anxiety. Interleukin-1 is connected to the production of corticotropin-releasing hormone and lymphocytes can synthesize ACTH (Glaser, 2005). Mice that demonstrated anxious behavior in the elevated plus maze and an open field test had heightened levels of interleukin-1, and tumor-necrosis factor-a, and interleukin-6 in their hippocampus (Dinel et al, 2011). Mice with transgenic overexpression of interleukin-6 in their central nervous systems exhibited higher levels of arginine-8-vasopressin and corticosterone when submitted to restraint stress (Raber, 1997). High levels of peripheral interleukin-6 have been implicated in the cause of brain inflammation and neurodegeneration, which are both associated with cognition and mood impairment (Dinel et al, 2011). Additionally, high anxiety-related behavior rats were found to have higher corticosterone and ACTH levels after a lipopolysaccharide (LPS) injection than low anxiety-related behavior rats (Salome, 2008). However, it is unclear whether this increase was due to the injection itself or the immune system responding to the injection with increased levels of interleukin-6 (Salome, 2008). Swiergiel and Dunn found that injecting mice with LPS and interleukin-1 caused an increase in anxiety-like behaviors in the elevated plus maze and open field test, as well as a decrease in overall locomotion, a confounding factor in these tests (Swiergiel and Dunn, 2007). Swiergiel and Dunn used these tests to try to distinguish animals that were unwell from those that were anxious. However, since there was a decrease in locomotion, their study was inconclusive. Thus the relationship between the immune system and anxiety thus remains unresolved and requires further investigation.

Mice as a Model for Anxiety

Mice can function as a model for human anxiety since they offer both face and construct validity. In order for a mouse to have facial validity the mice must be behaviorally and physiologically similar to humans (Clement et al, 2002). Both mice and humans will likely avoid a threat and respond to it with elevated heart rates and cortisol levels. For an animal model to have construct validity, the psychological and biological rationale underlying altered behavior should be similar to that seen in humans. A mouse's anxious emotionality can be quantified as behavioral inhibition or lack of exploration, and increased defecation and urination in a threatening location, such as a novel open field. Since pharmacological studies have confirmed that these factors correlate with the biological mechanisms of anxiety found in humans who identify as anxious, we appear to be justified in using them as markers of anxiety (Clement et al, 2002).

Enrichment

Mice in enriched cages receive additional sensory and physical stimulation that should enhance neural development (Simpson and Kelley, 2010). In order to test the effects of an immune challenge during a stage of neural development the larger study will either house mice in enriched or plain, non-enriched cages. Here it is assumed that the enrichment will stimulate neural development, as long as the energy from food for that investment are available to the mouse and not currently diverted to the immune system.

Housing enrichment could either increase or decreases anxiety. Physical enrichment that allowed exercise, play, and exploration was shown to increase rats' exploratory behavior in the open field task and elevated plus maze, implying a decrease in anxiety in novel environments (Simpson and Kelley, 2010). Nutcracker birds that were housed with enrichment for 92 days had reduced corticosterone concentrations in the final 25 days of enrichment (Fairhurst et al, 2011).

Similarly, enriched Indian field mice exhibited more exploratory behavior, lower levels of corticosterone, and higher levels of ACTH than their non-enriched counterparts (Varman et al, 2011). The cage enrichment probably decreased these animal models' anxious symptoms by allowing them exercise and acrobatic activity, which have been shown to reduce anxiety (Varman et al, 2011).

Enrichment has also been shown to increase anxiety and decrease boldness and exploration. Male Wistar rats raised in enriched cages had larger adrenals and increased adrenocortical function, indicating chronic stress (Moncek et al, 2004). However, these were solely physiological and anatomical results and thus inconclusive about anxiety as a whole. It is also possible that this study, and others that report similar results such as increased corticosterone levels, may have excessively enriched the cages or rotated the items too frequently, which could induce stress (Gurfein et al, 2012).

Food Restriction

It is possible that trade-off between different systems will only be observed when animals have limited resources. One way to simulate this in the lab is to restrict calorie intake. Calorie restriction did not affect Kunmin mice's exploratory behavior in the open field test when their diet was decreased to 80% and 65% of its normal size (Wu et al, 2003). Similarly, 70% calorie restriction 6 weeks before and then 2 weeks during testing did not affect thigmotaxic behavior in C57BL/6J mice in the open field test (Minor et al, 2008).

Present Study

In order to see if there is an effect of challenging the immune system, restricting feed, and enriching cages during development on anxiety-related behaviors in adult C57BL/6J mice, my

thesis will test anxiety through three behavior assays: the elevated plus maze, the open field test, and the novel object test.

One group of C57BL/6J mice, maturing in an 80% controlled feed, will receive an immune challenge and enrichment, another will receive enrichment, a third will only receive an immune challenge, and the final group will not receive enrichment or an immune challenge. We predict that mice that are given an immune challenge will demonstrate more anxiety-related behaviors than healthy mice since the literature indicated that the immune system could contribute to anxiety by constitutive activation of the HPA axis (Glaser, 2005; Dinel et al, 2011; Raber, 1997; Salome, 2008). Additionally, though there are inconsistencies in the literature, we predict that mice raised in an enriched cage will be less anxious.

METHODS

Animals

Subjects were male C57BL/6 mice bred by Jackson Labs (Bar Harbor, ME). The C57BL/6 strain is especially well suited for and widely used in the behavioral assays we conducted (Belzung and Griebel, 2001). Mice were assigned a number and given tail markings with a red marker to allow for identification.

At 3 weeks of age the mice arrived at the Penn State Centralized Biological Laboratory after being shipped over without their mothers. Half were placed in enriched housing while the other half remained in plain housing. Mice were kept with at least one other cage mate (a minimum of two mice per cage, with a maximum of four) in a reverse light cycle (lights off from 9 AM to 9 PM) in order to accommodate testing during the dark hours when mice are most active. Cohorts 1 and 2 were fed *ad libitum* while their food consumption was tracked by being weighed every day. Cohorts 3, 4, and 5 were given an 80% controlled feed in order to see if a reduced supply of energy would influence a trade-off.

