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TITLE:

Novel Object Recognition and Object Location Behavioral Testing in Mice on a Budget

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Memory, cognitive testing, object location, novel object recognition, mouse, hippocampus

SUMMARY:

Here we provide a protocol which includes comprehensive instructions for the economical establishment of murine object location and novel object recognition behavioral testing, including the design, cost, and construction of required equipment as well as execution of behavioral testing, data collection, and analysis.

ABSTRACT:

Ethologically relevant behavioral testing is a critical component of any study that uses mouse models to study the cognitive effects of various physiological or pathological changes. The object location task (OLT) and the novel object recognition task (NORT) are two effective behavioral tasks commonly used to reveal the function and relative health of specific brain regions involved in memory. While both of these tests exploit the inherent preference of mice for the novelty to reveal memory for previously encountered objects, the OLT primarily evaluates spatial learning, which relies heavily on hippocampal activity. The NORT, in contrast, evaluates non-spatial learning of object identity, which relies on multiple brain regions. Both tasks require an open-field-testing arena, objects with equivalent intrinsic value to mice, appropriate environmental cues, and video recording equipment and the software. Commercially available systems, while convenient, can be costly. This manuscript details a simple, cost-effective method for building the arenas and setting up the equipment necessary to perform the OLT and NORT. Furthermore, the manuscript describes an efficient testing protocol that incorporates both OLT and NORT and provides typical methods for data acquisition and analysis, as well as representative results. Successful completion of these tests can provide valuable insight into the memory function of various mouse model systems and appraise the underlying neural regions that support these

functions.

INTRODUCTION:

Effective cognitive tests isolate and assess the neural function of specific brain regions by examining behavior in a controlled environment[1]. In humans, specific tasks have been designed to assess the performance of targeted brain regions, such as the Wisconsin card sorting task for prefrontal function or the paired associates learning test of the Cambridge Neuropsychological Test Automated Battery (CANTAB) for hippocampal function[2,3]. These tests are designed to study the functions of specific brain regions in humans by assessing behaviors that result from the neural activity of those regions. The end goal of most biomedical research is the improvement of human health; however, many studies of brain function in health or disease cannot be ethically performed with human participants. For studies that cannot use human participants, small rodents such as mice are often the model of choice. Using mouse models allows for the direct control over experimental manipulations including alteration of gene expression, induction of injury or even modulation of circuit activity through optogenetic techniques. Behavioral testing of mice, similar to human testing, aims to assess the effect of experimental variables on brain function by measuring behaviors that rely on specific regions.

The hippocampus is an essential structure for memory formation in humans and rodents[4]. More specifically, the hippocampus plays a critical part in declarative memory involving relational representations, but not procedural memory, which relies on the motor centers of the brain[4]. Hippocampal memory function has been a focus of study across many fields of neuroscience because it is exquisitely sensitive to perturbation. Negative perturbations ranging from prolonged stress and aging to seizures and stroke are associated with hippocampal damage[5]. In contrast, positive interventions, such as social interaction, physical environmental enrichment, or exercise, improve hippocampal function[6-8]. Rodent studies with proper testing of hippocampal memory can reveal insight into the cellular and molecular mediators of memory as well as the effects of different environmental interventions on hippocampal function.

In rodents, several tests have been developed to study the hippocampus-dependent learning and memory[9-11]. They can be broadly subdivided into tasks that require a stimulus with emotional valence to elicit a change in behavior, and tasks that draw on the rodent preference to investigate novel stimuli[11]. Contextual fear conditioning, for example, pairs an unpleasant stimulus (foot shock) with an environmental context and then later tests memory for the context by measuring fear-induced freezing behavior[9,11]. The Morris water maze and its dry counterpart, the Barnes maze, use negative external reinforcement to promote spatial learning[4,11]. In each case, rodents seek escape from an aversive situation, being immersed in cold water or exposed on a brightly-lit platform, respectively. The radial arm maze, in contrast, relies on positive reinforcement as animals use natural foraging behavior coupled with spatial memory to retrieve small food rewards[4,11]. These tasks are widely used and have yielded foundational knowledge about hippocampal memory. However, negative and positive external reinforcements or fear-inducing stimuli like shock add an emotional component to these behavioral tests which in some cases may be undesirable. For example, the dorsal and ventral hippocampi are associated with distinct functions, spatial memory versus emotional regulation, respectively[12]. Tests that rely on an

emotional response to stimuli may not accurately reflect impaired spatial memory if ventral hippocampal emotional regulation functions are also affected.

The OLT is a simple and effective test that provides a measure of hippocampus-dependent spatial memory[13]. The task relies on an animal's intrinsic preference for novelty without additional external reinforcement and can therefore typically avoid complications associated with differential emotional responses[13]. The present protocol for OLT is presented for mice, but it is also effective in rats if the dimensions of the equipment are appropriately scaled. The protocol consists of acclimating a mouse to an open-field-testing arena and then allowing it to investigate 2 objects in relation to spatial environmental cues. The mouse is then removed from the arena, and during a delay (inter-trial interval or ITI), one of the objects is moved. After the ITI, the mouse is reintroduced to the arena and allowed to freely explore. In general, mice prefer novelty, and if they remember the location of the objects from their initial exposure, they will spend more time investigating the moved object. Animals with hippocampal lesions have impaired spatial contextual learning and consequently demonstrate no preference for objects in the novel location[14-15].

The OLT can be used independently or in combination with an additional test of memory that draws on neural activity from multiple brain regions, the novel object recognition task (NORT). The NORT is identical to the OLT until the test phase, when one of the objects is replaced by a novel object instead of being moved to a new location. As is the case with the OLT, mice with good memory of the objects will spontaneously prefer investigating the novel object. In contrast to object location memory, which relies heavily on hippocampal substrates, object recognition memory appears to rely on a variety of brain regions and the involvement of the hippocampus is unsettled. Many studies report that hippocampal lesions or inactivation do not affect novel object preference[10,13,16-17], while others find the opposite[18-19]. However, it is still a commonly used task to evaluate general memory function in rodents.

The protocol presented here delineates the steps involved in initiating and executing the OLT and NORT, both of which use an open-field-testing arena. Commercially available behavioral testing equipment can be cost-prohibitive, particularly for smaller labs. This protocol includes the design and simple steps to build arenas in-house at the minimal cost and without specialized tools. Furthermore, this protocol details the ideal behavioral testing area, including placement of arenas, contextual cues, and video recording system that sets the stage for implementation of the OLT and NORT protocols. Representative results for successful as well as flawed studies are presented, emphasizing the importance of optimizing all materials and procedures for each study.

[Place **Table 1** here]

PROTOCOL:

The following protocol has been approved by the Institutional Animal Care and Use Committee (IACUC) at the Ohio State University (OSU).

1. Building the Arenas

1.1. Order the materials outlined in **Table 1**: five sheets of acrylic, acrylic cement, and a 16-gauge hypo applicator.

1.2. Wear appropriate safety equipment according to manufacturer's instructions, which may include eye, skin, and other types of protection.

1.3. Remove the protective paper coating from the acrylic sheets.

1.4. Dry fit all the materials to confirm that sizes are correct (**Figure 1A**).

1.5. Assemble and load the syringe with acrylic cement.

1.6. Align the long edge of an outside wall (Part B) with a top edge of the base (Part C) and ensure that they are perpendicular to each other using a combination or machinist square.

1.7. Using the syringe, apply a small and steady bead of cement directly to the corner of the two pieces being joined.

1.8. Hold the two pieces (Parts B and C) in place until they are initially set (approximately 5 min).

Note: Typically, they will be 80% hardened in 24 h, but assembly can continue after 5 min.

1.9. Repeat steps 1.5-1.7 with the other outside wall (Part B) to the same base.

1.10. Attach the two inner walls (Part A), one at a time, to the base using steps 1.5-1.7.

1.11. Additionally, use the syringe to apply a small and steady bead of cement directly to the corner now being formed by the outside wall (Part B) and the inside wall (Part A).

1.12. Hold these pieces in place for 5 min.

1.13. After 24-48 h, proceed to setting up the behavioral testing environment.

2. Setting up the Behavioral Testing Environment and Equipment

2.1. Place environmental cues (as described in the discussion section) across from each other and facing the arenas in the testing area (**Figure 1B**).

2.2. Arrange the four testing arenas in a 2-by-2 manner either on the floor or sturdy table at appropriate distances from the cues and the camera to maximize visual input to the mice (**Figure 1B**).

2.3. Verify the line of sight using a meter stick propped from each arena floor over the wall towards the cues to confirm that these distances between the arenas and the cues are

appropriate.

2.4. Determine the optimal optical path length that allows video documentation of all four arenas by adjusting the height of the camera or the height of the table. (**Figure 1B**).

