

Video Article

Novel Object Recognition Test for the Investigation of Learning and Memory in Mice

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Abstract

The object recognition test (ORT) is a commonly used behavioral assay for the investigation of various aspects of learning and memory in mice. The ORT is fairly simple and can be completed over 3 days: habituation day, training day, and testing day. During training, the mouse is allowed to explore 2 identical objects. On test day, one of the training objects is replaced with a novel object. Because mice have an innate preference for novelty, if the mouse recognizes the familiar object, it will spend most of its time at the novel object. Due to this innate preference, there is no need for positive or negative reinforcement or long training schedules. Additionally, the ORT can also be modified for numerous applications. The retention interval can be shortened to examine short-term memory, or lengthened to probe long-term memory. Pharmacological intervention can be used at various times prior to training, after training, or prior to recall to investigate different phases of learning (*i.e.*, acquisition, early or late consolidation, or recall). Overall, the ORT is a relatively low-stress, efficient test for memory in mice, and is appropriate for the detection of neuropsychological changes following pharmacological, biological, or genetic manipulations.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55718/>

Introduction

The object recognition test (ORT), also known as the novel object recognition test (NOR), is a relatively fast and efficient means for testing different phases of learning and memory in mice. It was originally described by Ennaceur and Delacour in 1988 and used primarily in rats¹; however, since then, it has been successfully adapted for use in mice^{2,3,4,5,6,7}. The test relies on as few as three sessions: one habituation session, one training session, and one test session. Training simply involves visual exploration of two identical objects, while the test session involves replacing one of the previously explored objects with a novel object. Because rodents have an innate preference for novelty, a rodent that remembers the familiar object will spend more time exploring the novel object^{7,8,9}.

The main advantage of the ORT over other rodent memory tests is that it relies on rodents' natural proclivity for exploring novelty⁸. Therefore, there is no need for numerous training sessions or any positive or negative reinforcement to motivate behavior. This means that the ORT is much less stressful, relative to other tests^{10,11,12,13,14,15}, and requires significantly less time to run than other commonly used memory tests, such as the Morris water maze or Barnes maze, which both can take up to a week or longer. Consequently, the conditions of the ORT more closely resemble those used in studying human cognition, increasing the ecological validity of the test over many other rodent memory tests. Similarly, because ORT is a simple visual recall task, it has been successfully adapted for use in numerous species, including humans and non-human primates, to assess different inter-species aspects of declarative memory^{2,16,17}. Finally, the ORT can be easily modified to examine different phases of learning and memory (*i.e.*, acquisition, consolidation, or recall), to assess different types of memory (*e.g.*, spatial memory), or to assess different retention intervals (*i.e.*, short-term vs long-term memory).

The versatility of the ORT provides a platform for innumerable research applications. Studies can make use of pharmacologic agents to either disrupt or enhance memory. Varying the time of drug administration before or after training, or prior to testing can hint at the underlying neural mechanisms that lead to disrupted or enhanced memory^{6,18,19,20}. In a similar way, optogenetic technology can be used at these same various time points to look at the neural activation/inhibition that contributes to the different phases of learning and memory. The ORT is also appropriate for assessing differences in transgenic animals, in lesion studies, or in neurodegenerative models or in aging studies^{21,22,23,24,25,26,27,28}. The time between training and testing, known as the retention interval, can be altered to assess any of these changes on short- and long- term memory²⁶. Ultimately, the ORT can be used as a tool to study pharmacological, genetic, and neurological changes to learning and memory, or these tools can be used to study the basis of learning and memory in the ORT.

Protocol

All procedures performed here were submitted to and approved by the Animal Care and Use committee and were conducted following NIH guidelines.