Half the mice from each housing condition were vaccinated against the bacterium *Bordetella pertussis* through intraperitoneal injection of the approved human vaccine Adacel (200 ul), in conjunction with saline (200 ul). The other half from both housing conditions was only given an intraperitoneal injection of saline to serve as a control. The first vaccine was given when the mice were 28 days of age and the second vaccine at 42 days. Immunity to the bacterium was confirmed through the detection of *Bordetella* specific antibodies in the sera of vaccinated mice. 100uL of blood was collected by performing a submandibular bleed, using either a lancet, or a 22 gauge needle, on days 7, 14, and 21 after vaccination. Blood was collected in a serum separation microtainer and processed to collect serum. After vaccination and blood testing the mice rested for 6 weeks. For a schematic representation of this timeline, see Figure 1.

In total there were four treatment groups across five cohorts: 13 mice were vaccinated and enriched, 8 were vaccinated and not enriched, 12 were given saline and enriched, and 6 were given saline and not enriched.

Enrichment

Enriched cages in cohorts 1 through 4 housed a bridge, a chew ball, and a shelter for the mice to explore and burrow in. The enriched cage in cohort 5 was given the same materials with the addition of a running wheel in order to see if exercise would enhance their neuronal development.

Elevated Plus Maze

On the first day of anxiety behavioral tests, when the mice were 81 days old, the elevated plus maze was administered. The maze was placed in the middle of the mouse room on a table. It was elevated 32 cm above the table and had four perpendicular arms (56 cm long and 6.2 cm wide): two with walls and opposite from each other (called “closed arms”) and two without walls and opposite from each other (called “open arms”), as seen in Figure 2. The floor of the maze was made of white poster board. Each mouse received one five-minute trial in red lighting. At the beginning of the trial the mouse was placed in the center of the maze, at the intersection of the four arms. During the trial we recorded the number of times the mouse entered the closed and opened arms (all four paws in the arm equated to an entry), reared (climbed on the wall), stretch-attended (extended his head), groomed itself, and dipped its head below the maze. After 5 minutes, the mouse was placed back in its cage, the number of faecal boli and urine puddles were counted, and the floors and walls of the maze were cleaned with 70% ethanol. The trials were recorded onto a Macintosh Laptop computer in the hallway outside of the mouse room using a webcam directed at the maze inside. The videos were later analyzed to quantify the total amount

of time the mouse spent in the closed vs. open arms, the latency before each mouse entered an open arm, and how much movement between the different arms.

Open Field Test

The next day, when the mice were 82 days of age, the mice were given the open field test in the same red lighting. The open field was a 50.8 cm by 50.8 cm open arena with 20 cm high walls. It was divided into three rectangular rings with painter's tape: an outer ring along the walls of the box (7.3 cm wide), an inner ring (14.4 cm wide), and a central square (which is 15.8 cm by 15.8 cm), as seen in Figure 3. The field was located in the middle of the mouse room on a table. Each mouse received one five-minute trial. At the beginning of the trial the mouse was placed in the bottom left corner of the field, in the outer ring (this did not count as a "cross" into this ring). During the trial we recorded the number of times the mouse crossed into each ring, reared, and groomed itself. Once the trial was over, the mouse was placed back in its cage, the numbers of faecal boli and urine puddles were counted, and the floors and walls of the box were cleaned with 70% ethanol. The video recordings of the trials were later analyzed for the total amount of time the mouse spent in each ring of the field and the latency to enter the center of the field.

Novel Object Test

On the final day of anxiety tests, when the mice were 83 days of age, the mice were given the novel object test. For logistical reasons this test was not given to the first 2 cohorts of mice tested. The same apparatus for the open field was used again with the addition of a novel object, a plastic dinosaur that was 2 cm wide, 4.7 cm high, and 5.3 cm long, as seen in Figure 4. The novel object was taped to a piece of 15.4 cm by 12 cm whiteboard that was rotated around the inner ring and the center of the box each trial so that it had a different location for each mouse. The mouse was placed in the bottom left corner of the open-field at the beginning of each trial. During each

five-minute trial we recorded the number of times the mouse reared, crossed into each ring, groomed itself, and had all four legs on the piece of whiteboard with the novel object (called "approaches"). After 5 minutes, the mouse was placed back in its cage, the numbers of faecal boli and urine puddles were counted, and the floors and walls of the box were cleaned with 70% ethanol. The videos of the trials were later analyzed for the total amount of time the mouse spent in each ring of the field, the total latent time it took for the mouse to enter the center of the field, the total latent time it took for the mouse to have all four legs on the piece of whiteboard, and the total amount of time it spent on the whiteboard with the novel object. As the sample sizes for the 4 treatment groups for this particular assay were small, some caution is needed in interpreting the results.

Timeline

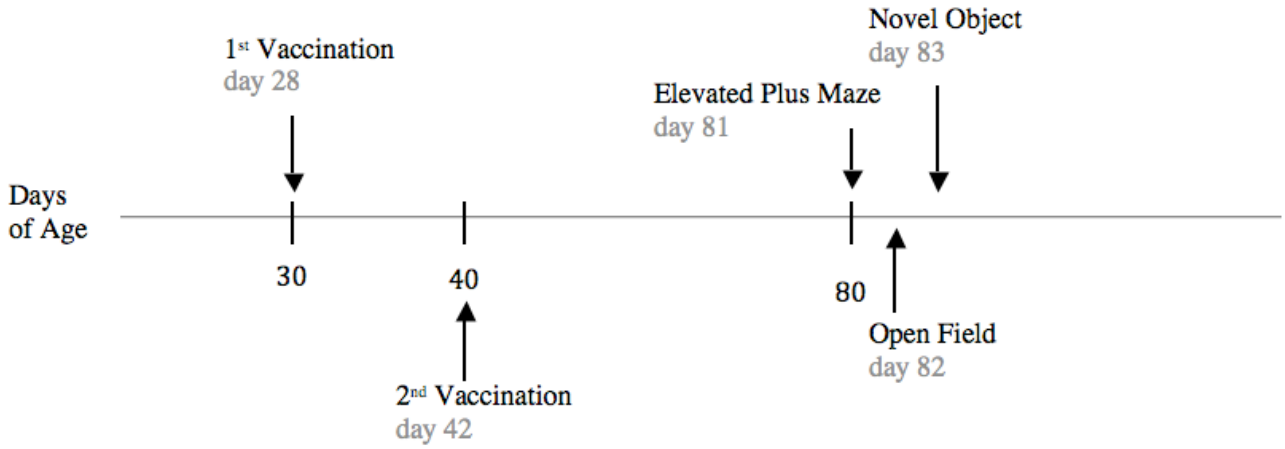

Figure 1. **Schematic representation of the timeline of vaccinations and temperament behavior assays administered to C57BL/6 mice.** Half the mice received these vaccinations while the other half received injections of saline. Similarly half the mice were exposed to enriched housing conditions starting at day 21 while the other half was not (i.e. a standard 2 x 2 design was used).