2.5. Connect the camera to a USB Extension Cable.

2.6. Using cable raceways, run the cable across the ceiling and down a wall to a computer running video capture software.

2.7. Hide the computer behind a curtain that will separate the mice in the testing area from the researcher (**Figure 1C**).

2.8. Assemble 4 each of at least 3 different objects that are approximately 2-5 cm in length and width and up to 10 cm in height to use for testing (**Figure 1D**).

[Place **Figure 1** here]

2.9. Validate these objects.

2.9.1. Obtain a minimum of 8 wild-type mice in the sex, strain, and age group representative of the experimental mice that will be used (*e.g.,* 6- to 9-week-old female and male C57Bl/6 mice).

2.9.2. Handle all mice daily for 1 min over the course of 3-5 days prior to testing.

2.9.3. Divide mice into groups of 4 and, if they are not already singly housed, move them into individual clean holding cages.

2.9.4. Bring them into the testing room and allow them to acclimate for at least 30 min.

Note: Presence of the experimenter in the room for these 30 min will reduce stress on the mice during the task, particularly if the experimenter is male[20].

2.9.5. After acclimation is done and the experimenter is ready to start, begin recording the video.

2.9.6. Place each mouse facing the walls of one corner of the arena (called the release corner) (**Figure 2A**).

2.9.7. Allow the mice to explore the arenas freely for 10 min.

2.9.8. Stop recording the video.

2.9.9. Return mice to their clean holding cages for a duration of 20 min.

2.9.10. Clean all arenas with animal facility recommended cleaning methods, such as wiping with 70% ethanol to minimize olfactory cues before the next use.

2.9.11. Using double-sided tape, affix 2 different objects near 2 non-release corners such that the objects are counterbalanced in the arena, and 6 x 6 cm from each wall of that corner (**Figure 2B**).

2.9.12. Start recording the video.

2.9.13. Place each mouse facing the walls in the release corner.

2.9.14. Allow mice to investigate the arena and objects freely for 10 min.

2.9.15. Stop recording the video.

2.9.16. Place mice back in their clean holding cages for a duration of 20 min.

2.9.17. Clean all arenas and objects with animal facility recommended cleaning methods such as wiping with 70% ethanol to minimize olfactory cues.

2.9.18. Repeat training trials with 2 new objects affixed in the same locations until all the objects (at least three different objects if conducting both the OLT and NORT) have been tested with each mouse.

2.9.19. Exclude objects that allow mice to sit on top of the object.

2.9.20. Analyze investigation time of each mouse with each object according to step 4.

2.9.21. Exclude objects that have a negative or positive intrinsic value.

3. Conducting the Behavioral Test

3.1 One week prior to testing: familiarization to individuals conducting the behavioral tests

3.1.1. Handle adult 6- to 9-week-old female and male C57Bl/6 mice daily for 1 min over the course of 3-5 days prior to testing.

3.2. Day 1: habituation sessions

3.2.1. Divide mice into groups of 4 and, if they are not already singly housed, move them into individual clean holding cages.

3.2.2 Bring them in to the testing room and allow them to acclimate to the testing room for at least 30 min.

Note**:** Presence of the experimenter in the room for these 30 min will reduce stress on the mice during the task, particularly if the experimenter is male[20].

3.3. After acclimation is done and the experimenter is ready to start, begin recording the video.

3.4. Place each mouse in the arena (one mouse per arena) facing the walls of the release corner (**Figure 2A**).

3.5. Allow the mice to explore the arenas freely for 6 min.

3.6. Stop recording the video.

3.7. Return mice to their clean holding cages during the inter-trial interval (ITI).

3.8. Clean all arenas with animal facility recommended cleaning methods such as wiping with 70% ethanol to minimize olfactory cues.

3.9. Repeat steps 3.3-3.9 two more times for a total of 3 habituation sessions for each mouse.

3.10. Return all mice to their home cages.

3.11 Clean all arenas with animal facility recommended cleaning methods such as wiping with 70% ethanol to minimize olfactory cues before use the next day.

3.12 Day 2: training trial, OLT, NORT

Note: The NORT is an optional test.

3.12.1 After 24 h, bring the same group of mice in to the testing room and allow them to acclimate to the testing room for at least 30 min as done before the habituation sessions on the previous day.

3.12.2. Conduct a training trial using 2 objects placed in the arena (**Figure 2B**).

3.12.2.1. Using double-sided tape, affix objects 6 x 6 cm away from 2 non-release corners such that they are counterbalanced in the arena.

3.12.2.2. Start recording the video.

3.12.2.3. Place each mouse facing the walls of the release corner as done during the habituation sessions.

3.12.2.4. Allow mice to investigate the arena and objects freely for 10 min.

3.12.2.5. Stop recording the video.

3.12.2.6. Place mice back in their clean holding cages for an ITI of 20 min.

3.12.2.7. Clean all arenas and objects with animal facility recommended cleaning methods such as wiping with 70% ethanol to minimize olfactory cues.

3.12.3. Perform the OLT.

3.12.3.1. Move one of the objects used in the training trial to a new non-release corner and affix the object 6 cm from each wall of that corner with double-sided tape (**Figure 2C**).

Note: The other object should remain where it was during the training trial.

3.12.3.2. Start recording the video.

3.12.3.3. Place each mouse facing the walls in the release corner.

3.12.3.4. Allow mice to investigate the objects for 10 min.

3.12.3.5. Stop recording the video.

3.12.3.6. Place mice back in their clean holding cages for an ITI of 20 min.

3.12.3.7. Clean all arenas and objects with animal facility recommended cleaning methods such as wiping with 70% ethanol to minimize olfactory cues.

3.12.4. Perform the NORT.

3.12.4.1. Replace the object that was not moved during the OLT with a novel object and affix the novel object 6 cm from the two walls of the corner with double-sided tape (**Figure 2D**).

3.12.4.2. Start recording the video.

3.12.4.3. Place each mouse facing the walls in the release corner.

3.12.4.4. Allow mice to investigate the objects for 10 min.

3.12.4.5. Stop recording the video.

3.12.4.6. Place mice back in their home cages.

3.12.4.7. Clean all arenas and objects with animal facility recommended cleaning methods such as wiping with 70% ethanol to minimize olfactory cues before next use.

[Place **Figure 2** here]

4. Analyzing Behavioral Test Data

Note: Analysis of video should ideally be completed by at least two independent, blinded experimenters.

4.1. Open the video file.

4.2. Apply a transparent circle that provides a border of 2 cm around each object over the screen to help determine active investigation. Use a video file image with a ruler placed in an arena to calibrate this grid.

4.3. Observe mouse behavior and record the times the mouse is actively investigating the object, which consists of its nose pointed at the object at a maximum distance of 2 cm from that object.

4.3.1. Record the time stamp the mouse starts to investigate an object and the time stamp when it stops investigating that object.

4.3.2. Repeat this for both objects in the arena for the duration of the trial.

4.3.3. Calculate the cumulative time the mouse investigated each object by subtracting the start time from the stop time for each instance of object investigation, and adding all of those values.

4.4. Calculate percent of total investigation time or the discrimination index with the following formulas:

4.4.1. Calculate percent of total investigation time =

$$\frac{(\text{time with novel location or object})}{(\text{time with novel location or object} + \text{time with familiar location or object})} \text{ x } 100$$

Note: A value above 50% indicates greater investigation of the novel location or object.

4.4.2. Calculate discrimination index =

$$\frac{\text{time with novel location or object} - \text{time with familiar location or object}}{\text{time with novel location or object} + \text{time with familiar location or object}}$$

Note: A positive value indicates more time investigating the novel object. A discrimination index of zero indicates equal time spent with both objects.

4.5. Graphically represent results and complete statistical analyses using a t-test or ANOVA as appropriate for the number of groups being compared.

REPRESENTATIVE RESULTS:

Figure 3 provides examples of typical positive and negative results obtained with male and female adult C57Bl/6 mice using this protocol[6]. Interpretation of OLT and NORT data always applies to the aggregate data of a group (see discussion below). Investigation time for a single mouse cannot be interpreted as memory or lack of memory. However, the performance of a group of mice (*i.e*., multiple samples) can be compared to other groups or to the fixed chance levels using statistical testing. During a typical training trial, groups of mice do not show a significant preference on average for either of the objects as they are both equally novel and do not have any intrinsically negative or positive value to the mice (**Figures 3A** and **3B**). If the aggregate data of a group of mice show significant preference for one object over another during training, these objects should not be used because that inherent preference/aversion will confound results in the subsequent trials. Additionally, the total investigation time of all the mice must meet a minimum standard (traditionally set at 20 seconds[21]) and should be compared to ensure that there is no baseline difference in investigation that may affect subsequent tests of memory.