1. Object Selection and Experimental Setup

1. **Select objects that are different enough to be easily discriminated by mice, but have a similar degree of complexity (texture, shape, color patterning and brightness, etc.) in order to minimize any potential induced object preference that may bias the results (see Ennaceur 2010 for a comprehensive description of object choice⁷).**
 1. Test for innate preference and discrimination (see Steps 2 & 3).
 2. Use objects that are mouse-sized or only slightly larger to encourage exploration (**Figure 1**). To reduce induced preference, ensure that mice are able to climb on both objects or neither object, though ability to climb on an object may increase interest in exploration (time spent just sitting on an object is not counted towards exploration time.)
 3. Have at least 2 copies of each object. However, to minimize potential odor cues during testing, have at least 3 of each object, duplicates for training and one for testing.
 4. Use objects made of non-breakable material, as loss of an object due to damage during experimentation could interfere with the continuity of testing, and potentially cause harm or injury to the animal.
 5. Thoroughly clean the objects before and between use (70% vol/vol ethanol is appropriate).
2. To minimize the stress of bright lighting, use diffuse, low lighting, with the center of the maze illuminated around 20 lux. Use a temperature and humidity similar to regular housing conditions. If mice are being moved from a housing room to a different room for the experiment, acclimate mice to their new room for at least 1 h prior to use each day.
3. Handle mice well prior to training. Because the test relies on the natural tendency of rodents to explore novelty, reduce any stress or anxiety from handling that may interfere with their desire to explore the arena, and subsequently, the objects. Ideally, handle mice 1 - 2 times/day for at least 1 min, 1 to 2 weeks prior to testing^{15,29}.
4. **For the main arena, use a square chamber (around 40 cm x 40 cm x 40 cm) made from white or black, non-porous plastic, contrasted to the color of the mouse. Alternatively, use a round arena ed. Specifically, when using anxious mice that may sit in the corners of a square arena, a round arena may be preferable to encourage exploratory behavior^{18,30}.**
 1. On training day, place 2 identical objects on the diagonal (*i.e.* one in the NW corner and one in the SE corner). If objects are too light and can be moved by the mouse, fasten them to the floor. Removable mounting putty works well.
 2. For testing day, use the 3rd copy of each object to place one familiar object and one novel object on the same diagonal as the training day (**Figure 2**).
 3. Counterbalance the use of each set of objects so that each object is used equally as a familiar object and as a novel object. Also, counterbalance the location of the novel object to each of the 4 corners of the arena. Be sure to note which diagonal is used for which animal so that the diagonal used on the training day is the same as that used on the testing day, for each mouse.
 4. Thoroughly clean the apparatus and objects to remove odor cues before and between use (70% vol/vol ethanol is appropriate).
5. Place the camera directly overhead of the apparatus for optimal view of exploration. Only use software with nose-point detection for accurate analysis. If using software, capture a background image with the objects in place prior to starting. In the software, set the area of exploration to be approximately 2 - 3 cm around the object.
6. To reduce experimenter interference, record the trial and score it later. Blind the scorer to the experimental conditions. If manually scoring during the experiment, ensure that the experimenter is more than 1 meter from the arena, and not visible to the mouse.
NOTE: Any time the mouse spends sitting on the object without active vibrissae sweeping or sniffing does not count as exploration time.
7. Define exploration as when the mouse's nose is pointed towards the object and within 2 - 3 cm of the object, with active vibrissae sweeping or sniffing. Do not count any time sitting on the object without indication of active exploration.

2. Necessary Pilot Experiments

1. **Testing for induced preference**
 1. For habituation, remove the mouse from its home cage and place it in the middle of the open arena. Allow the mouse to freely explore for 5 min.
 2. On Training Day (T1), place 2 different objects in opposite quadrants of the apparatus, (*i.e.*, NW and SE corners). Remove the mouse from its home cage and place it in the middle of the open arena. Allow free exploration for 10 min.
 3. Calculate the discrimination index. If there is no induced preference, the discrimination index should be at or near zero. Any objects that show preference should not be used for ORT.
2. **Testing for discrimination ability**
 1. For habituation, remove mouse from its home cage and place it in the middle of the open arena. Allow the mouse to freely explore for 5 min.
 2. On Training Session (T1), place 2 identical objects in opposite quadrants. Place the mouse in the center of the arena and allow the mouse to explore for 10 min.
 3. On Testing Session (T2), place one of the object used during T1 and one novel object in opposite quadrants (*i.e.*, NW and SE corners). Sixty min after T1, place the mouse in the center of the arena and allow free exploration for 10 min.
 4. Calculate the discrimination index. At a 60 min retention interval, the discrimination index should be above 0.25.