Apparatus

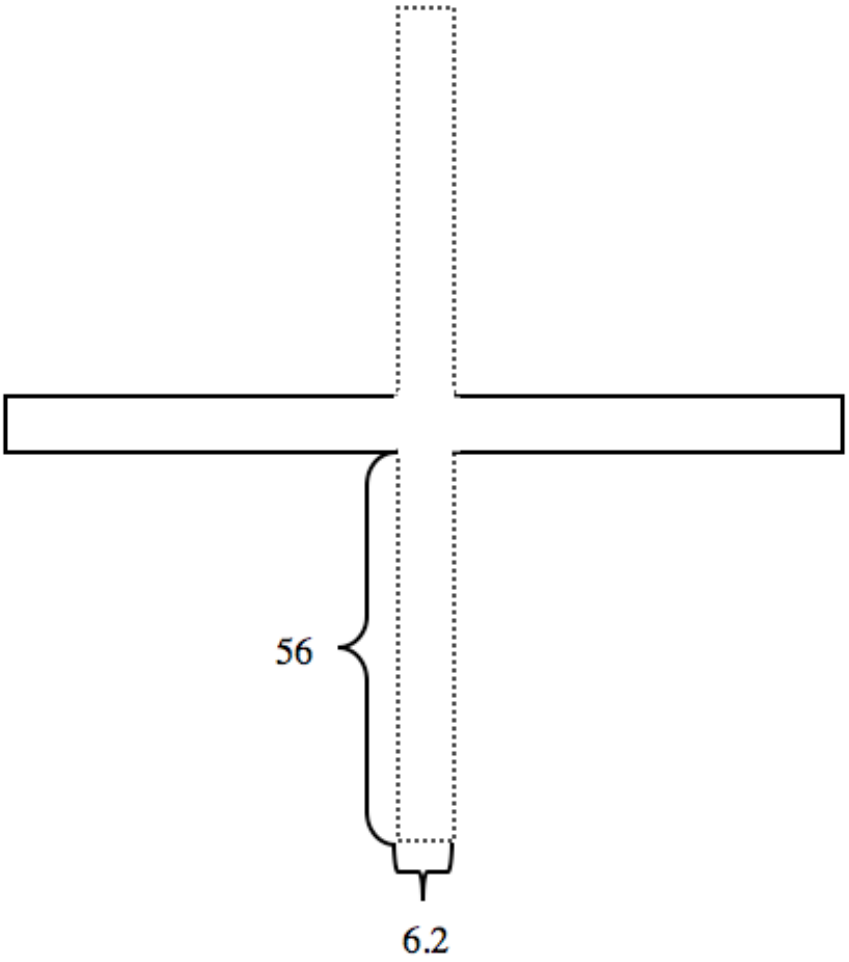

Figure 2. **Illustration of the elevated plus maze apparatus, as if seen from above, measured in centimeters.** The maze is elevated 32 cm and has four perpendicular arms (56 cm long and 6.2 cm wide): two open and opposite from each other and two with walls and opposite from each other. Walls are illustrated with dark lines. The open arms did not have any walls while the enclosed arms had black walls. The 5-minute trial was administered in red light.

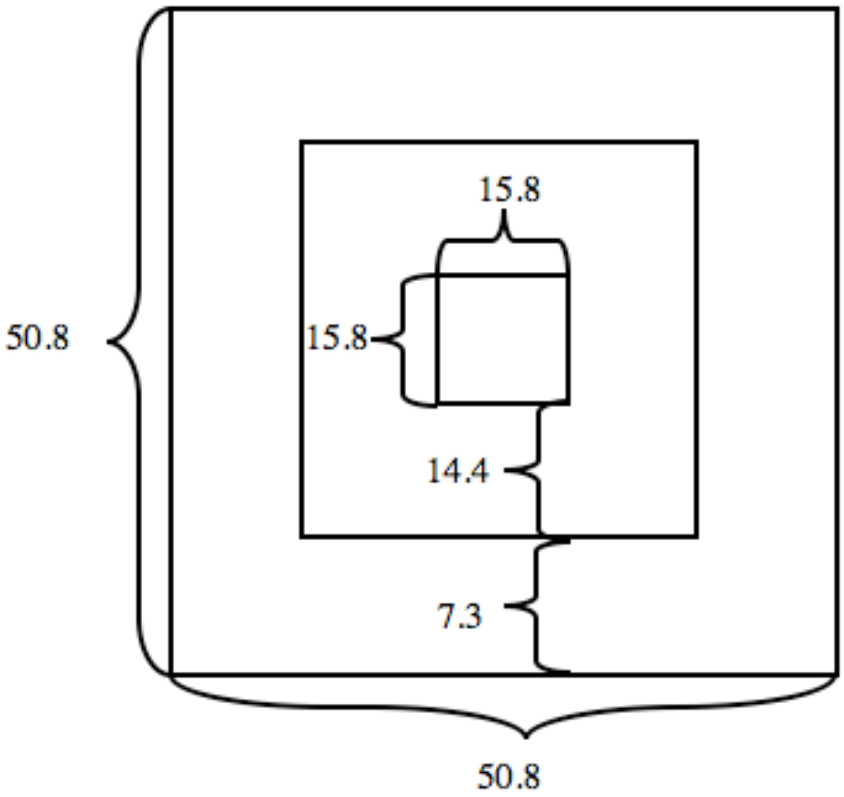

Figure 3. **Illustration of the open field apparatus, as if seen from above, measured in centimeters.** The open field is a 50.8 cm by 50.8 cm open arena with 20 cm high walls. It is divided into three rectangular rings with painter's tape: an outer ring along the walls of the box (wide), an inner ring (14.4 cm wide), and a central square (15.8 cm by 15.8 cm). The 5-minute trial was administered in red light.

