BUILDING A BETTER MOUSETRAP: NOVEL OBJECT LOCATION VERSUS NOVEL OBJECT RECOGNITION

By

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Submitted in partial fulfillment of the

Requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 2, 2022

BUILDING A BETTER MOUSETRAP: NOVEL OBJECT LOCATION VERSUS NOVEL OBJECT RECOGNITION

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ABSTRACT

Alzheimer's disease (AD) is often associated with patterns of chronic inflammation and cognitive dysfunction. Much of our lab's research involves studying how AD-like pathologies change and affect learning and memory. Our lab has developed a learning task in which object location memory (OLM) is tested in mice. Briefly, a mouse is placed into an arena with two identical objects for a training session. Four hours later, one of the objects is moved to a novel location, and the mouse is placed back into the arena for the testing session. Because mice exhibit a preference for novelty, memory is assessed as the amount of time the mouse spends exploring the moved object divided by the total time spent exploring both objects. Our goal is to identify testing parameters that make this task both accurate and efficient, as we will add this learning paradigm to a battery of other behavioral tasks in our lab to be used in future experiments. In this current study, the OLM protocol was performed twice according to two different experimental timelines that test the effects of adding an additional training session to the original protocol.

ACKNOWLEDGEMENTS

This project would not have been possible without the support and guidance of my supervisor Dr. Michael Chumley and mentor Dr. Gary Boehm. These professors have taught me essential foundations of research and theories of behavior. I am incredibly grateful to them both. I also want to thank the other members of my Honors Thesis committee, Dr. Shauna McGillivray and Dr. Giridhar Akkaraju, for their support throughout my time working on this project. I additionally owe great credit to graduate students, Kelly Brice and Paige Braden, and undergraduate, Shelby-Kay Miller. Each of them provided instrumental guidance regarding statistical analysis and the overall completion of this project.

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INTRODUCTION

The symptoms and pathologies of Alzheimer's disease (AD) have caused this neurodegenerative condition to gain status as a global health concern. AD is the single biggest cause of dementia and is primarily a condition of later life, nearly doubling in prevalence every 5 years after 65 years of age. Dementia is defined as acquired progressive cognitive impairment sufficient to impact activities of daily living, causing dependence, disability, and mortality. Current estimates suggest that about 44 million people live with dementia worldwide, and this number is predicted to triple by 2050 as the population ages (1). In fact, in England and Wales, dementia was the leading cause of death is 2015, accounting for 11.6% of all registered deaths.

Our lab has sought to study the primary pathologies of AD in order to gain knowledge and understanding about this disease. The hallmark pathologies of AD consist of amyloid beta plaques and neurofibrillary tangles (NFTs) (1). Our research with Alzheimer's consists of studying how these pathologies change in mice under varying conditions. This provides an advantage for understanding cellular and molecular changes with the human form of this disease. With a mouse, researchers can take tissue samples and analyze regions of the brain in order to see changes in real time. This is incredibly valuable research, as these are tasks that cannot be performed in humans.

Completing this type of research typically involves paradigms that can be learned by mice. However, researchers must use a mouse's behavior as an indicator of learning. This is fundamentally different from the variety of methods we can see learning in humans. Some of the common behavioral tests used in mice are the open field test, Morris water maze, and cued or contextual fear conditioning. However, all of these tests have significant disadvantages in analyzing learning of a specific task. First, the open field test measures anxiety, anxiety-related

behavior, and exploratory behavior. An interesting parameter of this test is known as thigmotaxis. This is a property that states: the more time the animal spends hugging the walls of the arena, the more anxious it probably is. Exploring the center of the arena, and how long it takes the animal to do so are used as measures of exploration and boldness (2). As one can see, though, this test does not give mice a task to learn. Therefore, it cannot give researchers data representing signs of learning or memory. Second, one could consider using Morris water maze to demonstrate learning. This test was developed to test the spatial learning behavior of rats or mice. The test apparatus consists of a circular water tank filled with opaque water, usually by adding powdered milk, and a hidden platform submerged a few centimeters under the water surface in one quadrant of the tank. The tank is surrounded by visual cues and trains the animals to locate the hidden platform. If the mouse cannot find the platform within five minutes of testing, it is rescued and placed on the platform to learn its position (3). This test, however, can take up to five or seven days. Because of the stress placed on the animals and the amount of time it takes to receive and analyze results, our lab chose to continue looking for another test to analyze behavior in mice. Cued or contextual fear conditioning is another possible task to use. In this experiment, animals are placed in a fear conditioning apparatus for about two minutes. During this time, a 30 second acoustic tone or light cue is introduced. In the last two seconds of the new stimuli, a mild foot shock is applied to the floor grid of the apparatus. This allows the animal to learn to associate the chamber with the impending foot shock. This learning is recorded through the percentage of time in which a mouse exhibits freezing behavior, which is the animal's normal response to fear. Therefore, the more freezing recorded, the more the mouse has learned the presented association task (4). However, this fear response can mask memory and learning, while it also allows for unnecessary stress on the animal. In looking at the