3. Experimental Procedure

1. Habituation

1. Remove the mouse from its home cage and place it in the middle of the open, empty arena. Allow for free exploration of the arena for 5 min. Once the home cage is empty, save it for use as a holding cage the next day.
 2. At the end of 5 min, remove the mouse and place in a holding cage. Do not return the mouse to its original cage, or this may affect the behavior of the remaining mice to be tested.
 3. Thoroughly clean the apparatus between mice using 70% vol/vol ethanol.
- NOTE: During habituation, anxiety-like behavior can be assessed by calculating time spent in the center (see Prut & Belzung 2003³¹). This is a useful metric when considering the length of time for T1. Higher anxiety mice may require a 10 min session to reach the minimum exploration criterion.

2. Training (T1)

1. Place two identical objects in opposite quadrants of the arena (*i.e.*, NE corner and SW corner).
2. 24 h after habituation, remove the mouse from its home cage and place it in the center of the arena, equidistant from the 2 identical objects.
3. Allow free exploration for a minimum of 5 min. If using a strain of mice that are known to have low locomotor or exploration activity, (*i.e.*, most mice do not reach a minimum of 20 s exploration of both objects by 5 min, as noted in the pilot experiments or the literature), extend the trial to 10 min for all mice in the cohort.
4. At the end of the trial, remove the mouse and place in the holding cage. Once the home cage is empty, save it for use as the holding cage on testing day.
5. Thoroughly clean the apparatus and objects between mice using 70% vol/vol ethanol.

3. Testing (T2)

1. Place one object used during T1 (*i.e.*, the familiar object) and one novel object in opposite quadrants of the arena. Use the same locations as used during T1 for each mouse.
 2. At the T1 to T2 interval of choosing, remove mouse from its home cage and place it in the center arena, equidistant from the familiar object and the novel object.
- NOTE: At a retention interval of 24 h, most mice will not be able to discriminate between the familiar and novel object (usually $-0.2 < d2 < 0.2$, as compared to a positive control³²). If testing for nootropic effects, use this time point to probe for memory enhancement. To probe for memory deficits, use a shorter retention interval of anywhere between 20 min to 4 h, depending on the strain of mouse.
3. Allow free exploration for 10 min. At the end of the trial, remove the mouse and place in the holding cage.

4. For both T1 and T2, score the first 5 min. If the mouse does not meet the minimum exploration time of 20 s for both objects, continue scoring past 5 min until total exploration exceeds 20 s.

4. Data Analysis

1. Exclusion Criteria

1. During both training (T1) and testing (T2), calculate the total exploration time for both objects for each session ($e1$ and $e2$). Most mice should reach a minimum exploration total for both objects of 20 s by 5 min.
2. Extend T1 and T2 time to 10 min for strains of mice that have low exploration and do not meet this minimum criterion by 5 min, as observed during pilot testing.
3. Score behavior for 5 min or beyond 5 min until they reach the 20 s minimum criterion.
4. If mice do not reach a 20 s minimum of exploration for both objects for either T1 or T2 at 10 min, exclude from analysis, as it cannot be confirmed they spent enough time exploring to learn/discriminate.

2. Absolute vs Relative Analysis

NOTE: Different formulas for analysis and their relationship to one another can be seen in **Table 1**.

1. Calculate $e1$ as the total exploration time during training for 2 identical objects, where $a1$ and $a2$ are the identical objects.
 $e1 = a1 + a2$
2. Calculate $e2$ as the total exploration time during testing for the familiar object (a) and the novel object (b).
 $e2 = a+b$
3. Calculate $d1$ as simply the time spent exploring the novel object minus time spent exploring the familiar object. The absolute discrimination measure ($d1$) does not take into account differences in exploration time between mice or treatment groups, though in certain circumstances, it may be a more sensitive measure².
 $d1 = b-a$
4. Calculate $d2$ as the time spent exploring the novel object minus the time spent exploring the familiar object divided by total exploration time. The most commonly used measure is a relative discrimination value often referred to as the discrimination index ($d2$), which is not influenced by differences in exploration time. This means all values will fall between -1 and $+1$.
 $d2 = d1/e2$
5. Alternatively, calculate the recognition or preference index ($d3$)³. This is the time spent exploring the novel object divided by the total time. This means all values will fall between 0 and 1 . It is often multiplied by 100 and used as percentage value.
 $d3 = b/e2*100$

3. Statistical Analysis

1. Using the mean discrimination values for each group, determine memory performance using one-way ANOVA. For further analysis, make two-way *post hoc* comparisons with the treated vs vehicle condition and positive/negative control groups.