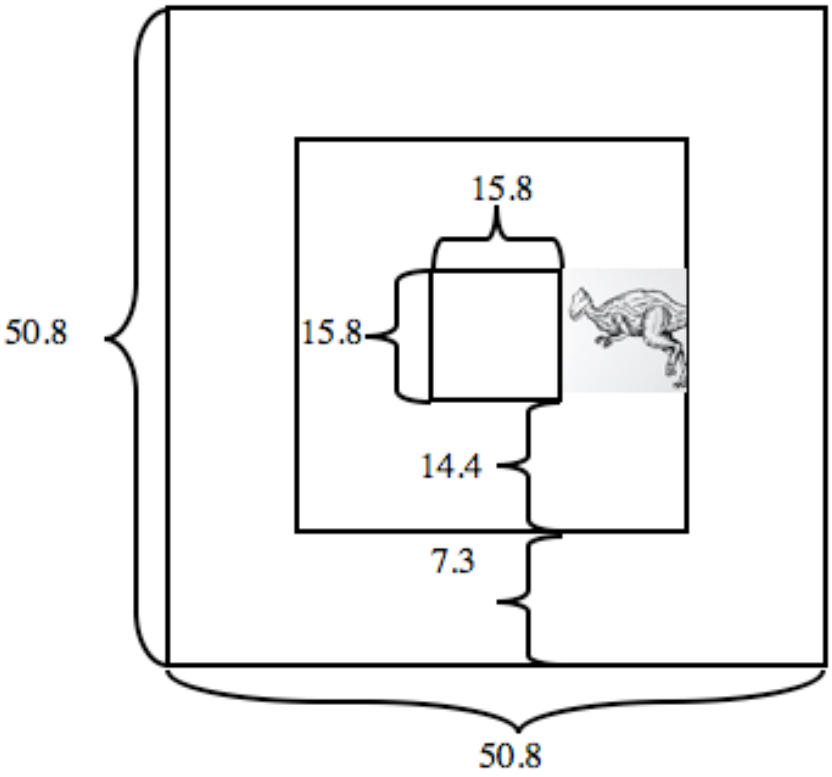

Figure 4. **Illustration of the Novel Object apparatus, as if seen from above, measured in centimeters**. The novel object, a plastic dinosaur (2 cm wide, 4.7 cm high, and 5.3 cm long) was attached to a piece of whiteboard (15.4 cm by 12 cm) that we rotated to a different point in the inner ring each trial. The 5-minute trial was administered in red light.

Data Analysis

The data were analyzed using StatView analysis software. All the values were tested for homogeneity of variance and normality and if they deviated from this then transformations were used. The different measures were then compared using ANOVA; general linear models were constructed with Vaccine and Enrichment as independent variables and a third term (an interaction between them) being used to explain the dependent variable data. Owing to the relatively small sample sizes, data from groups exposed to different husbandry conditions were pooled for the analysis, so I was not able to test for the effect of the addition of a running wheel in the final cohort. Similarly, as only the last 3 cohorts were tested in the Novel Object trials, this left one group (no vaccine, no enrichment mice) with only 3 mice, therefore the results from the Novel Object trials can only be considered tentative at this point.

RESULTS

Elevated Plus Maze

In the elevated plus maze we found a significant effect of enrichment on the number of entries into enclosed arms ($F_{1,35} = 15.65$; $p = 0.0003$), as seen in Figure 5a. The enriched mice entered the enclosed arms more frequently. There was no main effect of vaccination on this parameter ($F_{1,35} = 0.57$; $p = 0.45$) and no interaction between vaccination and enrichment ($F_{1,35} = 1.20$; $p = 0.28$).

There was a strong trend for an effect of enrichment on the number of entries into the open arms ($F_{1,35} = 3.76$; $p = 0.06$). Figure 5b shows this effect and excludes vaccination since it caused no main effect ($F_{1,35} = 0.93$; $p = 0.34$) and there was no interaction ($F_{1,35} = 0.21$; $p = 0.64$). Again, enriched mice had a higher number of mean entries.

Since the data for the number of stretch-attends was not normal we Log transformed it to normalize it. We found a significant main effect of vaccination ($F_{1,35} = 6.53$; $p = 0.01$) and a trend for a main effect of enrichment ($F_{1,35} = 2.98$; $p = 0.09$), but no interaction between the two ($F_{1,35} = 1.52$; $p = 0.23$). Figure 5c shows that the vaccine caused mice to stretch-attend more and enrichment caused mice to stretch-attend less.

As for the rest of the behavioral measures (number of head dips and rears), there were no significant main effects or interactions. There were also no significant main effects or interactions in the percent time spent in enclosed arms, percent time spent in open arms, and latency to enter open arms.

Open Field Test

In the open field test there was a strong trend for an effect of enrichment on the mean number of entries into the central square ($F_{1,35} = 3.60$; $p = 0.07$), as shown in Figure 6a. Although not a significant effect, this may indicate that mice housed in enriched cages enter the central

square more than mice raised in plain cages, it is possible that with more replicates this effect would become significant. While there was no main effect of vaccination ($F_{1,35} = 0.10$; $p = 0.27$) on the number of entries into the center, there was a strong trend for an interaction between enrichment and vaccination ($F_{1,35} = 3.27$; $p = 0.08$). Again, being cautious to interpret this trend, it suggests that there may be a greater effect of enrichment amongst the vaccinated mice than those that received saline.

Figure 6b illustrates a significant effect of enrichment on the mean number of entries into the outer ring ($F_{1,35} = 5.17$; $p = 0.03$). Enriched mice crossed the line between the outer and inner rings more frequently than mice that were housed in a plain cage. There was no main effect of vaccination ($F_{1,35} = 0.48$; $p = 0.49$), but there was a strong trend for interaction ($F_{1,35} = 3.42$; $p = 0.07$). This trend may indicate a greater effect of enrichment in the vaccinated group than the group that received saline.

Figure 6c shows a trend for an effect of vaccination on the mean number of rears ($F_{1,35} = 2.98$; $p = 0.09$). Mice that received saline reared more often than mice that were vaccinated. There was no effect of enrichment ($F_{1,35} = 0.58$; $p = 0.45$) and no interaction ($F_{1,35} = 0.15$; $p = 0.70$).