disadvantages talked about with the above three tests, researchers have always looked for a better mousetrap so to say, that is a simpler and less stress provoking task that can record learning and memory in mice. For this reason, our lab has chosen to focus on a different task, object recognition and location testing, that requires no external motivation, reward, or stress.

The Object Recognition Memory (ORM) and Object Location Memory (OLM) tasks have been widely used in the study of the neurobiological mechanisms underlying long-term memory formation, both by our lab and others. Before diving into object experimentation with mice and the difference between object location and object recognition testing, though, it is important to know that this testing has also been used with humans. OLM, specifically, is known to become gradually impaired through normal aging. This process accelerates in patients with mild cognitive impairment (MCI). However, OLM training in MCI patients has shown slight improvement in cognitive skills and tasks. Cognitive training has demonstrated promise as a future treatment due to its ability to use the brain's plasticity to strengthen and reorganize neuronal circuits implicated with task demands. It additionally lacks any risk in treatment. Many studies, though, only show moderate effects in both health older adults and MCI patients (5). While there are no concrete results on the effects of this treatment, it shows promise as a tool to detect early, preclinical cases of dementia. Therefore, this testing could be used as a paradigm to test for Alzheimer's in an early stage. Early diagnosis of Alzheimer's disease (AD) can have a major impact on the progression of the disease and possible treatments available. The earlier one can catch this disease, the greater amount of time patients will have to get their affairs in order and spend quality time with their family. We want to have a better understanding of OLM and how it works in order to help with the current research and diagnosis of Alzheimer's disease.

In looking at the OLM research done with humans, one can see that there are clear reasons whether to use OLM or ORM in a particular study. However, these tests are very similar. Both tasks involve handling the animals, habituating them to a training arena, and training and then testing with two objects. In training, mice have the ability to explore and familiarize themselves with the two objects. The day of testing is where one can see the biggest difference between ORM and OLM. In OLM, one object is moved to a novel location, and in ORM, one object is replaced with a novel object. What both of these tasks have in common is that they both rely on a rodent's preference for novelty. This means that in the OLM testing trial, a mouse will explore the displaced object for a greater time relative to the non-displaced object, and in ORM testing trial, a mouse will explore the novel object for a greater time compared to the familiar object. If these are found to be the final results of the test, this indicates that the mice learned and familiarized themselves with the previous location or object presented to them in the training trial (6). While these seem like very similar tasks, they call upon different regions of the brain, and therefore, use two different aspects of memory. In OLM, the hippocampus is used for encoding, consolidation, and retrieval. When the hippocampus is damaged, there is noticeable impairment of contextual or spatial aspects of an experience. However, memory of a specific object that was part of an experience remains functional (7). In ORM, though, several different brain regions appear to be critical, including the insular cortex (8), perirhinal cortex (8), and ventromedial prefrontal cortex (9). Additionally, the role of the hippocampus in ORM has remained somewhat controversial. There is evidence that damage to the hippocampus has little effect on the ability to recognize objects, while damage in brain regions outside the hippocampus has a far greater impact. On occasion, though, hippocampal damage can appear to impair

performance in the ORM task. This is likely due to ancillary abilities playing a particular role to support task performance, and this can vary based on the exact experimental setup (10).