During the OLT, memory for object location is reflected by mice spending on average significantly more than 50% of total investigation time with the moved object (**Figure 3A**). If the total investigation times of the individual mice vary greatly, results are better depicted as a discrimination index for the objects (**Figure 3B**). The significant increase in average discrimination index in **Figure 3B** indicates that the mice spent more time with the object after it had moved. Whether measured by increase in percent time or discrimination index, the increase in investigation of the object after it is moved suggests that the mice remember where the object was located during training.

The last trial of this protocol assesses object recognition memory. A representative example with one group of mice demonstrates a higher average percentage of investigation time (**Figure 3C**) as well as positive discrimination index (**Figure 3D**) compared to the fixed control values of 50% and 0, respectively. As with the OLT data, if there is significant variability in total investigation time between individual mice, the discrimination index is likely the better method to visualize this data. **Figure 3E** shows an example of a 2-group comparison in the NORT and some of the statistical complications that can arise in these tests (see discussion). While a one-sample t-test for group B shows investigation significantly above 50%, the same test for group A does not. This finding does not mean that A and B are different from each other. To determine group differences, a separate two-sample non-parametric Mann-Whitney test comparing groups B to A must be performed. A two-sample non-parametric Mann-Whitney test of this representative group data shows no significant difference ($p = 0.66$) between the two groups in percentage of novel object investigation time.

Both the OLT and NORT are highly sensitive to the intrinsic value of objects and thorough testing of object equivalence is necessary to ensure that there is no intrinsic bias that can confound results. **Figures 3F** and **3G** show an example of inappropriate object selection. In a pilot test with a sample size of 4, mice showed a trend towards spending less than 50% of investigation time

with object A when paired against object B (**Figure 3F**). When these objects were then used in a NORT with object A as the novel object and a larger sample size of 16, mice spent significantly less than 50% of investigation time with object A (**Figure 3G**). This aversion to the novel object is easily recognizable here as a technical flaw in the experiment and exemplifies why pilot testing of objects for inherent preference/aversion is essential.

FIGURE AND TABLE LEGENDS:

Table 1: Itemized list of materials and equipment required for behavioral testing.

Figure 1. Behavioral testing preparation. (A) Open-field-testing arena assembly with part A corresponding to the inner wall, part B as the outer wall, and part C as the base. The finished arena will have two outer walls (parts B) that run the entire edge of the base and two inner walls (parts A) that fit between the outer walls on the adjoining edges of the base (part C). All walls will rest on top of the base. (B) Representative arrangement of arenas on a 0.62 m high table, 60 x 90 cm environmental cues, lights, and a camera for a testing area that allows the capture of all four arenas simultaneously. (C) A curtain hides the experimenter and computer system from mice during trials. The overhead lights are on for the purposes of taking this photograph, but during testing, only the floor lamps are on. Also, one of the environmental cues has been removed for this photograph of the testing area, but during testing, there is a fourth cue in front of the arenas, facing the all black cue behind the table. (D) A representative object (and ruler for scale) that is appropriate for OLT or NORT testing with mice.

Figure 2. Arena configuration for trials. (A) Open-field testing arena without objects for habituation session. The black arrow indicates a release corner. This corner should be the same relative location in each arena and be consistent for every mouse being tested and for every trial. (B) For the training trial, two different objects are secured to the open field at 6 x 6 cm away from their respective walls. (C) For the OLT, one object is moved to a new location, also 6 x 6 cm away from the walls and not the release corner. (D) For the NORT, the object that was stationary in the OLT is replaced with a novel object while the moved object from the OLT is now the familiar object.

Figure 3. Behavioral testing data with wild type adult C57Bl/6 mice. (A) Comparison of percent total investigation time of the moved object during the training trial versus the OLT shows significant increase in investigation, after the object moved. ****$p < 0.0001$, paired t-test. (B) Representative results for moved object investigation during training and OLT trials displayed as a discrimination index similarly show a significant increase in investigation of the object after it is moved. ****$p < 0.0001$, paired t-test. (C) Percent total investigation time of the novel object in the NORT shows significant preference for investigating the novel object. **$p = 0.0024$, one-sample t-test *vs*. 50%. (D) Representative results for novel object investigation in the NORT displayed as a discrimination index similarly show preference for investigating the novel object. **$p = 0.0024$, one-sample t-test *vs* 0. (E) Representative results of a NORT analysis involving two different groups of mice. Group B differs significantly from 50% by one-sample t-test (**$p = 0.0024$), but group A does not ($p = 0.5837$). In a separate analysis, to compare groups, a two-sample Mann-Whitney test is used because of the uneven group sizes and no significant

difference in investigation is found (p = 0.66, ns). (F) Percent time with an object during validation trials in a small sample size shows a trend towards aversion to the object. p = 0.2159, one-sample t-test. (G) With a larger sample size and the object from (F) used as a novel object in a NORT, a significant aversion to the object is found, even though it is the novel object. *p = 0.0270, one-sample t-test. This is an example of a technical failure in object selection. Data are presented as mean ± SEM. Data from panels C-E are adapted from a previous publication[6].

DISCUSSION:

This protocol provides a cost-effective method to conduct object location and novel object recognition behavioral testing in mice. These tests enable the evaluation of hippocampal function as well as function of other cortical regions, such as the prefrontal cortex, involved in object recognition[10]. The OLT and NORT have the advantage of avoiding stimuli with strong emotional valence that are required for the Morris water maze, contextual fear conditioning, Barnes maze or radial arm maze. They also avoid the need for food deprivation as required for the radial arm maze. Furthermore, this protocol describes a simple two-day testing procedure that does not require extensive or complicated equipment for execution or analysis. One disadvantage of these tasks is that they do not allow for measures of learning or acquisition. A difference in novelty investigation could be due to poorer learning about objects during training, poorer memory for what was learned or both. Total time spent investigating objects is an important measure for ruling out any inherent differences in exploration drive but is not a measure of learning. If measures of learning are important for an experimental question, a water maze, Barnes maze, or radial arm maze would likely be preferable.

Custom building of behavioral arenas has the potential to save hundreds of dollars and bring object testing within the financial reach of a wide variety of labs. This protocol eliminates many obstacles and streamlines the process of fabrication to make in-house arena construction more accessible to scientists with no specialized training in acrylics. It is important to note that purchasing colored acrylic sheets that will contrast with the mice, such as white acrylic for black mice and black acrylic for white mice, will facilitate data acquisition and analysis, especially when using commercially available analysis software. Ordering cut-to-size sheets with “routed edges” eliminates the need for a table saw (**Table 1**), and the use of acrylic cement removes the need to drill and countersink pilot holes. Screwing in fasteners, drilling, and cutting acrylic often causes it break, chip and crack due to its brittle nature. Because the cement is a solvent, it will flow into the area being joined, dissolving and softening any acrylic it encounters. Thus, it should not be applied to each piece separately as if it were a traditional glue. Unlike glue, the cement will not fill negative spaces or adhere to surfaces. This is the primary reason to order “routed edges” as this will ensure a smooth and flat edge, creating a much better bond. When the cement dries, it will have fused the two acrylic sheets into a single piece in a process called “solvent welding”. Much like metal welding, the finished product is a single piece, but the welded area will always remain the weakest location. As such, once the arenas are in use, care should be taken to avoid direct impact or extreme stress at those junctures.

This protocol also shows how to set up 4 arenas for simultaneous testing of up to 4 mice (**Figures 1** and **2**). The opaque walls of the arenas prevent mice from seeing one another during testing,

but there is still a possibility that having other mice in the room causes odor or noise distractions that can impair testing. Habituation trials as detailed here can help mitigate this concern, as mice are exposed to the multi-animal room conditions before behavioral testing. However, if distractions from other mice or experimenter noise associated with handling other mice is a strong concern, one arena with one mouse can be used, as well, though it will increase the time required to complete the OLT and NORT with multiple mice. More than 4 arenas could theoretically be used as well, but most cameras do not have a wide enough field of view to show that many arenas with good resolution.

The dimensions and distances provided here are general guidelines for mouse behavioral testing in a typical testing room that is 16 x 16 x 16 m in dimensions (**Figures 1B** and **1C**). Set up of appropriate environmental cues, arenas, and video recording equipment must be optimized for each environment. Cues can consist of large shapes or patterns (typically in black and white) that enable mice to spatially orient themselves during the OLT. Instead of placing cues at different locations across from each other, cues can also be mounted to the walls of the testing area. This protocol recommends dividing the testing room with a curtain to hide the researcher and computer during behavioral testing. During all habituation, inter-trial intervals, and active trials, researchers should close the curtain to separate themselves from the testing area. If this is not practical, the computer can remain in view of the mice, but the researcher must move out of view during the task. If the researcher is present, the mice may try to rely on her or him as a spatial cue.