While there were no significant main effects of vaccination ($F_{1,35} = 0.94$; $p = 0.34$) or enrichment ($F_{1,35} = 1.89$; $p = 0.18$) on latency to enter the center, there was a significant interaction between the two ($F_{1,35} = 6.56$; $p = 0.01$), as seen in Figure 6d. Of the vaccinated mice, the non-enriched mice took much longer to enter the central square than the enriched mice.

There were no significant main effects of vaccination or enrichment and no interactions in grooming bouts and time spent in the center.

Novel Object Test

The only significant trend found in the novel object test was an interaction between vaccination and enrichment in the time spent in the outer ring ($F_{1,20} = 7.6$; $p = 0.01$). There were

no significant main effects of vaccination or enrichment and no interactions in the other measures analyzed. However, as fewer mice were used here, caution is needed when drawing conclusions.

GRAPHS

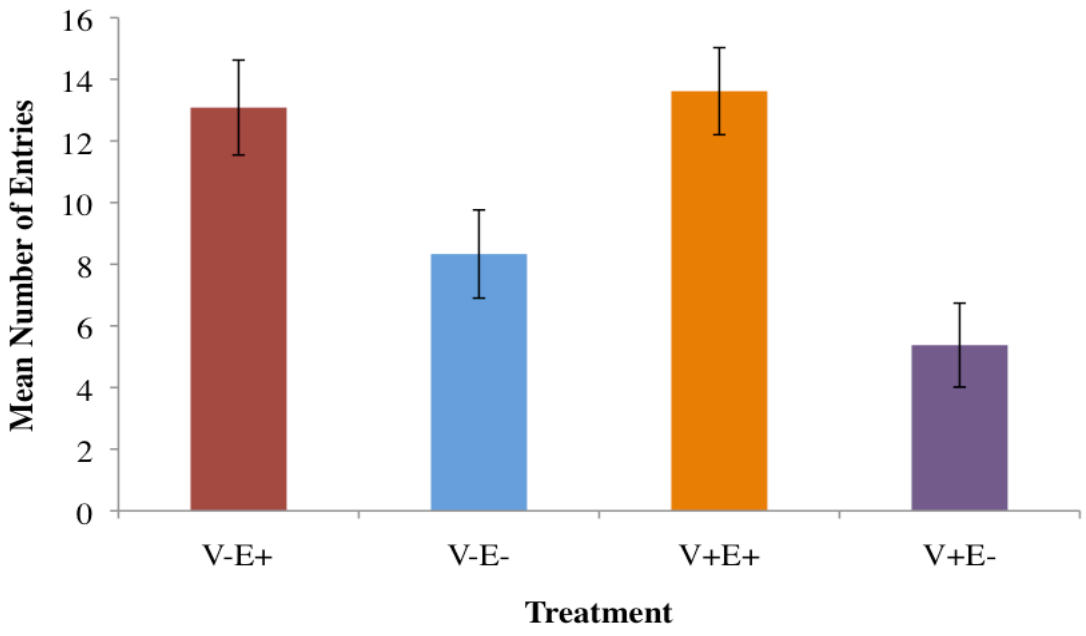

Figure 5a. **Number of enclosed arm entries in the elevated plus maze during a 5-minute trial.** Across the four treatment groups of male C57BL/6 mice (12 given saline and enrichment (V-E+), 6 saline and no enrichment (V-E-), 13 vaccination and enrichment (V+E+), and 8 vaccination and no enrichment (V+E-)) there was a significant effect of enrichment ($F_{1,35}$ = 15.65; p = 0.0003).

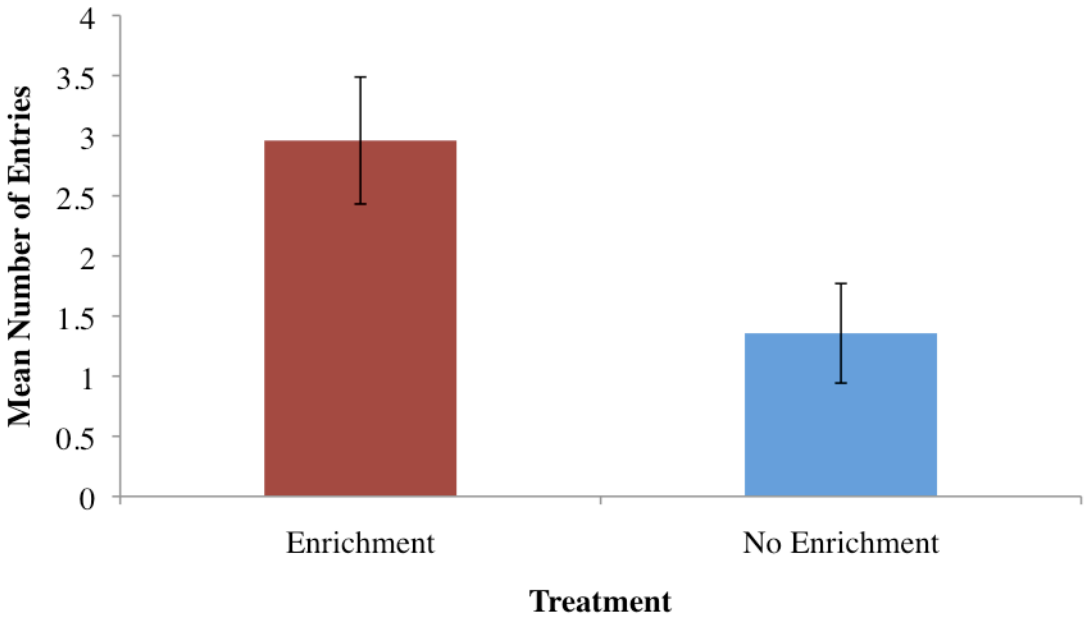

Figure 5b. **Number of open arm entries in the elevated plus maze during a 5-minute trial**. The 25 male C57BL/6 mice that were in the "enrichment" group included both vaccinated (13) and not vaccinated (12) mice. Additionally, the 14 mice that were in the "no enrichment" group were both vaccinated (8) and not vaccinated (6). There was a strong trend for an effect of enrichment ($F_{1,35} = 3.76$; $p = 0.06$).

