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Investigating memory updating in mice using the Objects in Updated Locations (OUL) task.

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Abstract

In the laboratory, memory is typically studied as a de novo experience, in which a naïve animal is exposed to a discrete learning event that is markedly different from its past experiences. Most real-world memories, however, are updates - modifications or additions - to existing memories. This is particularly true in the aging, experienced brain. To better understand memory updating, we have developed a new behavioral paradigm called the Objects in Updated Locations (OUL) task. OUL relies on hippocampus-dependent spatial learning and has the advantage of being able to test both the original memory and the updated information in a single test session. Further, OUL relies on incidental learning that avoids unnecessary stress that might hinder the performance of aging animals. In OUL, animals first learn the location of two identical objects in a familiar context. This memory is then updated by moving one object to a new location. Finally, to assess the animals' memory for the original and the updated information, all animals are all given a test session in which they are exposed to four copies of the object: two in the original training locations, one in the updated location, and one in a novel location. By comparing exploration of the novel location to the familiar locations, we can infer whether the animal remembers the original and updated object locations. OUL is a simple but powerful task that could provide new insights into the cellular, circuit-level, and molecular mechanisms that support memory updating.

Keywords

Object memory; reconsolidation; updating; aging; hippocampus

INTRODUCTION:

Memories are not static, fixed representations of past experiences. Instead, memories are continuously revised in the face of new, relevant information. The brain has an enormous capacity to change existing memories to incorporate new information, a process broadly termed "memory updating." The ability to update memories is critically important; as humans and other organisms rely on memory to guide behavior and anticipate future outcomes, maintaining memory relevance in the face of changing circumstances is necessary for survival. Further, most memories (including those affected in human diseases) are not brand-new experiences but are actually updates to existing memories. Despite its

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fundamental importance, memory updating is not well-characterized at a molecular, cellular, or circuit-level in the brain. Further, although memory appears to grow inflexible with age (Bizon, Foster, Alexander, & Glisky, 2012; Kwapis et al., 2019; Schoenbaum, Nugent, Saddoris, & Gallagher, 2002), the mechanisms that underlie age-related impairments in memory updating are currently unclear. To improve our mechanistic understanding of memory updating across the lifespan, we have developed a novel paradigm termed the Objects in Updated Locations (OUL) task that can assess memory updating in a simple but sensitive and powerful manner (Kwapis et al., 2019). This task is based on the object location memory (OLM) task (see Vogel-Ciernia & Wood, 2014) and therefore relies on hippocampus-dependent spatial memory. Further, as OUL is a type of incidental learning, rather than appetitive or aversive learning, it avoids unnecessary stress that might hinder performance in aging rodents. Finally, OUL is capable of assessing both the original memory and the updated information in a single test session, making it high-throughput and capable of assessing potential interactions between the original and updated information.

This unit provides a thorough explanation of the steps necessary to perform the OUL task in both young and old mice. This task should work well for rats and other rodents and could even be adapted for use in humans. The OUL procedure includes five phases: handling, habituation, training, updating and testing. Following handling and habituation to the context, mice are trained to learn the locations of two identical objects in the now-familiar context (locations A1 and A2; Fig. 1A). 24h after the final training session, mice are given an update session in which one object is moved to a new location (A3; Update group). A control group (No Update group) is again presented with the objects in the training locations. Finally, 24h after the update session, mice are exposed to four objects: two in the original training locations (A1 and A2), one in the updated location (A3), and one in a new location (A4). To assess memory for the original and updated locations, the experimenter can compare exploration of the object in the novel location A4 to the objects in locations A1, A2, and A3. As rodents have an innate preference for novelty, mice that remember the original and updated locations will preferentially explore the object in a new location relative to a location they remember from the training or updating session. The researcher can therefore calculate a discrimination index for objects A1, A2, and A3 that can be compared across experimental conditions to determine whether memory for the original information (objects A1 and A2) or the updated information (A3) is affected by the experimental manipulation.