All behavioral testing should be completed in a temperature- and humidity-controlled environment with dim, but even illumination at around 310 lux and minimal extraneous sound or strong environmental odor cues, such as perfumes on experimenters. Between each trial and testing day, all arenas and objects should be cleaned with animal facility recommended methods of sterilization such as wiping with 70% ethanol or unscented bleach wipes to minimize olfactory cues. As with any behavioral task, handling mice for several days before testing is necessary to familiarize them with the individuals who will be performing the OLT and NORT and reduce stress during testing[20,22]. As mice can experience acute stress due to unfamiliar individuals in the vicinity of the testing area, it is also recommended that all behavioral testing should be completed by the same individual(s). The testing parameters and conditions detailed in this protocol have been optimized for 6- to 9-week-old adult C57Bl/6 mice and would be most useful in revealing memory impairments due to injury in this age group or memory impairments due age itself in older mice. If the aim is to test for memory improvements in young mice, a longer ITI ranging from 1 hour to 1 day would be more appropriate to avoid ceiling effects on performance in the easier 20-min ITI version. Indeed, ITIs can range from 5 min (for immediate recall) to several hours or days (for remote memories), depending on the specific needs of the experiment and the strains and ages of the mice. Importantly, regardless of the length, all ITIs should be consistent between sessions in an experiment. As different strains and ages of mice exhibit differences in behavior and learning, the timing for each trial and interval, testing area arrangement, and objects used can be modified according to the particular strain of mice, their age, and the specific injury/disease/intervention model being tested[9,21,23-25].

While the hippocampal dependence of the spatial memory functions tested in the OLT are well-established, the NORT may or may not rely on the hippocampus. Interpretation of data from the NORT should take this caveat in to account. The determining variables, for whether the hippocampus is involved, in object recognition memory are not agreed upon yet, but could include ITI length or saliency of spatial cues[18]. Notably, the presented protocol uses spatial OLT before the NORT, which may bias mice towards using hippocampal processes in the NORT. Thus, it is important to note that the order can be reversed, or each task can be run independently, depending on the experimenters' questions and needs.

Object selection is a critical aspect of both the OLT and NORT[22,24]. Ideal objects are heavy enough not to be easily displaced by a mouse and made of material, like glass or metal, that a mouse cannot damage by chewing or scratching. Wooden, foam, or soft plastic objects are not appropriate as they are easily deformed and are difficult to keep odor-free. Furthermore, the objects used in the trials should all be relatively similar in size, texture, odor and material. **Figure 1D** gives an example of an appropriate object that could be used. This orange plastic figurine of a chick is filled with sand to give it enough weight and sealed to prevent leakage of cleaning reagents or other odor-causing agents. Because of the shape of the top, mice are unable to climb on top of or sit on this object. For the OLT or NORT, this object is best paired with another object of similar size, weight, material, color and complexity, such as a similar plastic figurine of a rabbit. To ensure that object investigation truly reflects preference for novelty, all objects must be validated for equivalent intrinsic value with a minimum of 8 mice in the same strain, sex, and age of the experimental group as described in section 2.9 of the protocol. Additionally, the objects should be randomized in terms of which object is the novel or moved object between mice in the same study to further ensure that inherent characteristics of the objects are not affecting preference. Object placement can also greatly affect the success or failure of the OLT and NORT. Objects must be counterbalanced in the arenas and not be too close to the walls. A corner crowded by an object is an attractive place for mice to hide and this will confound measures of investigation.

An important prerequisite to data collection is defining "active investigation", which is when a mouse engages an object with its nose pointed at the object no more than 2 cm away. A mouse moving over the top of the object or looking past the object does not qualify as active investigation. Furthermore, since the OLT and NORT depend on a mouse remembering either the spatial location or actual features of the object, there must be sufficient study of these during the training trials. Thus, the researcher must define a minimum investigation time and exclude any subjects that do not meet that baseline level of investigation, traditionally set at 20 seconds[21].

Quantification of object investigation time can be accomplished in various ways either with or without expensive analysis software. If manual scoring is used, as described here, the most comprehensive data collection can be achieved by recording the time stamps on the video when the mouse is investigating the object. Recording the start and stop times of object investigation during the three individual trials creates a permanent log of unbiased time stamps and facilitates accurate manual calculation of data as opposed to alternative methods such as using a stop

watch to additively record the total time a mouse investigates each object.

Commercial software packages are also available for scoring object investigation. Commercially available software can provide a wealth of data beyond the hand scoring data described here, including total distance traveled, amount of time spent in certain areas of the arena, and speed of movement[16,23]. Software, once properly calibrated and confirmed to reliably detect object investigation, can also yield data much faster than manual scoring. This faster data yield may, in the long run, yield net savings compared to the human-hours required for manual scoring. However, most software packages for behavioral analysis have high up-front costs that can be prohibitive for many labs and manual scoring can often be accomplished by student researchers, making the hourly costs of behavioral scoring minimal. Commercial software also frequently comes with restrictions on how many users can access the software concurrently, limiting the data throughput and time savings. Though there are many advantages to these software packages, they are not necessary to gather the essential information of time investigating objects from the OLT and NORT. The ability to manually acquire this data makes novel object tasks more financially accessible to a wider variety of researchers.

An additional feature of this protocol is that the first session of habituation is essentially a trial in an open field, which can yield data on activity and anxiety levels. Activity levels can be quantified using total distance traveled or average speed, if tracking software is available. Similarly, with tracking software, anxiety measures can be derived from time spent in the center of the arena or distance traveled in the center. These data can also be acquired manually by overlaying the arena video with a grid and quantifying crossings. This open-field data can help rule out gross motor deficits or excessive anxiety that might interfere with object investigation later.

The statistical tests used in this protocol are representative of traditional methods of analysis. When comparing 2 groups of mice, a two-sample, two-tailed t-test is recommended to test the significance of the difference in percent time investigating the moved or novel object between groups. If the sample sizes are uneven, have exceptionally high variability, or show uneven variability between groups, a non-parametric test is recommended instead, as many of the underlying assumptions of the t-test will be violated in these conditions. When comparing 3 or more groups of mice, ANOVAs are recommended to test whether group has a significant effect on percent time investigating the moved or novel object. Error-corrected post-hoc tests, such as Tukey's test or Bonferroni-corrected pairwise comparisons, can then be applied to test the difference in percent investigation time between each pair of groups. With the OLT, an additional way to analyze data is to test for a significant change in percent time investigating the moved object from training to OLT trials. With only one group of mice, this test would take the form of a paired, two-tailed t-test, testing for significant changes in percent time with the moved object from training to OLT in each mouse (keeping in mind that these are paired analyses and not independent measurements, since each mouse yields two data points). With two groups of mice, a repeated measures two-way ANOVA would be used, with the repeated measure being the trial (training versus OLT) and group assignment being the second factor. Post-hoc tests for differences between treatment groups should compare the groups to each other within trials.

Alternatively, if evidence of memory in the OLT or NORT is all that is being tested, a one-sample t-test of percent time investigating an object versus the fixed value of 50% can be used. A percent time significantly higher than 50% suggests memory for the object. A percent time significantly below 50% suggests aversion (for some reason), and object choices should be re-evaluated. However, the one-sample t-test cannot reveal whether two or more groups differ from each other. For example, in an experiment with two groups of mice, if Group A spent 60% of the time with the novel object for $p = 0.049$ by one-sample t-test comparison to 50%, and Group B only spent 59% of the time with the novel object for $p = 0.051$, Group A is significantly different from 50% (and group B is not). However, it is erroneous to conclude that these two groups are significantly different from each other. A two-sample t-test comparing A and B can easily reveal that these two groups are statistically indistinguishable. If the end goal is to compare the memory performance of two or more groups, those groups must be statistically compared to each other, not only compared to an external standard. A similar guideline applies for a comparison of time spent with a moved object between training and OLT. In this case, finding a significant difference in percent time with a moved object between training and OLT in one group and not in another does NOT show that these groups are statistically different. Groups must be post-hoc compared against each other within each trial, not just across trials within group.

It is important to keep in mind that the investigation time of any individual mouse cannot be used as evidence for or against memory. Rather, memory for object location or identity in these tasks can only be concluded based on aggregate data that is statistically compared to either another group's aggregate data or the fixed chance levels (50% for time, 0 for discrimination ratio). The sample size needed will depend heavily on the effect size of a particular manipulation and variability in behavior, both of which in turn will depend on the mice being used. Age, sex, and manipulations all impact variability. In the example data presented in **Figures 3A** and **3B**, $n = 14$ subjects were used, yielding an effect size of 0.68 and power of 0.65 for a paired t-test with $\alpha = 0.05$. If a power of 0.8 were desired for this comparison, a sample size of 18 would be required.