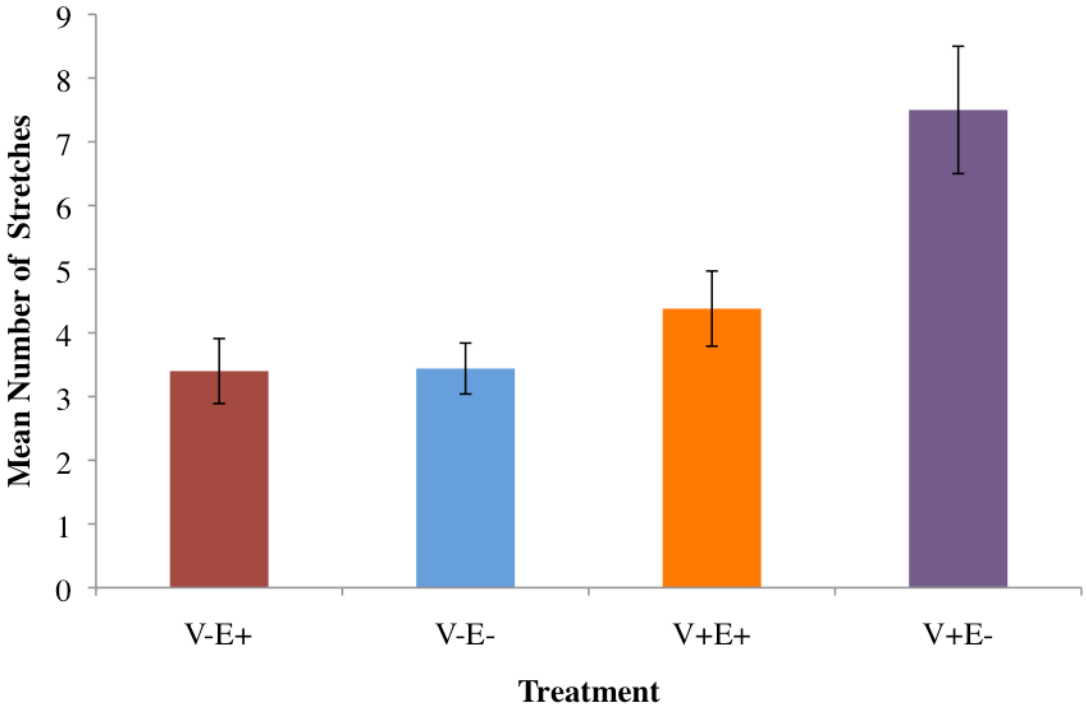

Figure 5c. **Number of stretch-attends in the elevated plus maze during a 5-minute trial after the data was Log transformed.** Across the four treatment groups of male C57BL/6 mice (12 given saline and enrichment (V-E+), 6 saline and no enrichment (V-E-), 13 vaccination and enrichment (V+E+), and 8 vaccination and no enrichment (V+E-)) there was a significant main effect of the vaccine ($F_{1,35} = 6.53$; $p = 0.01$) and a trend for a main effect of enrichment ($F_{1,35} = 2.98$; $p = 0.09$).

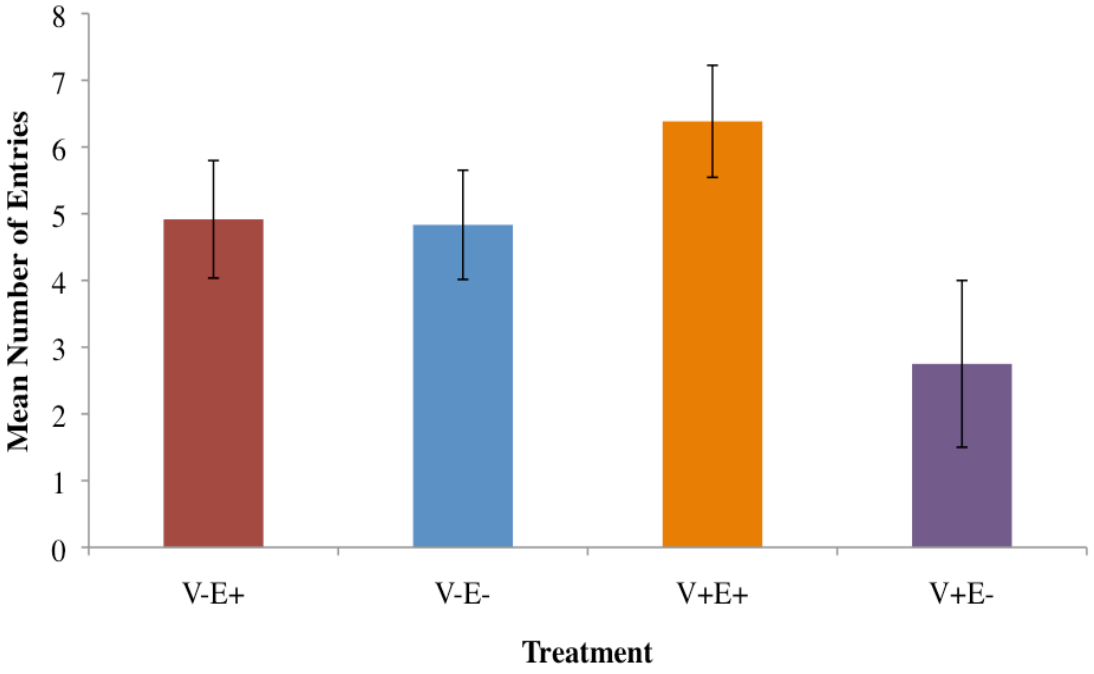

Figure 6a. **Number of center square entries in a 5-minute open field test.** Across the four treatment groups of male C57BL/6 mice (12 given saline and enrichment (V-E+), 6 saline and no enrichment (V-E-), 13 vaccination and enrichment (V+E+), and 8 vaccination and no enrichment (V+E-)) there was a strong trend for an effect of enrichment ($F_{1,35}$ = 3.60; p = 0.07) and an interaction ($F_{1,35}$ = 3.27; p = 0.08).