In determining which task to use to assess learning and memory in mice, our lab must choose the particular brain regions we would like to eventually study with this paradigm. In the earliest stage of Alzheimer's disease, it is believed that certain areas of the brain are especially vulnerable to specific degenerative processes and neuronal dysfunction. The role of the hippocampus in memory processes is likely one of the first brain regions to be affected by the onset of AD (11). Therefore, as our lab desires to understand early effects of Alzheimer's disease, we will target the hippocampus through the OLM task. While we are currently only using the OLM paradigm to analyze behavior and not hippocampal regions of the brain, one could instill manipulations within this task where studying tissues would also be helpful. For example, when running an OLM experiment with experimental mice injected with LPS and control mice injected with saline, if there is a significant difference in behavior in the OLM test, one can analyze the hippocampus to see how LPS affected the hippocampus. The hippocampus could be extracted and examined under a microscope, and one would likely find a higher presence of amyloid beta in the experimental group hippocampus compared to the control group. This paradigm additionally requires one brain region, the hippocampus, to be analyzed. ORM, in comparison, requires examination of multiple tissues, and it is unclear as to the level of importance of some of the involved tissues. With the OLM paradigm, if there is a significant difference in hippocampal presentation between the two groups, this would confirm that the hippocampus is the cause of impairment shown by the results of the experiment.

In addition to wanting to target the hippocampus, though, the ORM paradigm presents an additional difficulty, as researchers must work around a mouse's innate object preference. Mice

have an innate presence for novelty, and therefore, if the mouse recognizes the familiar object, it will spend more time exploring the novel object. However, if objects are extraordinarily different from one another in regard to texture, shape, color, and pattern, a mouse may prefer one object over another because one object is simply more interesting. There is research to support that rodents have complex visual systems that can support categorization-like processes. Visual categorization of objects is thought to rely on perceptual procedures that compare specific object information, such as size, shape, or color, to the rest of the environment (12). As objects are visually categorized in different manners, it can be easy for preference of one object over another to occur. This is why in the ORM testing, it is crucial to test for innate preference and discrimination between the two objects before beginning the experiment. Another way to help avoid this bias is to select objects that are different enough to be easily discriminated by mice but also have a similar degree of complexity, texture, shape, color, patterning, and brightness. This will help reduce any unwanted object preference (13).

This potential bias in the ORM task has given our lab another reason to opt for the OLM paradigm, as the same objects are used in training and testing. However, choosing the object that will be used in the experiment is still critical. Object choice is often considered to be one of the most important and underappreciated aspects of creating a successful protocol. After all, the results of this experiment are based off exploration of the objects themselves. In order to use an object in an experiment, it must meet two important requirements. First, it must cause no fear response to a mouse. Second, the object must be able to be adequately explored during training and testing (6). The object also needs enough density and height so that mice cannot climb on top of or knock the object over. A mouse climbing on an object does not indicate exploration. If this were to occur, a mouse would be using the object to further explore the environment rather than

exploring the object itself. Some protocols choose to use velcro to hold down the objects to avoid them falling over (14). However, what is often overlooked is that velcro gives off a scent that provides an extra environmental cue for the mice to pick up. This could unknowingly affect the results of the experiment, and variables, such as this example, should try to be eliminated.

There are additional variables that should also be taken into account to help the protocol run well. The variables I am referring to could affect animal stress throughout the experiment. Any conditions in the testing room could lead to increased animal stress and can impact the results of the experiment. Ways to do this include minimizing the amount of people in the testing room to one or two people, using the same researchers to perform this experiment to prevent changes in scent within the room, avoiding use of perfumes or colognes, utilizing the same cages and arenas throughout the experiment, and decreasing the chance of loud noises within and around the testing room (6). It is additionally important to note that there could be stress with injections if they are involved in the experiment, regardless if the injected substance is saline or another drug.

A mouse can also have a different response to the experiment depending on mouse strain, age, sex, training duration, and retention duration (6). First, in this OLM testing, male C57BL/6 mice are used. However, if this test had been performed with Swiss albino mice, for example, it would not be unusual to see different results. Second, there are age associated effects with performance in this experiment. Younger mice, aged three months, have shown less deficits in visuospatial memory than middle aged mice, aged to 12 months (15). Third, different studies have shown varying performance in the OLM paradigm that correlates with the sex of the mouse. In a particular experiment, the development of spatial abilities was tracked across the early ages of development in male and female mice exposed that were exposed to early life stress. Male