Representative Results

A general experimental setup for the ORT is shown in **Figure 2**. On habituation day (T0) mice are placed in the empty arena for 5 min. Twenty-four hours later, mice are placed back in the chamber with 2 identical objects and allowed to freely explore for up to 10 min (T1). On testing day (T2), the mice are again placed in the arena, but with one familiar object and one novel object, and allowed to explore for up to 10 min. The retention interval, the time between T1 and T2, can be changed, depending on the ultimate goals of the experiment. In the representative data, because the inhibitors are expected to enhance memory, the mice are tested 24 h after T1, a time in which vehicle treated mice should show no discrimination.

Phosphodiesterase 2 inhibitors have been shown to enhance learning and memory in the ORT. As compared to vehicle, administration of the PDE2 inhibitors Bay 60-7550 or ND7001 significantly enhanced memory in a dose-dependent manner, when given 30 min prior to training (T1)⁶ (**Figures 3a** and **3b**). When administered at different time-points relative to training and testing, the PDE2 inhibitor Bay 60-7550 significantly enhances memory when given 30 min prior to training, immediately after training, and 30 min prior to recall (**Figures 4a** and **4b**). This suggests that PDE2 inhibition enhances memory during acquisition or early consolidation mechanisms and during recall⁶. Mice used in this experiment were experiment naive male ICR mice, 6 - 8 weeks old.

When considering the design of the experiment, there are a number of factors that must be considered. The strain of the mouse can greatly affect both exploration time and the retention interval for positive object discrimination. Sik and colleagues analyzed a number of commonly used strains, including C57BL, Swiss, BALB/c and 129/Sv mice³⁰. They showed that there is a significant difference in total exploratory time between the strains, with Swiss and Balb/c having the highest exploratory time and C57BL and 129/sv with the lowest exploratory time. This affects the absolute discrimination value ($d1$). Additionally, the retention interval shows a significant decrease over time, with 129/Sv mice having the lowest $d2$ value of the four strains at 1 h (**Figure 5**). For the 129/Sv strain, it could be due to their low level of exploration and not necessarily their lack of recognition. It should be noted that in this study, the researchers used a shorter T1 and T2 (3 min per trial), which resulted in only two of the four strains reaching the 20 s minimum time of exploration suggested here. This minimum criterion is often considered the minimum amount of time needed to learn and explore the objects⁴. The study cited here demonstrates the importance of having such a minimum criterion.



Figure 1: Sample Objects for Use in the ORT. The objects used in Lueptow et al. are fairly simple, but have a few distinguishing features⁶. All items are slightly larger than a normal mouse and can be easily climbed on. Shown from left to right is an upside-down beer tasting glass, a toy building block with tape for texture, an ice pack, and a toy building block with a protruding eye attachment. Objects were stuck to the floor with removable mounting putty, so as not to move or tip during exploration. [Please click here to view a larger version of this figure.](#)

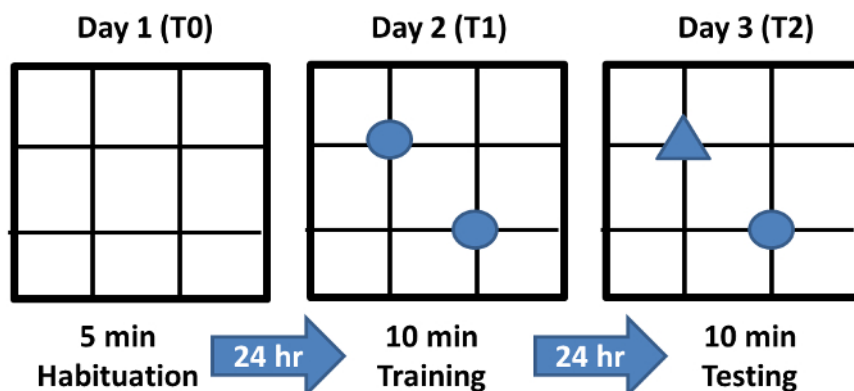


Figure 2: Experimental Setup for the ORT. The ORT takes place over 3 days. The first day is habituation (T0), in which a mouse is allowed to explore the open field for 5 min. Day 2 is training (T1), in which the mouse allowed to explore the arena with 2 identical objects placed along the diagonal. Testing (T2) takes place 24 h after T1 (this retention interval can be made shorter or longer, depending on experimental conditions). Mice are allowed to explore the arena with one of the familiar objects and one novel object, placed along the diagonal. [Please click here to view a larger version of this figure.](#)