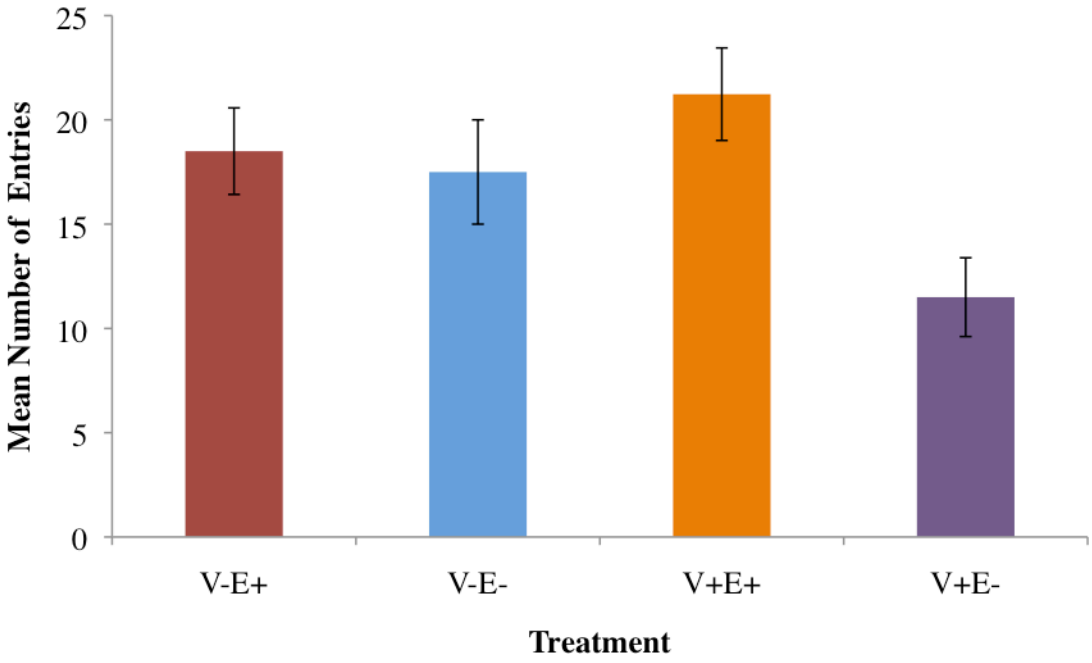

Figure 6b. **Number of outer ring entries in a 5-minute open field test.** Across the four treatment groups of male C57BL/6 mice (12 given saline and enrichment (V-E+), 6 saline and no enrichment (V-E-), 13 vaccination and enrichment (V+E+), and 8 vaccination and no enrichment (V+E-)) there was a significant effect of enrichment ($F_{1,35}$ = 5.17; p = 0.03) and a strong trend for the interaction ($F_{1,35}$ = 5.17; p = 0.03).

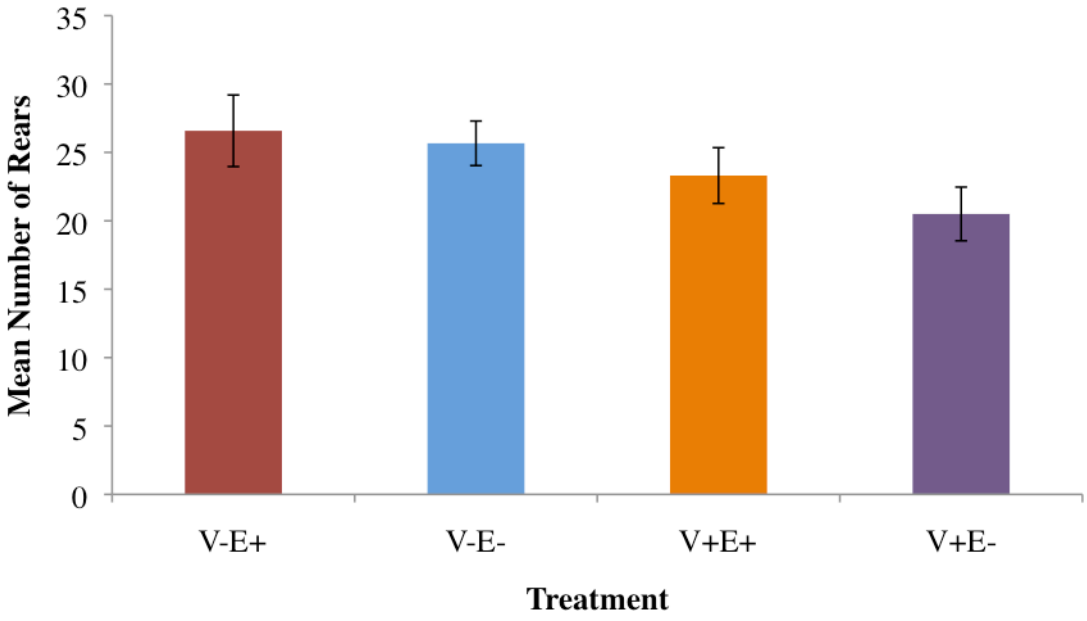

Figure 6c. **Number of rears in a 5-minute open field test.** Across the four treatment groups of male C57BL/6 mice (12 given saline and enrichment (V-E+), 6 saline and no enrichment (V-E-), 13 vaccination and enrichment (V+E+), and 8 vaccination and no enrichment (V+E-)) there was a significant effect of enrichment and a strong trend for the interaction ($F_{1,35}$ = 5.17; p = 0.03).