mice showed significant impairments in the OLM task compared to the control group. However, female mice only showed impairments in the OLM task immediately following weaning and during peri-adolescence. These effects did not persist into early adulthood. These results indicate that males are more pre-disposed to stress compared to females, and this can alter the results in an OLM experiment (16). Finally, additional aspects to take into account with this experiment is how long the training duration and retention period should be. Training duration represents the amount of time a mouse has during each training sessions and also takes into account the number of training sessions a mouse is given. A retention period is the length of time between training and testing in the experiment. These are big questions because the length of both the training duration and retention period is the length of time a mouse is allowed to learn the OLM task. It has been stated that a single, ten-minute training session is sufficient to generate short and long-term memory. However, the ten-minute training session is said to be ill suited for examining long-term memory. It has also been shown that a three-minute training session is not sufficient enough to result in either short-term or long-term memory (6). The retention period can also vary in length depending on the type of memory being tested. If one wants to target short-term memory, the retention period should be close to 90 minutes. If one desires to target long-term memory, though, the retention period should be close to 24 hours (6). With hundreds of studies using the OLM paradigm, there are a variety of different training durations and retention periods that have been successful. It is often most helpful to undergo trial and error in combination with research when performing this experiment to see which time durations give the best results for one's particular experiment.

Once our OLM experiment was completed, data was acquired that used a mouse's explorative activity to calculate a discrimination ratio that is compared across different

experimental conditions. We performed two slightly different OLM paradigms to test whether giving mice an additional training session before testing will help them better learn this task. If the overall research of this paradigm is successful, both memory impairments and memory enhancements can be examined with the OLM task. This protocol can make an incredible difference in the study of neurodegenerative diseases and could lead to discoveries that wouldn't be possible by studying these diseases in humans alone.

METHODS

Subjects

Male C57BL/6J mice between the ages of 3 and 6 months, bred in the Texas Christian University vivarium from a stock obtained from The Jackson Laboratory (Bar Harbor, ME) were used in all experiments. All animals were treated in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU and in accordance with the guidelines described by the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010). Animals were housed in 12.5 cm x 15 cm x 25 cm polycarbonate cages. All experimental groups were subjected to the same 12-hour light/dark cycle with lights on at 06:00 and off at 18:00. Food and water were available continuously and cages were cleaned regularly.

Experimental Groups

Mice were placed into an experimental or control group. The experimental group received 200 μL saline injected intraperitoneally daily for 7 consecutive days. Control animals

received a scruff to account for possible stress associated with handling alone during the injection procedures.

Experimental Set-Up

Figure 1.

The experiment room was a dedicated behavior room that was not used to house animals. This was done to minimize auditory and odor cues throughout the experiment. A fan was placed on the highest setting within the room to serve as white noise and further minimize auditory stimuli. The room was illuminated using a set of overhead lamps that gives equal lighting to all parts of the arenas. To record a mouse's exploration throughout the experiment, a video camera was positioned above the two arenas. The feed was transmitted to a computer that was situated behind a curtain within the same experiment room. This allowed the bright light from the computer screen to be diminished throughout the experiment (Figure 1). The arenas were 12" x 8" x 8" testing chambers positioned side by side. This allowed our program to record the exploration of two mice at the same time. About a half cm of bedding was also added to the floors of these arenas. A green, laminated triangle and a yellow, laminated circle was placed against opposing sides of the chambers to serve as visual cues. Cue locations were counterbalanced between the two arenas. Throughout training and testing trials two identical 50 mL conical tubes filled with silver beads were used as the target objects (Figure 2). Each trial, researchers performed data collection

Figure 2.

using the Noldus Ethovision XT tracking software. The software was setup according to manufacture instructions.

Experimental Timelines

To help determine an optimal testing protocol, the OLM paradigm was run twice, each with slightly different protocols. In both experiments, days 1 through 7 consist of daily saline injections for the experimental group or a daily scruff for the control group. In the first experiment, training and testing are performed on day 8. In the second experiment, however, an additional training session occurs on day 8 followed on day 9 with training and testing performed similarly to day 8 in the first experiment. This gives the mice two training sessions, allowing the mice more time to explore the arena and learn the original locations of the objects. A detailed diagram of this timeline can be found below (Figure 3).