BASIC PROTOCOL 1

BASIC PROTOCOL TITLE

Objects in Updated Locations (OUL)

Materials:

70% (v/v) ethanol

10% (v/v) ethanol

Subject mice: e.g., C57BL/6J, aged 2-6 months (young) or 18-20 months (old)

Marking pen (e.g. Securline surgical skin marker)

Isolated experimental room outside of the colony room

Overhead lamps

LUX meter (e.g. URCERI MT-912 from Amazon)

Empty holding cage

OUL testing chamber: white rectangular open field 60.96 x 45.72 x 26.67-cm (24 x 18 x 10.5-inches, See Fig. 1B) with vertical black marking strip (recommended material for chambers: opaque white Plexiglas)

Automated video recording and tracking system (e.g. Ethovision)

Computer capable of running video acquisition system (e.g. Dell Precision T3620 with graphics card)

Camera-mounting bracket to allow camera to face straight down

Video cables and adapters as needed to run video equipment

Handling sleeve (e.g. Fisher Scientific #19-170-904)

Paper towels

Stopwatches without beepers

200-mL tall-form beakers (e.g. Pyrex 1060-200) filled with hydraulic cement (four per chamber) Two (or more) gooseneck lamps with incandescent 60W light bulbs or equivalent LED bulbs

NOTE: All protocols involving live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow regulations for the care and use of laboratory animals.

Protocol steps — Step annotations:

Animal preparation

1. Purchase animals in advance so they are delivered at least one week before beginning the procedure to allow the animals time to recover from the shipping stress. Transfer animals to their standard housing cages. Group housing (two to four mice per cage) or single housing can be used. Avoid combining male mice from separate shipping containers, as this can produce fighting.

   This protocol can be used with young mice (8 weeks to 6 months old) or modified for use with old mice (18-20 months old). We have successfully used both male and female mice in this task and have observed similar performance for both sexes.

2. Prepare a detailed experimental plan with all of the relevant information, including animal number, treatment group, tail marking information, chamber number, and location of the updated object. Counterbalance the update location (left or right) and the box assignment across all genotypes, treatment and sex.

3. If using group housed mice, mark the tail of each animal to easily distinguish between mice.

   This step is essential, as it allows the experimenter to rapidly identify and place each animal in the correct chamber throughout each phase of the experiment. If markings fade, make sure to remark after the last day of habituation. As the process can be stressful, it should be avoided on training, updating and testing days. The experimenters conducting the procedure should be able to identify mice while remaining blind to the animals' experimental group assignments.

Room preparation

4. Behavioral experiments should be conducted in a quiet room, separate from the main housing room. The behavior room should also be separated from any areas with odors or sound, such as surgical areas and the main colony room.

   Either nitrile or latex gloves should be worn at all times when handling animals, objects, or cleaning the arenas. The type of glove used (nitrile, latex, etc.) should remain consistent throughout the experiment.

5. Illuminate the OUL chambers using a set of overhead, gooseneck lamps.

   For this learning task, standard fluorescent ceiling lights are too bright. Adjust the lighting on the overhead lamps until the light meter reading taken from the floor of the testing chamber(s) is between 16 and 20 LUX. Pointing the lamps at the ceiling produces diffuse, weak lighting ideal for this task.

6. Set up recording camera, computer and software according to the manufacturer's instructions

   Recording software must allow for live tracking of animal's movement during each session for habituation and to record videos for future offline analysis of training, updating, and testing sessions.

Testing arena preparation

7. Attach a marking strip in the middle of one wall of each chamber to help the animals orient within the context (see Fig. 1B,C). Keep this marking strip in the same location throughout the experiment.

8. Ensure that area surrounding the testing arenas have sufficient orientation cues. If putting a curtain around chambers, provide extra-maze cues such as cut-out shapes to help the animal orient within the room.

9. Mark floor of each chamber with a dark marking pen to indicate the predetermined positions of the objects (see Fig. 1B). Marking the bottom side of the floor is preferable so that the mark is less visible to the mouse and will not wipe off when the chambers are cleaned.

10. Clean the chambers with 70% ethanol the day before an experiment and allow arenas to air dry overnight. No bedding is used in the testing chambers in this protocol. During the experiment, chambers are cleaned with 10% ethanol between animals.

Phase 1: Handling the animals:

CAUTION: The overhead room lights should be on for handling procedures.

11. Place a handling sleeve on one arm, covering the area from the wrist to the elbow.

    Both the gloves and the sleeve should be cleaned with 70% ethanol before handling each animal.

12. If rodents are group housed, set out an empty cage to hold animals after handling.

13. Transport animals in their home cages from colony room to experimental room on a cart.

    The ride should be made as smooth and quiet as possible by pushing the cart slowly and carefully. Shielding the cages with a light-blocking curtain is optional. Covering the cart can help calm anxious mice and may be necessary if running behavior during the dark cycle to avoid light cues.

14. Remove lid and water/feeding apparatus from the cage to allow access to the mice.

15. Pick up each mouse grabbing its tail and immediately setting it on the handling sleeve.

    Mice should be handled gently but confidently. Do not chase mice around with your hand. Practice until you can quickly and reliably grab the tail. Never dangle the mouse by its tail, which causes stress and may trigger biting.