This discussion is framed around p-values and significance cutoffs because these are the measures and analyses most typically reported for OLT and NORT data, and therefore are likely to familiar to both experimenters and reviewers. This reliance on p-values has been heavily criticized as statistically invalid[26]. However, though alternative analysis methods exist and are endorsed in some journals[27], none have been broadly adopted by the behavioral and biomedical fields as standard[26].

In summary, this protocol effectively tests memory in mice at minimal costs. Recommendations for appropriate modifications to the protocol are included to ensure successful implementation with any small rodent model. Application of this protocol to specific injury or therapeutic intervention models can reveal valuable functional relevance that complements the cellular and molecular mechanisms being studied.

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The authors have nothing to disclose.

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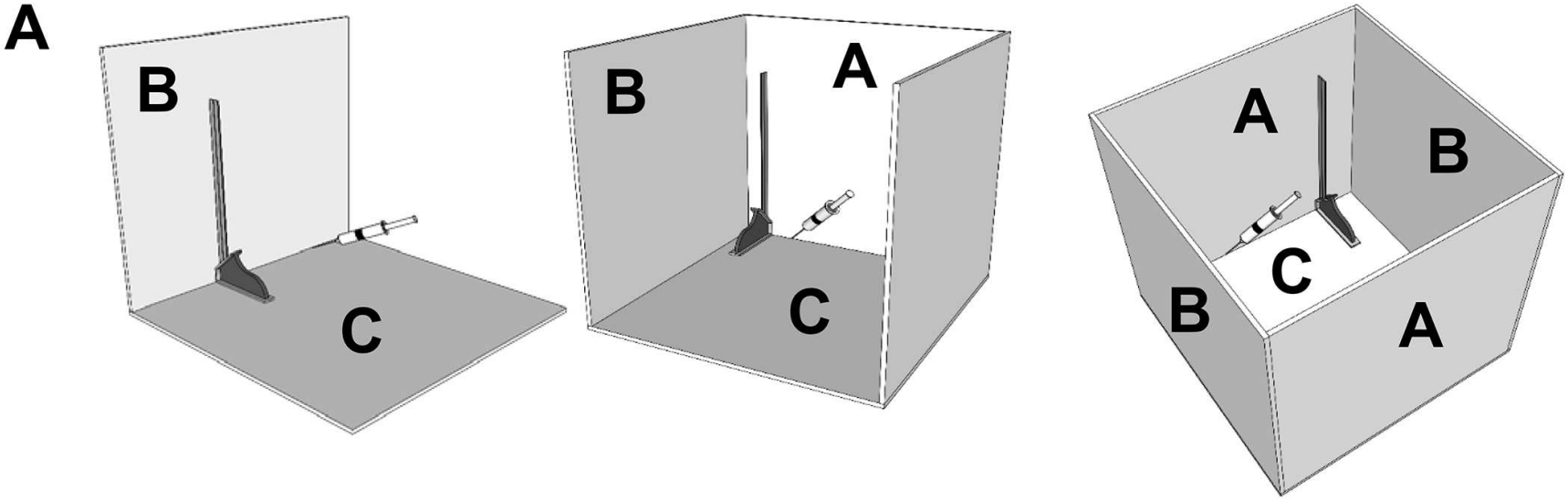