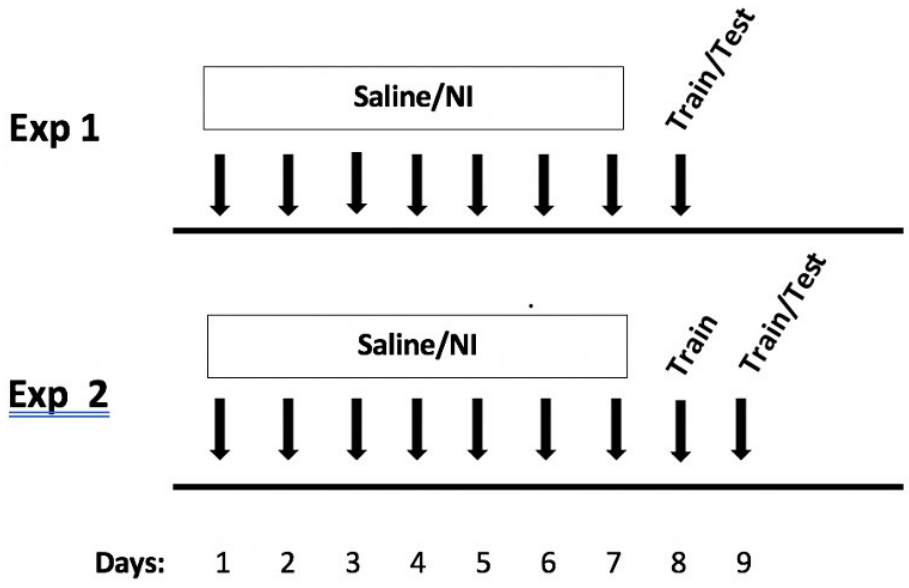

Figure 3. Two Experimental Timelines. These timelines should be read from left to right. NI = no injection. This represents the control group that received a scruff alone to account for the stress of injection procedures. In experiment 1, training and testing occurred 4 hours apart on day 8. In experiment 2, an additional training session is given on day 8. Day 9 consists of training and testing sessions that are also 4 hours apart.

Training and Testing Sessions

During a training session, a mouse is placed into an arena with two identical objects for 5 minutes. Four hours after the onset of training, memory is tested by returning the mouse to the same arena for 5 minutes. However, one of the objects has been moved to a novel location in the arena for the duration of the testing trial. In our experiment objects presented during the training trials were placed in the two corners of the same wall. In the testing trial, one object was moved to a centered position by the opposite wall of the arena (Figure 4). Arenas and objects were cleaned with water between each training and testing trial. At the end of the experiment, the arenas were cleaned with 70% ethanol.

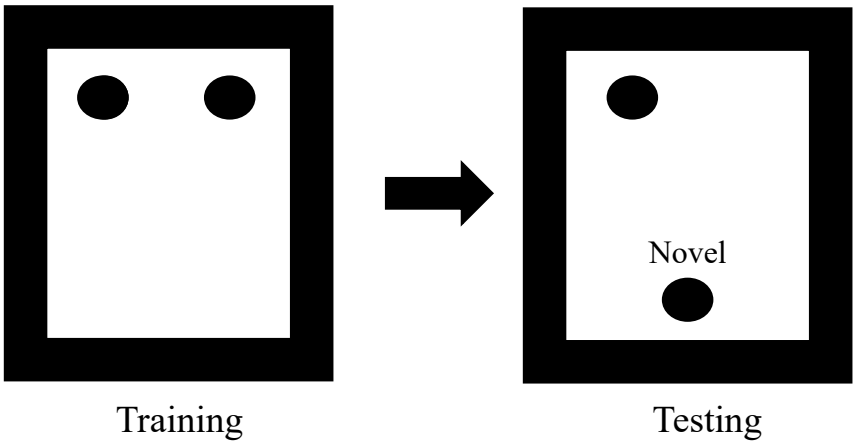

Figure 4. This demonstrates how object locations change when transitioning from training to testing trials. The objects were counterbalanced to control for mouse preference of placement or visual cues in the arena.