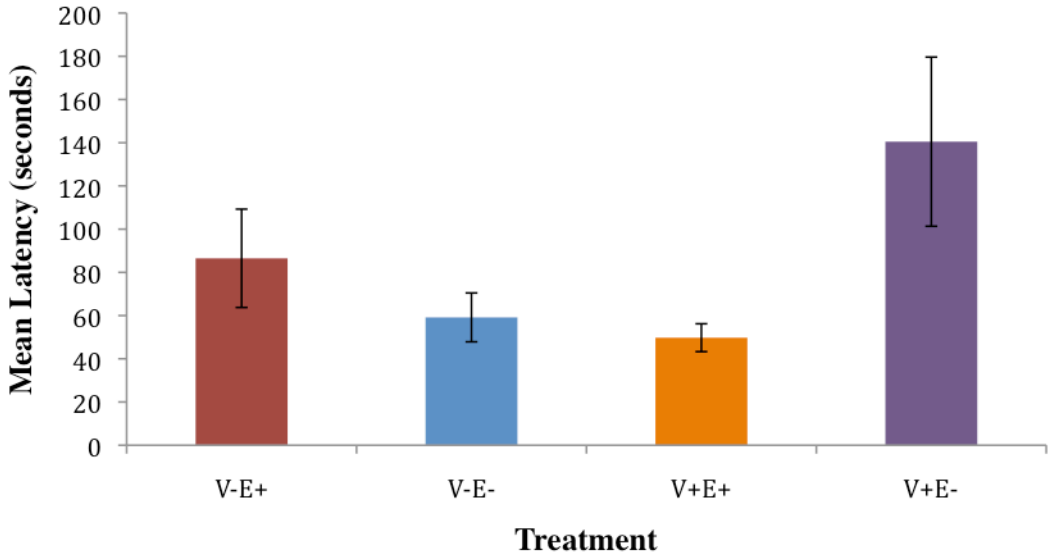

Figure 6d. **Latency to enter central square in a 5-minute open field test.** Across the four treatment groups of male C57BL/6 mice (12 given saline and enrichment (V-E+), 6 saline and no enrichment (V-E-), 13 vaccination and enrichment (V+E+), and 8 vaccination and no enrichment (V+E-)) there was a significant interaction between the vaccination and enrichment ($F_{1,35} = 6.56$; $p = 0.01$)

DISCUSSION

Enrichment

In the elevated plus maze enriched mice showed fewer anxiety-related behaviors. Enriched mice entered the enclosed arms more frequently than mice raised in plain cages did. Though these are the more protected arms, which could mean these mice were experiencing thigmotaxis (preference for vertical surfaces as a natural defensive strategy), the enriched mice also had a higher number of mean entries into the open arms of the maze. Entries were defined as crosses from one arm to the next. Therefore, the enriched mice were altogether more active and exploratory. Additionally, there was a trend for a main effect of enrichment causing mice to stretch-attend less in the maze. Stretch-attending, which is when the mouse extends their body forward and points their nose in the air, is a risk assessment and thus an anxiety-related behavior. This ethological trend supports the conclusion that enriched mice were overall less anxious. Furthermore, it validates that the mice raised in plain housing were not just less locomotive, but also behaved anxiously.

In the open field test there was a strong trend of enriched mice entering the central square more frequently than mice raised in plain housing. Additionally, enriched mice significantly entered the outer ring more frequently than plain mice. One outer ring entry meant that the mouse had to leave the walls of the arena, cross into the inner ring, and then cross back into the outer ring. In the open field test leaving the outer ring signifies a lack of thigmotaxis since everywhere inside of the walls is exposed open space. It is therefore a submission of protection and a non-anxious, or bold and exploratory, behavior. In sum, the enriched mice displayed less anxiety-related behaviors in both assays.

Exposure to enrichment increased general activity patterns and reduced anxiety-related behavior in the mice. This finding is in accordance with the literature since we find that

enrichment makes other animals bolder (Simpson and Kelley, 2010; Fairhurst et al, 2011; Varman et al, 2011). Additionally it is in accordance with our hypothesis that mice raised in enriched cages would be less anxious.

Vaccination

Vaccination was shown to have some overall effect on general movement behaviors in the mice. Mice that received the vaccine exhibited significantly more stretch-attending in the elevated plus maze trials, which is a marker of anxiety. Discordantly, vaccinated mice tended to rear less in the open field test, which is a sign of boldness. However, this trend was not significant, so the finding could be due to a Type I error since multiple statistical tests were used (Jackson, 2012). Additionally, vaccinated mice would not be expected to rear less in response to abdominal pain from the intraperitoneal injection they received because this pain has actually been shown to increase rears (Wright-Williams et al, 2007; Fox, et al, 2000). Rearing, when the mouse climbs up a wall, involves extension of the body, which could be a post-operative response.

Discounting the latter trend, the results indicate that there could be changes in temperament associated with whether mice were vaccinated or not.

Interactions

In the open field test there was a significant interaction in total latent time to enter the central square, the most exposed area of the open field. Of the vaccinated mice, the enriched mice entered the central square much earlier on in their trials than the non-enriched mice. There was also strong trend for an interaction between enrichment and vaccination on number of entries into center of the arena. Enriched mice entered the central square much more often than plain mice amongst the vaccinated treatment groups, while this difference was only slight in the group that

received saline. There was also a strong trend for an interaction between vaccination and enrichment in the measure of the number of entries into the outer ring. Again, there was a greater increase in rings crosses by enriched mice in the vaccinated group than the group that received saline. Crossing in and out of the outer ring is not very typical of anxiety since anxious mice would be expected to exhibit thigmotaxis and stay in the outer ring. Thus, overall, the vaccine increased the effects of the enrichment on the mice to exhibit bold behaviors. This rejects our hypothesis mice with challenged immune systems would demonstrate more anxiety-related behaviors than healthy mice, since the literature indicated that the immune system could stimulate an anxious response in the HPA axis. It is possible that instead of creating a trade-off between neural development and immune response, the vaccine heightened neural development, causing the mice to be bolder, more exploratory, and less anxious. This result has not been previously reported.

The only significant trend found in the novel object test was an interaction between vaccination and enrichment in the time spent in the outer ring, but this could be due to a Type I error since multiple statistical tests were done (Jackson, 2012).

Conclusions

The results from the different assays show that anxiety related behaviors were changed both by exposure to a vaccine and to enrichment. Enrichment increased bold behaviors in the elevated plus maze and the open field test while vaccination increased an anxious behavior in the open field test. In both tests vaccination increased the effects of enrichment.

Implications could not be drawn from the novel object test data since the sample size was too small and since the numbers across treatment groups were not completely even. In hindsight the novel object test, the controlled feed, and the form of enrichment should have been kept the

same for every cohort, but this was not logistically possible for the larger study to which this thesis contributed. These changes would create larger sample sizes since there would be consistent conditions across cohorts, allowing for more conclusions to be drawn (Chow et al, 2007). Because of this small sample size there was only one significant finding in the novel object test, which was also probably due to a Type I error. The other data was natural log transformed, but still no trends were observable. In future experiments treatment groups should ultimately have equal numbers to avoid bias of results (Sapsford and Jupp, 2006).

Future experiments will address how vaccination and enrichment influence the learning and memory abilities of the mice, and how these relate to anxiety measures. The effects of vaccination and enrichment on anxiety will have to be taken into consideration when administering a Barnes maze or other forms of learning and memory tests. Since enriched mice were less anxious, it is likely that enriched animals would explore more during a learning and memory test, allowing them to adapt to the test faster than non-enriched animals.

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Research Experience

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