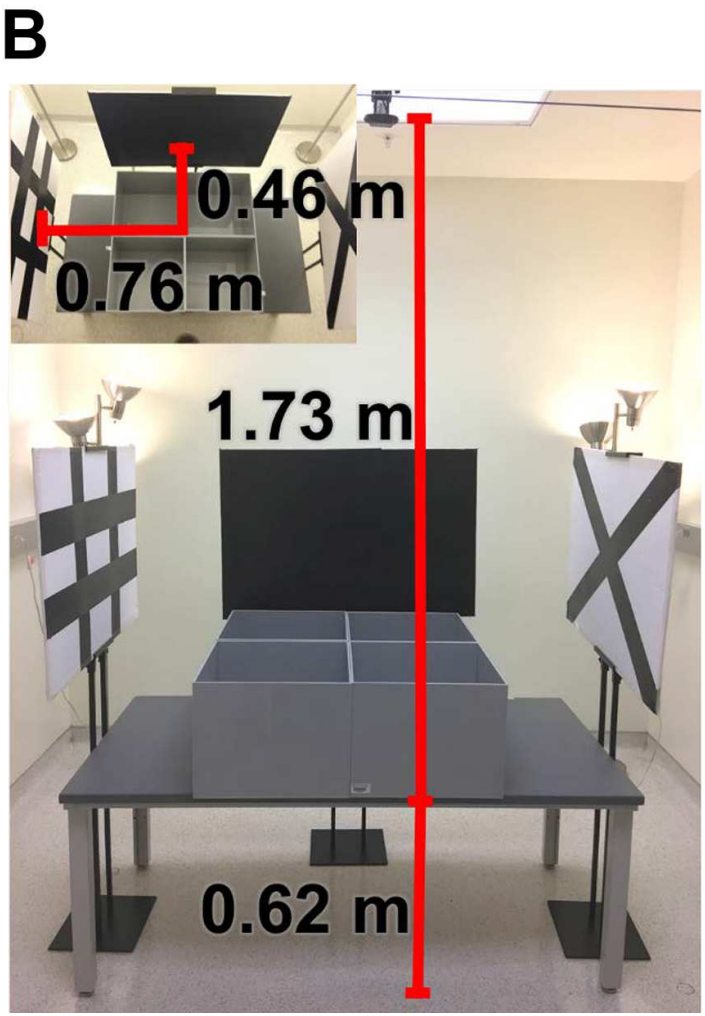

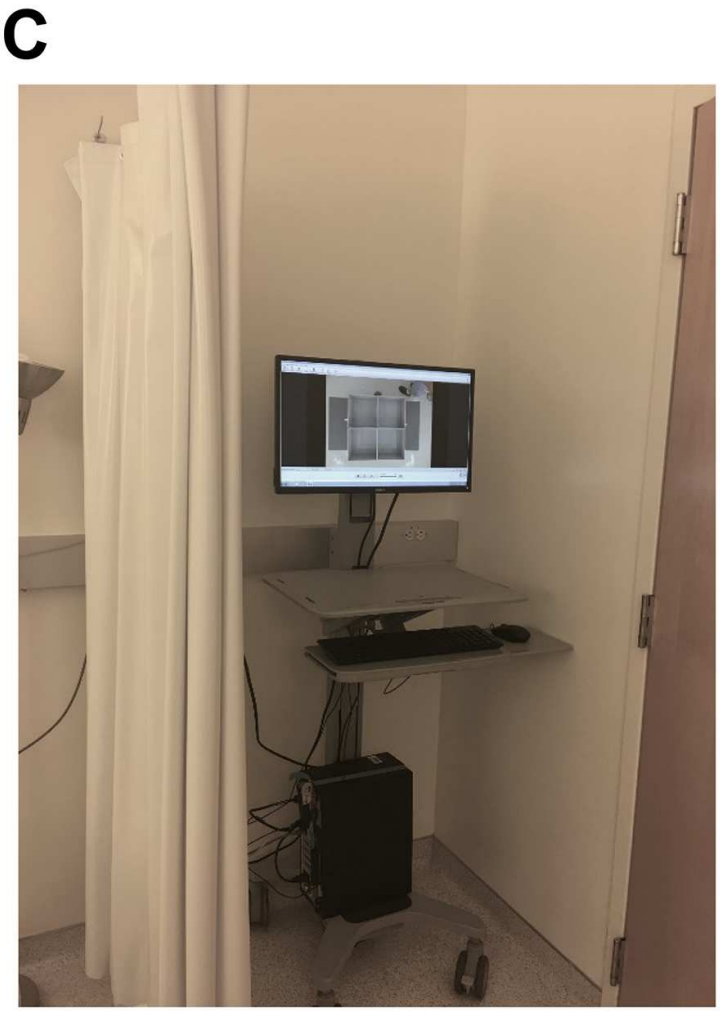

Figure 2

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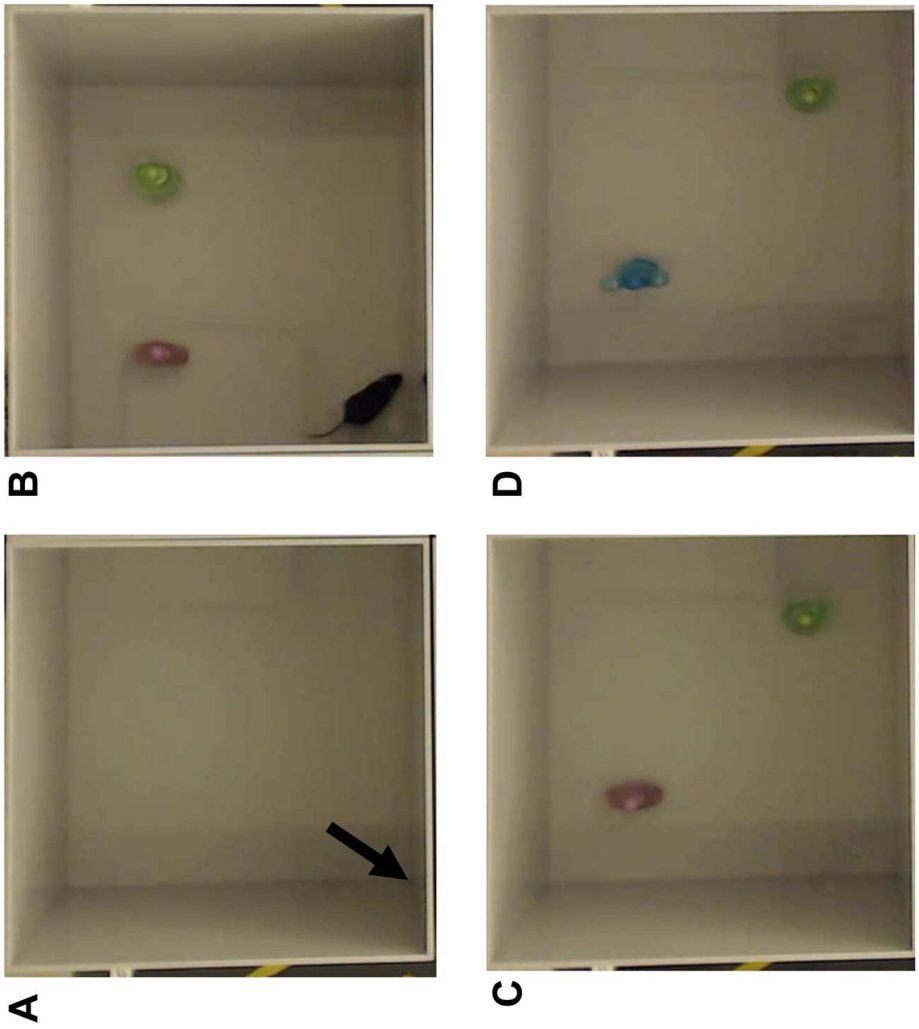

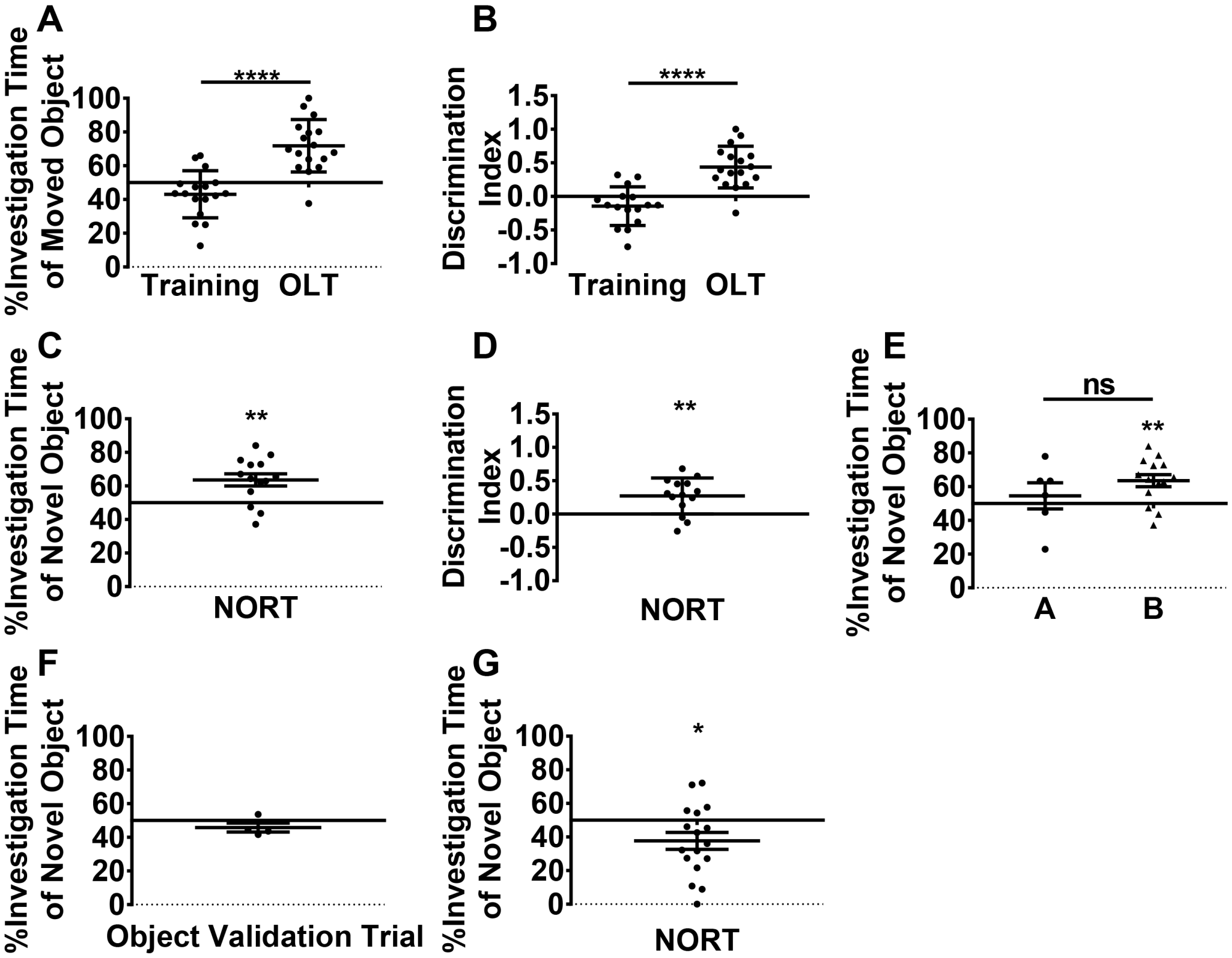

Table

Description
Part A: Acrylic sheet - Opaque White (0.635 cm x 40 cm x 40.64 cm)
Part B: Acrylic sheet - Opaque White (0.635 cm x 40 cm x 41.91 cm)
Part C: Acrylic sheet - Opaque White (0.635 cm x 41.91 cm x 41.91 cm)
Acryllic Cement (1 pt.)
16 Gauge Hypo Applicator
Combination Square
HD Webcam
Video Capture Software
USB 2.0 Extension Cable
Cable Conduit

Option	Quantity
Routed Edges	2
Routed Edges	2
Routed Edges	1
NA	1
NA	1
NA	1
NA	1
NA	1
NA	1
NA	1

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Name of Material/ Equipment	Company
Sign White - 9% Translucent and Opaque Colored Cast Acrylic (Chemcast)	TAP Plastics
Sign White - 9% Translucent and Opaque Colored Cast Acrylic (Chemcast)	TAP Plastics
Sign White - 9% Translucent and Opaque Colored Cast Acrylic (Chemcast)	TAP Plastics
TAP Acryllic Cement (1 pt.)	TAP Plastics
16 Gauge Hypo Applicator	TAP Plastics
Debut Video Capture Software Pro Edition	NCH Software
Stanely SAE/Metric Comb Square	Grainger
Logitech Pro Stream Webcam C922x	CDW
Belkin 16' USB 2.0 Active Extension Cable	CDW
Panduit Pan-Way LD Surface Raceway (8')	CDW
Spatial/Environmental Cues	Variable
Objects for testing	Variable
70% ethanol	Variable
Double-sided tape	Variable

Catalog Number	Comments/Description
NA	1/4" x 15.75" x 15.75" with Routed Edges
NA	1/4" x 15.75" x 16.5" with Routed Edges
NA	1/4" x 16.5" x 16.5" with Routed Edges
NA	
NA	
NA	
6R171	
4429927	
570691	
300902	
NA	
NA	
NA	
NA	

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To the Editor of JoVE:

Thank you for inviting us to submit a revised draft of our manuscript "Novel Object Recognition and Object Location Behavioral Testing in Mice on a Budget" to the Journal of Visualized Experiments. We are grateful for the reviewers' insightful comments on the manuscript and have edited the manuscript to address their concerns.

Overall, we agree with the general comment from the reviewers regarding our previously restrictive categorization of NORT as completely hippocampus-independent. To address this, we have added more elaboration on the complexities of the brain region-dependency of the NORT, especially in the introduction and discussion sections. We have also accounted for the possible role of the order of the OLT and NORT in encouraging use of spatial strategies in the NORT by adding a note in the protocol itself and a recommendation in the discussion. To address the concern over emphasizing the importance of object selection and validation, we have made several changes to the protocol and discussion. Notably, we have moved the validation step earlier in the protocol and have expanded our discussion section to include the references provided by Reviewer 3. We have also modified Figure 1C and streamlined the discussion of that figure which hopefully provide a clearer explanation of appropriate objects.