Data Collection

Data analysis is performed by Noldus Ethovision XT tracking software. A criterion is used to score exploration of each object during the training and testing periods. Exploration consists of the interaction time a mouse has with an object when the mouse's nose is within 2 cm of the object and is pointing directly at the object. If the mouse is not approaching the object and

the nose accidently comes within 2 cm of the object, the mouse climbs on top of the object, the mouse is looking over the object, or the mouse is engaged in a repetitive behavior within 2 cm of the object, such as digging or biting the object, this does not count as exploration. As this technology does not have 100% accuracy, two researchers hand-score exploration and make adjustments to data when necessary. Once the data is collected, it is analyzed through a discrimination ratio. It is calculated through the following formula: [(time exploring the object in novel location) / (time exploring object in novel location + time exploring object in old location)] * 100. A discrimination ratio above 50%, which is chance, indicates preference for the object in the novel location. This discrimination ratio is used to evaluate the experimental and control subjects to determine results and conclusions.

RESULTS

Experiment 1: No significant difference in percent exploration of the novel location in the experimental group.

We used data produced by the Noldus EthoVision XT Software, including adjustments for any noticed technological errors throughout the experiment. In examining our calculated discrimination ratios, control mice appear to explore the novel location significantly more than the experimental group that received saline injections. However, this was not a statistically significant difference ($t(17) = 1.440$, $p = 0.168$, NS). Nonetheless, the control group demonstrated a discrimination ratio greater than 50% and the experimental group demonstrated a discrimination ration less than 50%, suggesting the control mice could discriminate between the old and new object location while the saline injected animals could not (Figure 5).

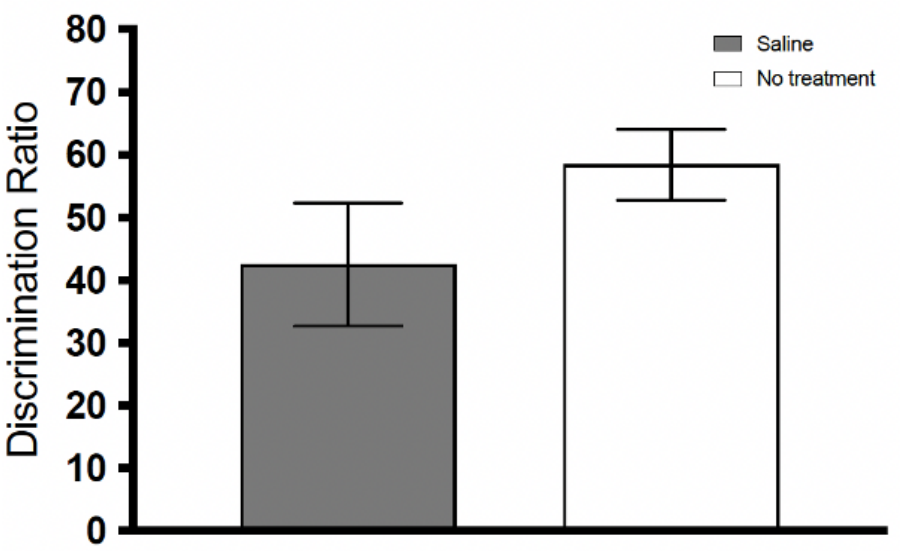

Figure 5. Experimental mice injected with saline display a discrimination ratio below 50% and control mice display a discrimination ratio above 50%. No significant differences were observed. Bars represent +/- SEM (N's 9-10).

Experiment 2: No significant difference in percent exploration time of the novel location in the experimental group.

In this experiment, both control and experimental mice received two training sessions instead of one. The idea of adding a training session is to give the mice more time to learn the old location and see if this makes a difference in their ability to discriminate between the old and new object location. However, we observed similar effects as we did in Experiment 1. Our calculated discrimination ratios indicate that control mice appear to explore the novel location more than the experimental group in testing (Figure 6). Again, however, this difference was not statistically significant ($t(18) = -0/905$, $p = 0.337$, NS. As seen in experiment one, the control group exhibited a discrimination ratio greater than 50% and the experimental group displays a discrimination ratio less than 50%. Overall, this indicates that the control mice performed the task slightly better than could the saline injected mice.