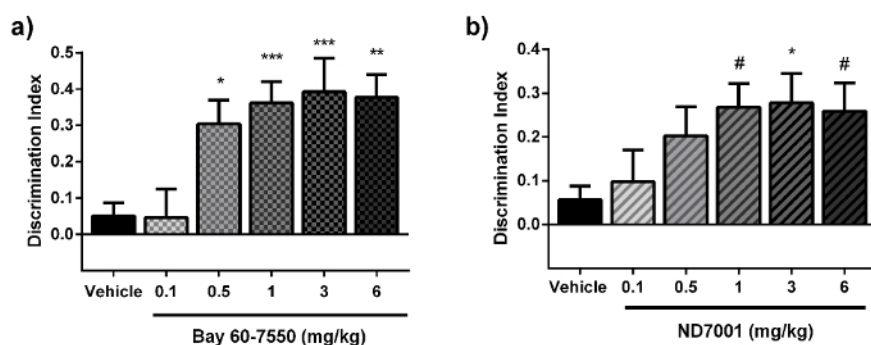


Figure 3: Dose-dependent Enhancement of Memory in the ORT. (a) Bay 607550 and (b) ND7001, when given 30 min prior to training, improved memory in a dose dependent manner when given, as seen by an increase in the discrimination index (d2). Bars represent means \pm S.E.M.; n = 10-18 per group. p = 0.05, *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle. This figure has been copied from Lueptow *et al.*, 2016⁶ with kind permission from Springer Science and Business Media. [Please click here to view a larger version of this figure.](#)