    Once on the sleeve, allow each mouse to explore while gently holding the tail. During the first handling, mice may try to jump from your arm, so it is important to maintain a firm but gentle grip on the tail.

16. Hold each mouse for 2 min and then place it into the empty holding cage (if group housed) or back into its home cage (if individually housed).

17. Repeat the process until all mice in a cage are handled. Return mice from the holding cage back into their home cage and replace on the cart. Repeat for all mice in a given experiment.

18. This handling procedure should be repeated once a day for 4 days.

    Take care to perform the experiment at the same time each day and to counterbalance the order of each condition to avoid circadian

confounds. All experimenters that will be habituating, training, updating, and testing mice should participate in handling, as the experimenters should remain constant throughout the experiment.

Urination and defecation typically occur the first few days of handling. This fear response should diminish by the third day of handling as mice become more comfortable with being picked up and transported. As mice become more relaxed, it is possible to handle two mice at once by grabbing the tail and turning the mouse onto the palm of your hand. The mouse should remain on your hand and it is no longer necessary to continue holding the tail.

Phase II: Habituation to the context

19. Prepare the behavior room by turning off the overhead lights and turning on the gooseneck lamps above the chambers. Adjust the lighting to approximately 16 to 20 Lux (as measured from the floor of the testing chambers. Ensure that chambers are evenly lit and centered under the video camera. Make sure the image is focused and clear.

20. Prepare the chamber(s) by cleaning each with 10% (v/v) ethanol (in water)

An experimenter may use one behavioral chamber or multiple chambers (up to four) at once. Each mouse is placed in a separate chamber and this chamber assignment must remain the same for the duration of the experiment.

21. Transport the animals to the experimental room in their home cages on a cart

High-anxiety mice can be placed in the room an hour prior to handling to reduce stress. Mice that fail to habituate to the context across days, show high levels of urination or defecation in the context, or excessively avoid being picked up may benefit from this additional resting period.

22. Prepare the first cage (or cages) containing the mice designated for the first round of habituation. Place the cage on a holding table near the chambers and remove the food, water, and lid to allow for easy access to animals. The rest of the cages should remain on the cart until they are ready to be habituated.

23. Transfer the first round of mice from their home cages to the correct chambers.

When running multiple chambers at once, it is most efficient to pick up and transfer two mice at once.

When placing mice in the chamber, it is best to lower your hand to the floor and tilt your hand to encourage the mouse to step from your hand into the chamber. This avoids unnecessary stress. Mice should be placed in the center of each chamber.

24. Start the video recording and live-tracking software. If necessary, start a stopwatch to time the session.

25. Allow the mice to explore the chambers for 5 min while recording.

    Experimenters must remain quiet during this time to avoid disturbing the animals.

26. Remove the mice and place them back into their home cages.

    To remove the mouse, gently grab its tail and turn it into your hand. It is often helpful in these large chambers to place one hand in front of the mouse to draw its interest and quickly grasp its tail with the opposite hand.

    If single housed, return the mouse to its home cage. If group housed, have a holding cage (typically a clean, empty home cage with a lid) prepared if additional mice in the cage are unhabituated. The holding cage can be placed on a table in a quiet spot within the experimental room, ideally near the cages of animals that have already been trained. Do not mix habituated and unhabituated animals.

27. Remove any feces and clean the chambers with 10% (v/v) ethanol before the next group.

28. When all groups have been habituated, clean chambers with 70% (v/v) ethanol and allow to air dry overnight.

29. Repeat habituation once daily for 6 days.

    The 6-day habituation protocol is based on previous work in both OLM (Vogel-Ciernia, 2014) and OUL (Kwapis et al., 2019). During habituation, the speed and distance traveled should be analyzed each day to ensure that movement reduces over the first few days but stabilizes at a low level by the final day of habituation. We strongly recommend tracking movement during habituation both to ensure sufficient habituation and to assess potential movement differences between experimental conditions.

    The last 2 days of handling can overlap with the first 2 days of habituation. On days when animals are scheduled for both handling and habituation, all mice should be handled first and then all animals should be habituated.

    If mice will be receiving intraperitoneal or subcutaneous injections during the experiment, they should also be habituated to the restraint and injection procedure. In this case, we recommend that each animal is transported to the injection site and briefly scruffed to mimic the injection procedure following the final 3-4 days of habituation.

Phase III: Training the animals

30. Prepare the experimental room exactly as in habituation. Adjust the lighting, clean chambers with 10% ethanol, and prepare the video recording setup.