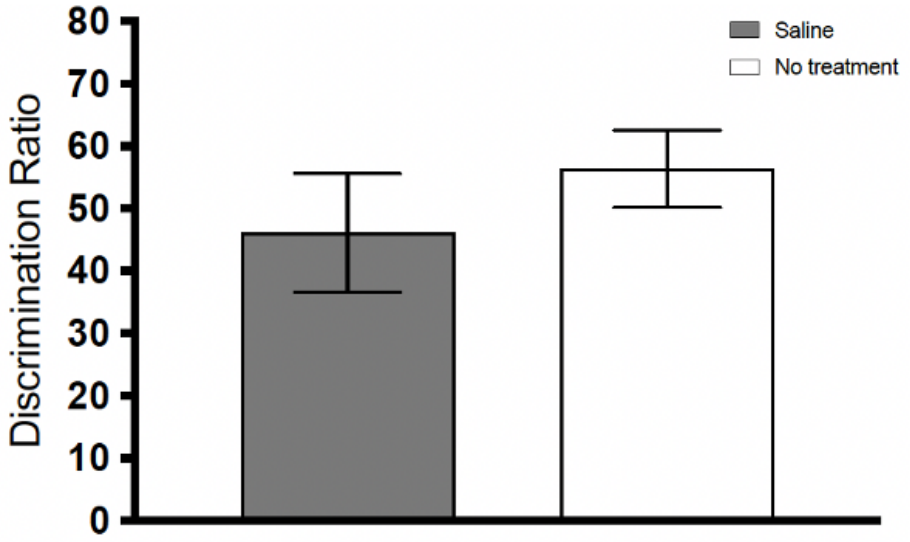

Figure 6. Experimental mice that were injected with saline mice display a discrimination ratio below 50% and control mice display a discrimination ratio above 50%. No significant differences were observed. Bars represent +/- SEM (N's = 10).

Experiment 2 (Revised): Increased exploration of the novel location in both experimental and control groups.

We decided to re-examine the recorded feed of each testing trial in experiment 2. By doing this, we found that two animals in the experimental group displayed abnormal exploration behavior. They tended to stay in corners or hug the walls throughout their entire trial. They also seemed to be uninterested in exploring the objects in the arena. We decided to remove these mice from data analysis due to their exhibited abnormal exploration even though it was not indicated statistically to do so. Removing these animals led to the experimental and control groups achieving the same discrimination ratio, both above 50% (Figure 7). Again, no significant differences were observed ($t(16) = 0.021$, $p = 0.984$, NS). This indicates that the control and saline injected mice performed similarly during the testing session.

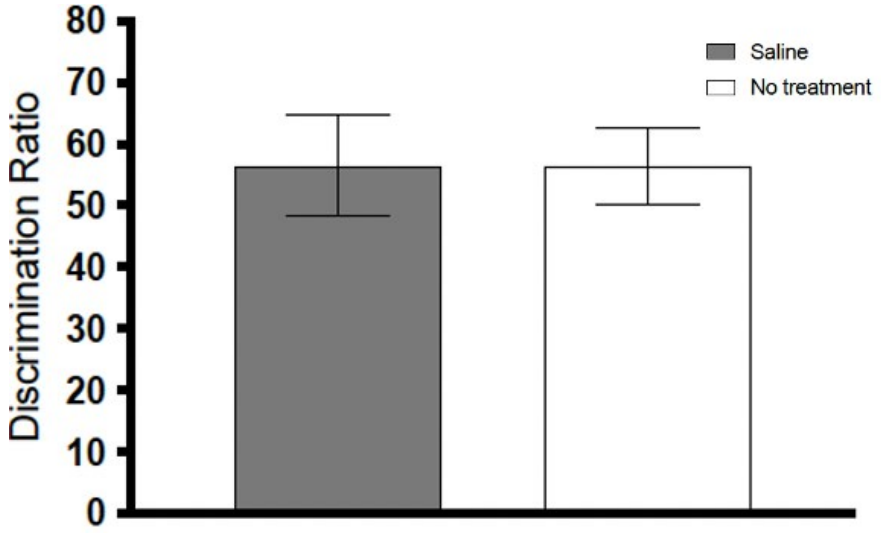

Figure 7. After removing two animals due to non-performance, the control and saline treated groups now display the same discrimination ratio, both above 50%. No significant differences were observed. Bars represent +/- SEM (N's 8-10).