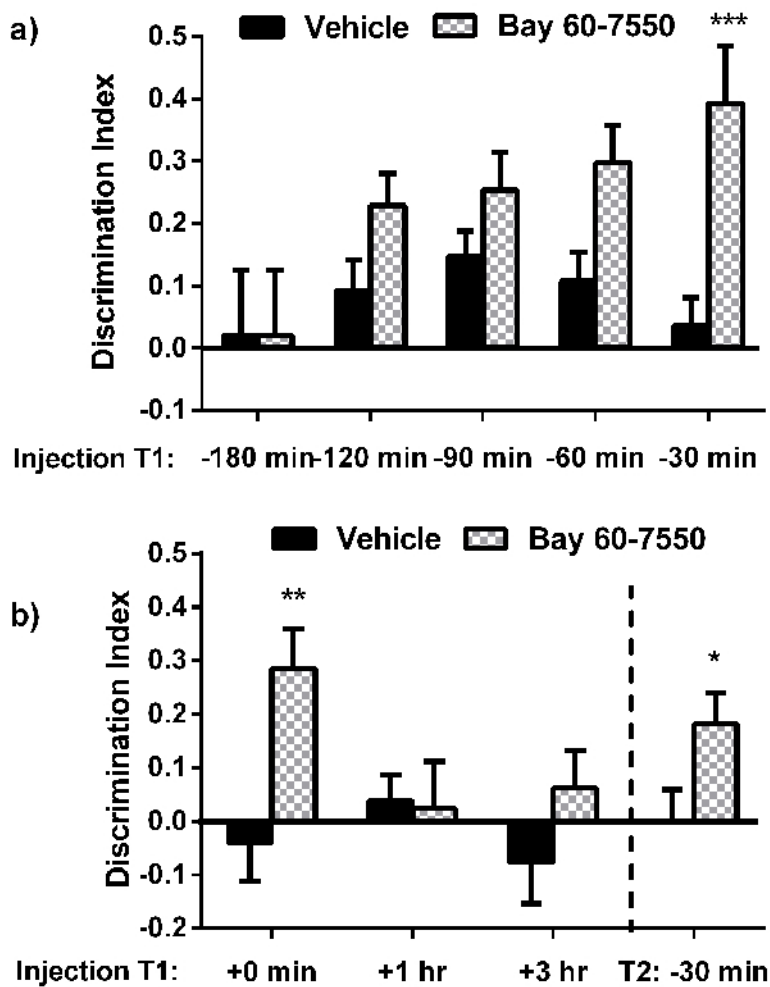


Figure 4: PDE2 Inhibition During Acquisition, Early Consolidation, or Recall Significantly Enhanced Memory in the ORT. (a) Bay 607550 (3 mg/kg) significantly enhanced memory when given 30 min prior to training (acquisition). (b) Bay 607550 (3 mg/kg) given immediately after training (consolidation) or 30 min prior to testing (recall) significantly enhanced memory, as seen by an increase in the discrimination index (d2). Bars represent mean \pm S.E.M.; n = 10 - 18 per group. *p <0.05, **p <0.01, ***p <0.001 versus vehicle. This figure has been copied from Lueptow *et al.*, 2016⁶ with kind permission from Springer Science and Business Media. [Please click here to view a larger version of this figure.](#)

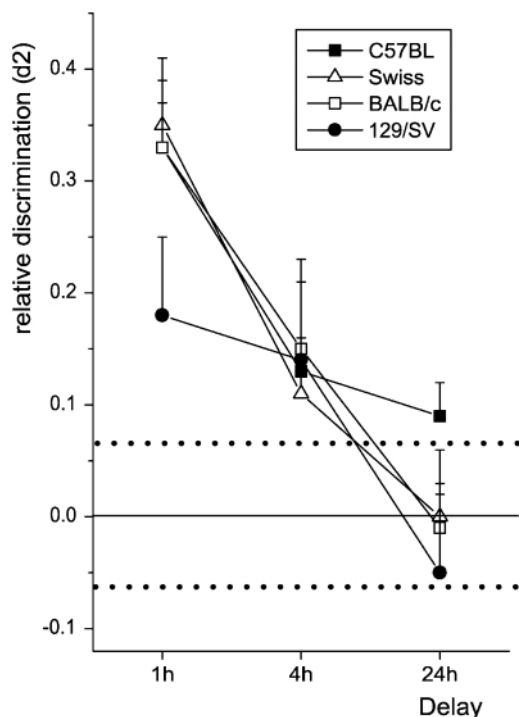


Figure 5: Strain Dependent Differences in Memory Across Retention Intervals. The performance of C57BL, Swiss, BALB/c and 129/Sv mice on the relative discrimination index (d2) in an object recognition task at different delays. All strains discriminated between the objects at the 1-h interval, but not at the 4 and 24 h interval. Values represent mean (\pm S.E.M.). Area between the dotted lines indicates the S.E.M.-range of the virtual group (mean: 0, S.E.M.: 0.06). This figure has been copied from Şık *et al.* 2003³⁰ with kind permission from Elsevier.

Exploration	Discrimination
$e1 = a1 + a2$	$d1 = b - a$
$e2 = a + b$	$d2 = d1/e2$
	$d3 = b/e2*100$

Table 1: Formula for Data Analysis in the ORT. *e1* is the total exploration time during training. *a1* and *a2* is the time at each identical object during T1. *e2* is the total exploration time during testing, where *a* is the familiar object and *b* is the novel object.

Discussion

The ORT is an efficient and flexible method for studying learning and memory in mice. When setting up an experiment, it is important to consider a number of variables that may affect the outcome. As discussed in the representative results, the strain of mouse will affect both exploration time and retention interval. A decrease in exploration time may skew or mask results in an absolute discrimination analysis^{2,3,5,30,32}. Certain strains of mice may have lower discrimination values at shorter retention intervals, such as 1 or 4 h, which could mask outcomes if looking for memory impairment. Alternatively, some strains may have high discrimination values at longer retention intervals, such as 24 h, which may mask effects of memory enhancement^{4,5,30}. In addition to strain differences, other biological factors are important to consider, including age, gender, and disease states (see references for further reading^{20,21,22,23,24}). Therefore, the retention interval must be carefully considered, and a time-course analysis is likely necessary to determine the most appropriate interval. Finally, it is also important to carefully evaluate the objects used in the assay⁷. They should be pre-tested to rule out any object preference and always used in a counterbalanced way to minimize any induced object preference.