31. Transport the animals in their home cages to the experimental room on a cart as before.

To reduce anxiety, mice can be placed in the experimental room on the transportation cart up to an hour prior to training. We recommend giving a rest period of at least 30-min to 1h before beginning training. The cart can be placed in a holding room near the experimental room or in the experimental room itself as long as the cart is far enough away from the training setup to not disturb the animals being trained. If using a holding room, ensure that it is isolated and quiet, close to the experimental room, and that the lighting conditions are similar to those in the experimental room.

32. Clean the training objects and chambers with 10% ethanol using paper towels. Dry the objects with a clean paper towel (or allow to air dry) before placing them in the chamber.

For OUL, we use 200-mL tall-form beakers filled with cement. The objects are presented upside down, all facing the same direction. The cement is important as it prevents the mice from knocking the objects over or moving them.

33. Place the objects in the correct spots in each chamber. For training, object locations are identical in all chambers, with objects placed in the top two locations (closest to the marking strip). Make sure to place objects directly over the premade marks on the bottom on the chamber (see Figure 1B, 1C).

34. Open the video recording software. Prepare the file you will be using for the experiment with significant components: date, experiment number, and all other identifying information in the file name.

It is important to record the full session length for training, updating and testing, as these videos will be used to score object exploration from the moment each mouse is placed in the chamber. To ensure the entire session is recorded, the experimenter should begin recording before the first animal is placed in the chamber and an additional ~30s should be added to the recording duration to allow enough time to transfer all mice to their chambers.

We recommend using 1-3 10-minute training sessions for OUL. A single 10-min training session consistently produces observable long-term memory in a young mouse (Stefanko et al., 2009, Kwapis et al., 2019). For older mice that have age-related memory impairments (e.g. at 18 months old), a single 10-min training session is not sufficient to produce robust long-term memory, however, and 3 daily 10-min training sessions is recommended to produce memory for the original training locations. In order to make direct comparisons with young mice, the researcher may choose to use the 3 x 10-min training protocol in young mice, as well, which also works well in the OUL paradigm.

35. Check video feed to make sure that the image is focused and clear.

    It is important that both the animals and the objects are easy to see. Additionally, you need to be able to clearly see the animal's nose as it will be important for analysis later on.

36. Place the first cage (or cages) on the holding table near the chambers and remove the food, water, and lid to allow easy access to animals. As before, the additional cages should remain on the cart.

37. Start the video recording.

38. Place mice in the chambers as described in habituation. Take care to place mice in the center of each chamber, away from the training objects.

    Placing the mice away from the training objects will eliminate the chance of object bias that could occur if the animal is placed in front of one object or the other.

    Animals should explore the chamber and spend approximately the same amount of time exploring each object.

39. Start a stopwatch if necessary and allow the mice to explore the chamber for the appropriate amount of time while the video is recording.

    Experimenter(s) must remain quiet during this time to avoid disturbing the animals.

40. When the time has expired, remove each mouse as during habituation and place back into the home cage or, if group housed, place into an empty holding cage until all mice in the cage have been trained.

    Do not mix trained and untrained animals together.

41. Clean both the objects and the chamber(s) with 10% ethanol and dry them with a clean paper towel.

    Remove any feces in the chamber before wiping down with 10% ethanol.

42. Place objects back into chamber(s) in preparation for the next animal

43. Repeat the training process for all animals. Return the animals back to their colony room on the cart when all groups are trained.

44. Clean the chambers and objects with 70% ethanol after completion. Allow them to air dry overnight.

Phase IV: Updating the animals

45. Prepare the experimental room exactly as for training and habituation. Clean both the objects and the chambers with 10% ethanol as described for training. If necessary, adjust the lighting and video recording setup to ensure a clear picture.

46. Transport animals in their home cages to the experimental or holding room on a cart. Leave home cages on the cart until it is used in testing.

As with training, we recommend a rest period of 30-min to 1h before training.

47. Place objects in chambers according to your pre-planned Excel sheet. Make sure to place objects directly over the premade marks on the bottom on the chamber (see Fig. 1B, 1C)

For mice in the No Update control condition, the beakers should remain at the same locations used during training. For mice in the Update condition, one beaker should remain in the training location and the other should be moved to a new location at the bottom right or bottom left of the box (see Fig. 1A). The novel location should be counterbalanced across experimental conditions and across chambers.

48. Prepare the video recording software and associated data file as before.

Set the video with additional time to account for time needed to transport mice into the chambers.