In the rest of this letter, we address the reviewers' specific concerns.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have made the necessary corrections to the manuscript, figures, and table.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have corrected the citation in the figure legend and uploaded a link to the editorial policy to our Editorial Manager account.

3. Figure 3: Please define error bars in the figure legend.

We have added the appropriate definition at the end of the figure legend.

4. Table 1: Please fix the typos in the table.

The typos have been corrected.

5. Please provide an email address for each author.

We have provided email addresses for every author.

6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

All units in the manuscript, table, and figures are now in SI units.

7. Please remove the commercial pricing from the manuscript. Though the emphasis of the paper is on the economical method of creating the apparatus, the wholesale listing of prices in the manuscript and in the Tables is not appropriate for publication here. Is there any other way to discuss the cost economies?

In an effort to preserve the aim of this manuscript while avoiding listing wholesale prices, we have eliminated table 1 and removed the specific companies and cost comparisons in the main text. We hope that this still helps readers make an informed decision about building versus ordering arenas.

8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Logitech Pro Stream Webcam, Belkin, NCH, etc.

We have omitted the original Table 1 and removed all commercial language from the manuscript and Table 2 (now the new Table 1). They are still referenced in the Table of Materials and Reagents.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the protocol text to avoid use of personal pronouns.

10. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The protocol has been streamlined to only contain actions in the imperative tense. Intervening text has been moved to the discussion or simplified as a note. Specifically, the long bodies of text describing statistical methods are now in the discussion section. Notes and additional comments previously in section 1 are now incorporated into the appropriate part of the discussion section.

11. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Several notes have either been moved to the discussion section or modified to be an action (see comment 10).

12. 1.7: Please specify which two pieces.

The appropriate 2 pieces are now specified in the text.

13. 3.1: Please specify the age, gender and strain of mice.

The age, genders and strain of the mice have been added.

14. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3

actions per step and a maximum of 4 sentences per step. Please move the discussion about the protocol to the Discussion.

We have extensively modified the protocol section as detailed in our responses to comments 10 and 11.

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the appropriate text for filming.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

The appropriate sub-steps for filming have been highlighted.

17. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

We have corrected the reference to include full journal titles and volume numbers. We could not include issue numbers for all references as some references do not have issue numbers.

Reviewers' comments:

Reviewer #1:

-Line 97- The OLT & NORT are identical until the test phase, when the objects are replaced/moved.

We have modified the text to say “test phase” instead of “ITI”.

-Section 1- Very well written. I would suggest presenting an alternative approach using brackets (i.e., Makerbeam) for any scientists that may not feel confident with the cement procedure. You could highlight that your procedure is the cheapest while presenting the alternatives. Also, there is no mention of any safety precautions that should be followed with the cement. Are there any requirements for protective clothing, ventillation, etc.. ?

We agree that a viable alternative to solvent welding is the more traditional use of brackets. The use of brackets, like Makerbeam, or other fasteners would result in stronger and more durable arenas. However, from our experience, the drilling of pilot holes and use of fasteners with 0.25” thick acrylic is troublesome for multiple reasons. First, the diameter and depth required for drilling pilot holes entails a level of precision that can only be obtained with a drill press. Further, with a thickness of 0.25” overtightening a fastener risks cracking the entire piece as acrylic sheet is both hard and brittle. This can be alleviated by using a thicker 0.375” acrylic sheet which we have previously used for other behavioral test equipment; however, these thicker sheets are only available in clear. Therefore, one would need to sand, prime and paint the arena to eliminate any incidental visual stimuli if one choose to use 0.375” thick acrylic. This

method requires familiarity and confidence with power tools which is not always accessible in a biology/neuroscience lab. We therefore restrict our presentation to the solvent welding technique that requires no specialized equipment or skills beyond what a typical lab would already have on hand.

-Section 3.11- Most animal facilities are now requiring sterilization with unscented bleach germicidal wipes or clidox S (or comparable) in addition to an alcohol cleaning step. I would suggest adding this into the procedure everywhere where there is an alcohol cleaning step.

We have added "Clean all arenas with animal facility recommended cleaning methods such as wiping with 70% ethanol to minimize olfactory cues before next use." to all appropriate steps in the protocol to account for different cleaning procedures.

-Section 3.12.1- Authors state objects should not be too close to the walls, but don't define the desired distance until much later in the manuscript (Fig 2 legend). I would add the 6cm x 6cm recommended spacing here.

We have added 6 cm x 6 cm to the text.

-Section 3.12.6- The authors mention the ITI can be varied, which is true. However it should be emphasized that varying this ITI changes the type of memory that is probed by the test. I.e., Short, intermediate, long, and remote memories.

We agree with this comment and have expanded our discussion of ITIs and the importance of their length in the discussion section.

-Section 3.14- The authors present the procedure as OLT and then NORT. A variation is NORT and then OLT. It would be good to suggest varying which test is done first to ensure that the order of the testing does not influence performance in either test.

We agree with this comment and have added a section in the discussion regarding the reversal of testing order or execution of the OLT and NORT separately.

-Section 4- This is extremely important for the performance of section 3. I would change the order and move Section 4 to Section 3 and vice versa.

We agree with this comment and have made the appropriate changes to the manuscript (see overall changes above).

-Section 5- Manual scoring is cheaper than an automated system, but the authors should present at least a high-level analysis of time spent analyzing data manually vs. automated systems, especially considering >=2 scientists must score the videos. I agree the up-front costs of implementing manual scoring are much less than using an automated system, but once validated, an automated system can save a significant amount of time (and money) especially when conducting large studies. Is there an inflection point where once an experiment hits a certain size and/or if a lab performs a certain number of experiments per month/year, it is more cost effective to implement an automated scoring system?

The reviewer brings up a valid point regarding upfront cost savings of manual scoring versus the relative time-savings as this cost is spread over several experiments. This is a difficult issue to address as each lab considering behavioral testing will have different needs in terms of number of experiments, number of mice, and number of people involved in execution and analysis. This difficulty is compounded by the

complicated nature of defining the cost of a researcher's time, especially since this also depends on each researcher's efficiency and training level. For example, undergraduate student researchers receiving academic credit can frequently be trained to score videos and the "cost" of their time is different than the time of a postdoctoral fellow or research scientists. To address this concern without delving too far in to the many variations entailed, we have added a statement regarding the potentially time-consuming nature of manual analysis and a recommendation that each lab should evaluate the cost of purchasing software versus the cost of the researcher's time.

-Section 5.2- How is the video calibrated such that the scientists scoring the data know when a mouse is <= 2cm from an object?

Thank you for bringing this omission to our attention. Typically, the area can be demarcated on the screen during video analysis by placing a clear sheet with a circle around each object that denotes a 2 cm boundary. We have added the appropriate text to the protocol to clarify this process.

-Section 5.3.1- While in theory, >50% indicates preference for an object, what is the typical variability of the novelty preference? Can the authors also provide guidance for a practical score indicating novelty preference that would be above noise? Can the authors perform a power analysis of the data they present in the manuscript to inform signal:noise and minimum number of subjects?

The reviewer brings up an important discussion point here that we did not address in the original manuscript. The OLT and NORT can only reveal memory in the aggregate. Any individual mouse's score cannot be interpreted as memory or lack of memory because any individual mouse could show a percent time investigating an object that varied from 50% for a variety of reasons. Memory can only be concluded by looking at a group of multiple mice and performing some kind of statistical analysis to determine whether the difference that emerged is likely to occur from chance variation. We have modified some of the data analysis section (now in the discussion primarily) to reflect this distinction more clearly.

We have also added a power analysis of the data presented in figure 3A and B to the discussion of data analysis, though we also note that the sample sizes and power will all depend heavily on effect size and variability which can vary in different sets of mice (of different ages, sexes or exposed to different treatments, for example).

As for what constitutes noise, in our re-framing of the data analysis, we emphasize that no individual data point can be interpreted alone, but rather grouped data must be used and compared statistically to determine whether memory is detected or whether differences are detected between groups. We are not aware of an agreed upon fixed level of object preference that is considered a threshold for being meaningful. We therefore restrict ourselves to providing the guidelines for finding statistically significant differences by relatively standard methods within behavioral neuroscience.

-Section 5.4.4- I agree with the point the authors are making here, but it is statistically incorrect to predicate the argument around p-values. I would stick to means and confidence intervals here and remove the references to p-values.

While we agree that reliance on p values is statistically invalid, it is the measure most commonly reported for these tasks and is even frequently required by journals to publish results of tests like these. To make our recommendations as useful and practical as possible, we would like to retain the p-value discussion, but with some added discussion of the reviewer's point.