DISCUSSION

In examining the data, the revised results we received from experiment 2 was our goal. As the experimental mice were injected with saline, there should be no affect in their ability to learn. In the past, our lab has experienced difficulty in successfully getting our mice to learn this paradigm in a shorter time frame. We have had success when the protocol is extended over 4 to 5 days. However, our goal is to make this experiment as efficient and accurate as possible so that it can be used with a battery of other behavioral tasks in our lab. The more paradigms we have to use, the more we are able to confirm and replicate perceived conclusions. Therefore, we ideally want this task to be performed in one to three days, but we have experienced some difficulty with this timeline. As we have even had trouble with control mice, who display no memory or learning impairment, learning the task, this particular experiment is designed to ensure that there is no additional stress between mice who receive saline injections and mice who only receive a

scruff in a wild-type mouse model. We want both groups to learn equally to later add other variables and stimuli to this experimental protocol in the future.

In looking to improve future experiments, there are a few aspects of our current protocol that may need to be adjusted to yield more successful results. First, based on the revised results of experiment 2, it is appropriate to develop a criterion to remove mice that display abnormal explorative behavior from data analysis. This will take research and likely trial and error to create a criterion that works best for our lab. I have currently found a criterion that states that animals that do not explore both objects for more than 3 seconds in total during training and testing must be excluded from analysis. In addition, animals that have discrimination indexes +/- 20 in training are considered to have a significant location bias during training and are also excluded from analysis (6). However, I also have found a different approach to this criterion. Some researchers prefer that mice reach a minimum exploration time of 20 seconds for both objects. It is rare, though, that mice meet this exploration requirement within a five-minute training or testing period. To combat this, scoring continues past 5 min until the total exploration exceeds 20 s. It can then also be recorded which specific animals explored objects and met this requirement quicker than others (13). These are two different approaches to determining a criterion of exploration within our novel object location protocol, and it will take experimentation with both to determine which works best for our lab.

Another aspect of this protocol that is important to examine is the objects that we used in this experiment. As I have mentioned previously, animals have an innate object preference to certain textures, patterns, and colors (13). While this is more important in object recognition testing, as researchers are using two different objects in their trials, it is also important to think about in object location testing. In both experiments, mice are choosing to explore the objects in

comparison to exploring or moving throughout the rest of the arena. Our object, a 50 mL conical tube filled with beads to weigh the object down, displayed one texture, which was plastic, and two colors, which were blue and grey. These objects did not display a great variation in texture, color, or pattern. It may benefit future results if the objects used have a greater variety in color and texture, as the mice would be more inclined to explore them for a greater period of time. However, one must be careful to not use object materials that mice could chew. It is also important that these objects are easy to clean between each trial. There are many aspects that are involved in choosing objects to be used with this experimentation and using the most appropriate objects to attract greater interest from mice will drastically affect the success of our object location testing paradigm.

A third consideration for improving this behavioral task is the concept of habituation. In a habituation session, a mouse is allowed to simply explore the testing arena without the presence of objects for the same duration as a training or testing trial. While we thought about including habituation sessions in this protocol, we decided to do without them due to the amount of time it would add to our protocol. We believed that an extra training session instead would fulfill the role of a habituation session by giving the mice more time to explore both the objects and the arena. However, there is an added benefit of giving mice habituation sessions. During habituation, anxiety-like behavior can be assessed by calculating time spent in the center of the arena. This is useful data when determining the appropriate duration of the training sessions in the experiment. If the mice display higher anxiety by not spending as much time in the center of the arena, they may require a longer training session to reach a set exploration criterion and appropriately learn the task (13). The caveat with adding habituation sessions to our protocol is

that an additional day would be added to the experimental timeline. It will be important to assess the pros and cons of adding habituation to our future protocol.

Lastly, we want to examine the location of our objects throughout training and testing. In training sessions, both objects were placed in corners on the same side of the arena. However, in testing, one object was moved to a middle position on the opposite wall. This object could potentially cause additional anxiety to the mice due to its location. Mice, especially ones that display higher anxiety, often like to stay near walls or corners in an arena because they feel safer and not out in the open. The fact that the new location is more out in the open might cause the mice additional anxiety when they choose to explore it. This could lead to lower exploration of the object in the new location simply because of its position. To avoid this in the future, our lab will need to look at possibly selecting a new location to help yield more accurate and successful results in future experiments.

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