While the ORT is fairly simple and fast to use, it does have limitations. Because there is only one training session, it is not possible to analyze potential differences in the rate of learning. However, because training and recall are only one session each, it allows for studying individual phases of learning and memory, such as consolidation or recall. Additionally, the group sizes needed to achieve statistical significance with proper power tends to be fairly high (often 15 - 20 mice/group), and often, 2 or more mice must be excluded due to lack of adequate exploration during T1, T2, or both. However, the time needed to implement the assay is quite short when compared to other tests of memory, which allows for higher throughput overall. In terms of the neurobiology that underlies ORT, unlike some of the other tests of memory, which can be clearly attributed to one brain region, ORT appears to make use of a few brain regions and neurotransmitter systems, including the hippocampus and perirhinal regions^{16,17,33,34,35,36,37,38,39}. This makes it potentially difficult to interpret, in terms of underlying neurobiology, but also offers a rich area of research for further understanding.

While running ORT, some issues may arise, such as a lack of exploration among certain strains or cohorts of mice, an innate object preference resulting in skewed performance, or a lack of effect due to an improperly chosen time point. Therefore, it is very important to run pilot

experiments to identify and correct any potential issues. Certain strains of mice have higher innate levels of anxiety, which could potentially impact locomotor activity and/or exploration time. Increasing the number of exposures or duration of exposure to the arena prior to training may help lower anxiety and encourage exploration³⁰. For a longer habituation, increase exploration to twice per day for 5 min each time (6 h interval) for 3 days. If mice are not reaching the minimum criterion by 10 min, again, mice may be anxious, in which case habituation time and/or handling should be increased for all mice. Other causes of anxiety may be from a stressor in the housing room or in the experimental room that should be addressed (noise, odors, temperature, lighting, etc.). If stress and anxiety are ruled out, mice simply may not be interested in the chosen objects, in which case new objects should be used. When assessing memory impairment, if control mice are not discriminating between the objects at the chosen interval, choose a shorter retention interval. When assessing memory enhancement, if control mice are discriminating between objects, choose a longer retention interval. If using pharmacologic agents with no effect, a time course may be used to determine if it is effective at a different stage of learning (e.g., acquisition, early or late consolidation, or recall).

The importance of running pilot experiments cannot be over stated. Improper experimental design may lead to either false-positive or false-negative results. If mice have an induced preference for a specific object, this will result in the mice spending more time at that preferred object, completely altering the paradigm and preventing the expression of learning or memory. Additionally, certain strains of mice or mice with certain genetic mutations may have diminished visual abilities, which could potentially affect discrimination ability, independent of any cognitive changes. Therefore, mice should be tested at a short-term time point (*i.e.*, 1 h or less), at which point their cognitive abilities would allow discrimination.

One common modification to the ORT is to use a novel location, rather than a novel object. This allows assessment of more spatially dependent memory. During T2, instead of replacing a familiar object with a novel object, move one of the familiar objects to a new location within the arena. This requires the addition of spatial cues around the arena. Simple, large shapes or patterns (e.g., 8" x 12" white sheet of paper with 4" thick black stripes; 8" x 12" white sheet of paper with large, black circle) should be used. If possible, use curtains to surround the maze. Curtains minimize external room cues that could change throughout the course of the experiment, and allow consistent, replicable placement of maze cues. The remaining setup and analysis is similar. All the equations for analysis can remain the same, subbing in time spent at the novel location for time spent at the novel object. Similar to the ORT, an increase in the time spent at the object in the new location relative to the object in the same location is an indication of memory.

It is important to note that the currently available software systems may not be ideal for scoring exploration. First, they do require 3-point detection (nose, body, and tail) in order to correctly identify when the nose is near the object, and not all software packages come with 3-point detection. Secondly, the mice do occasionally sit on or near the object without actively exploring the object (as noted by lack of vibrissae sweeping), and the software is not able to make such discriminations. Therefore, it is recommended to record the videos and have them hand-scored by a blinded experimenter at a later time.

The future applications of the ORT are fairly far reaching. There is ample opportunity to dissect the molecular cascades and/or neural circuitry involved in different phases of learning and memory (e.g., acquisition, early and late consolidation). It can also be used as a screen for potential nootropic drugs, or when looking for treatments for neurodegenerative disorders. It may also be useful in identifying the role of various genetic mutations on learning and memory. Because of its relative ease of use, lack of stressful conditions for the mice, and fairly short assay length, it can be a robust first step in identifying cognitive changes or a primary tool for analysis. Overall, its potential applications are numerous.

Disclosures

The author has nothing to disclose.

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