-Line 409- Same as above- provide more guidance of what is a real signal, rather than referencing the theoretical >50% as novelty preference.

This concern is discussed above.

-Line 421- Emphasize that one cannot combine the 2 statistical approaches suggested in the figure legend.

We have added this emphasis to the figure legend as well as the results section to specify that the two statistical approaches are separate methods of analysis.

-Line 424- Authors state NORT is highly sensitive to intrinsic value of objects. Authors should indicate that OLT is also highly sensitive to objects.

This statement has been added to the discussion section where we have also expanded our discussion of object selection and validation.

-Line 511- Dimensions indicated in text are," 16m x 16m x 16 m". I think the authors meant, "16 in x 16 in x 16 in", which is closer to the size of the acrylic panels indicated in the methods.

The original text is actually correct because those dimensions refer to the size of the testing room itself, not the arenas. To clarify this, we have modified the sentence to state that the 16m refers to each dimension of the room.

-Figure 3- Any data on performance of OLT with objects that do not have equivalent intrinsic values to mice?

While we recognize that providing results for an OLT with objects of differential intrinsic values could further demonstrate the importance of object validation and selection, we have not tested objects with inherent value differences in an OLT.

-Figure 3F- Are n=4 animals enough to validate an object given the variance observed in the other panels?

We originally recommended a minimum of 4 animals for validation, requiring that any hint of object preference is carefully heeded. Our test with n = 4 revealed a subtle aversion to one object that became significant with n = 16. In retrospect, it would be more prudent test at least 8 mice to make subtle preferences/aversions more evident.

-Table 1- I would recommend adding the CleverSys system to the comparison table.

In the revised manuscript, we have omitted this table per the Editor's request to remove all specific vendors from the manuscript.

-Table 2- Stanley (SAE/metric comb square) is misspelled.

We have corrected the spelling.

Reviewer #2:

The authors are overly conservative about the brain regions involved in NLT and NOR. PFC and Hippocampus are both involved.

We agree with this comment and have made broad changes to the manuscript (see overall changes above).

Add to the discussion of the criteria to choose the test objects. Heavy, non-displaceable objects are best for NOR and NLT tasks. Avoid Wooden objects which mice give preference to. Objects that are made of Plexiglas, glass, or metals are preferred

We appreciate this suggestion and have added a section in our discussion that addresses this issue in greater detail.

The authors have should discuss criteria for excluding data, e.g. when exploration time is <5s .

We agree that this was an omission in our original manuscript. We have added criteria for excluding data in the discussion and protocol sections of the revised manuscript.

The problems with using an arena to study more than one animal at a time need further consideration. The animals can be distracted by a conspecific or if studying multiple animals at the same time adds to the noise from multiple lab personnel. Adding animals to a multianimal arena at different times can affect data collections

The reviewer raises an important point regarding multi-animal arenas. We have added this to the discussion.

Reviewer #3:

While the concept of offering an affordable alternative to commercially available behavior products is wonderful, and the authors offer ingenious method for doing so, the actual protocol for running OLT/ORT misses a few important points, especially in terms of troubleshooting or adapting the protocol for different cohorts of mice. Additionally, the authors should more explicitly refer to other protocols that go into greater detail, so that readers can figure out how to adapt the protocol to their specific needs and still be successful in running the assay (such as Ennaceur 2010 for more specifics on object selection; Ennaceur and Delacour 1988 for the original protocol, as well as the minimum exploration criterion; also Leger et al 2010 or Lueptow 2017 for further troubleshooting and overall experimental design).

We thank the reviewer for the additional references. We have expanded our discussion to be more specific with regard to troubleshooting or modifying the protocol.

The authors repeatedly state that ORT is "hippocampal-independent", which isn't exclusively true. Some researchers have found hippocampal lesions to interfere with ORT, especially when the task is shifted to encourage a more spatial strategy, such as use of spatial cues. This is an important point to make, as it is possible that in their combined protocol, which first calls for OLT and then ORT, may be encouraging a more spatial strategy, as the OLT is conducted first, and spatial cues

are used. Specifically line 100-101 should address the possible role of hippocampus in ORT (as well as in the discussion-line 482).

We agree with this comment and have made appropriate changes to the manuscript (see overall changes above).

Lines 65ff: Be careful with the use of "positive" and "negative" here in the context of reinforcement and punishment, as these terms have specific connotations within the field (i.e., positive refers to adding a motivating factor that alters the likelihood of a specific behavior, while negative removes an unpleasant factor to influence the likelihood of the behavior.) Specifically, I would try to reword parts of the paragraph to avoid the confusion (especially line 71- here MWM is relying on negative reinforcement for motivation, essentially, but say this instead of "negative stressor"; also line 76- negative and positive reinforcement)

We thank the reviewer for this suggestion and have replaced negative or positive "stressor" with "reinforcement" as appropriate.

1. Are there any cheap alternatives for a round arena? Some labs prefer to use a round arena to encourage locomotor behavior in mice that may be high anxiety, in order to discourage sitting in the corners of a square arena.

The reviewer asks an interesting question. A circular arena would have certain advantages over a rectangular open field in terms of discouraging sitting in the corners. Also, its construction would be much simpler. Unfortunately, all commercially available materials are cost prohibitive as a cast acrylic tube 18" in diameter is $630 per foot, making a 16" high round arena just under $1000 in total materials. Additionally, acrylic tubing of that size would only be available in clear. Therefore, one would need to sand, prime and paint the arena to eliminate any incidental visual stimuli, requiring specific experience with materials and construction that is not a prerequisite for the current protocol.

2. (from part 4 or line 309ff) As the authors state, running the pilot studies to discover potential object preference/aversion is very necessary. The object selection is critical to success of this assay and should be done prior to any other testing. Therefore, the section should be moved before the section on habituation, and further emphasis should be given to the importance of this step. Also, it would be helpful to either further discuss necessary considerations in object selection, or explicitly direct readers to a paper that does. One important consideration is whether or not mice can climb on the object. Using more mouse-sized objects that allow for climbing may encourage exploration, but it should be noted that time sitting on the object is NOT included in the overall exploration time (I see it's briefly mentioned in 5.2, but is worth further emphasis)

We agree with this comment and have made broad changes to the manuscript (see overall changes above). In addition, we have added exclusion criteria in the protocol and discussion for when mice climb on top of objects.

3. Objects and locations should be counterbalanced (this should be mentioned in 3.12.1)

We have added this statement to the text.

4. I'm not completely convinced that the pictured objects are the best representative objects. They are greatly different in size, and the shorter one might more greatly encourage climbing. It also

has more textural features, which could affect preference. (see Ennaceur 2010 for further discussion)

We agree with this comment and have made necessary changes to the protocol and discussion sections of the manuscript as well as Figure 1C. While we recognize the reviewer's concern regarding this object, in our experience with our mice, they have not been able to successfully climb and perch atop this object. However, this does not preclude the possibility that other labs with different types and sizes of mice may experience mice climbing atop shorter objects. To prevent this from affecting behavioral experiments, we recommend in the protocol and discussion sections thorough validation of objects with mice that are representative of the age, strain and gender to be used in actual experimentation.

5. There is no mention of a minimum criterion level for exploration of the objects. If a mouse does not sufficiently explore the object, how can you verify that any "learning" has been achieved? Generally 20s has been used as a minimum level of necessary exploration, though somewhat arbitrarily. However, some value should be defined.

We agree that this was an omission in our original manuscript. We have added a statement regarding minimum criterion for investigation of objects in the discussion and protocol sections of the revised manuscript.

6. Is there a reason to run OLT and then ORT? As mentioned earlier, perhaps the use of cues and spatial memory to train and test prior to the ORT could bias the learning or memory strategy for the ORT to be more hippocampal-dependent. Have the authors considered running ORT first and then OLT?

We agree with this concern and have added a section in the discussion regarding the reversal of testing order or execution of the OLT and NORT separately.

7. Data analysis: It should be noted that while both methods for data analysis are acceptable, if there is great variability in the exploration time between mice, it is perhaps better to control for those differences by using the discrimination index.

The data analysis section has been moved to the discussion section and greatly expanded to account for all of the reviewers' comments. Furthermore, a statement regarding the use of a discrimination index to better depict OLT and NORT data when total object investigation times vary greatly between individual mice has been added to the results section.

8. Discussion: one additional critical component of successfully running this assay is in choosing an appropriate ITI. The authors mention it briefly, but I think a slightly expanded discussion (or reference to another paper that discusses it) would be important. Someone running the test for the first time may not understand the importance of the ITI and miss the correct window to see an effect, especially if they only try one ITI in their cohort.

We agree with this comment and have expanded our discussion of ITIs and the importance of their length in the discussion section.

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