We recommend an update session duration of 5 minutes, which is sufficient to update the existing object location memory in young mice. Aging mice (18 months old) show updating impairments at this duration, allowing the experimenter to investigate age-related impairments in memory updating. Longer and shorter times have not yet been tested but a shorter update session could potentially be used to create a “subthreshold” update that is not sufficient to update memory on its own. A longer update session could potentially be used to create a stronger and more persistent memory for the updated information.

49. Move the first cage(s) to the holding table near the chambers and remove lid, water, and food from the cage for easier access to the mice.

50. Start the video recording.

51. Place mice in the chambers as described in habituation and training. Take care to place mice in the center of each chamber, away from the objects.

52. Allow the mice to explore the chamber for the designated time while the video records.

It is important for the experimenter(s) to remain quiet during this time.

53. Remove the mice and place into the home cage or holding cage as described for training.

Do not mix mice that have received the update session with those that have not yet been updated.

During the OUL task, it is critical that manipulations affect the memory updating phase without affecting the original training phase. It is

therefore recommended that manipulations are applied at least 24h after the final training session to avoid affecting the consolidation of the original memory. Manipulations should be fast-acting so that they can be applied either just before or, ideally, just after the update session.

54. Clean both the objects and the chamber(s) with 10% ethanol. Allow them to air dry.

Remove any feces in the chamber before wiping down with 10% ethanol.

55. Place objects back into chamber(s) in preparation for the next animal. Make sure to place objects in the correct locations according to the predetermined Excel sheet.

56. Repeat the updating process for all animals. Return the animals back to their colony room after all updating is complete.

If the experimenter will be giving a post-update injection, it is best to do this immediately after the update session for each group. In this case, immediately after updating, cart each group to the injection room and administer the injection before returning the home cage(s) to the colony.

57. Clean the chambers and objects with 70% ethanol after completion. Allow them to air dry overnight.

Phase V: Testing the animals

58. Prepare the experimental room exactly as described in training and updating. Clean the chambers and objects with 10% ethanol.

59. Transport animals in their home cages to the experimental or holding room on a cart as before.

As with training, we recommend a rest period of 30-min to 1h before training.

60. Place the objects in the chambers. For the testing phase, each chamber gets four objects, one in each pre-marked location (see Fig. 1).

61. Open the video recording software. Prepare the file you will be using for the experiment with all relevant information as before.

The testing duration is 5 min. Set the video to record for additional time (~30s) to account for time it will take to transport mice into the chambers.

62. Remove lid and water/feeding apparatus from the cage for easier access to the mice.

63. Start video recording

64. Place mice in the chambers as described previously.

65. Allow the mice to explore the chamber for the allotted time while the video is recording.

    Experimenter(s) must remain quiet during this time to avoid disturbing the animals.

66. Remove the mice as previously described and transport each back to its home cage if single housed. If group-housed, have an empty holding cage available as needed.

    Do not mix tested and untested animals together.

67. Clean all objects and the chamber(s) with 10% ethanol. Dry them with a clean paper towel.

    Remove any feces in the chamber before wiping down with 10% ethanol.

68. Place objects back into chamber(s) in preparation for the next animal.

69. Repeat the testing process for all animals. Return the animals back to their colony room on the cart.

70. Clean the chambers and objects with 70% ethanol after completion. Allow them to air dry before the next use.

Collect the data

71. Movement, speed, and distance during the habituation sessions can be exported directly from the automated tracking system. This can be analyzed in Excel to ensure normal habituation for all groups.

72. Training, updating, and testing data should be analyzed offline using a computer that allows you to play the recorded videos.

    If using a computer for hand scoring, a keyboard with a laptop/short keypad (e.g. an Apple keyboard or laptop keyboard) is required for accurate scoring. A previously published MATLAB code (Vogel-Ciernia, 2014) can be used for scoring OUL. Alternatively, you can use two stopwatches to record the amount of exploration of two objects within a given session. For test sessions, which have four objects, each chamber will need to be scored twice: once for the top two objects and once for the bottom two objects.

    Although you can use an automated program for scoring object exploration, such as Ethovision or AnyMaze, we have found that this results in greater variability. Our best automated scores were obtained by drawing a “donut” around the object (covering approximately the outer quarter of the object (~1.5cm) and an equivalent amount of space surrounding the object) and only counting time when the animal’s nose was inside this ring and pointing to the center of the circle.

73. To accurately, reliably measure object exploration, we follow strict guidelines as previously described (Vogel-Ciernia & Wood, 2014). Behavior is only counted as exploration when the mouse's nose is within 1cm of the object, pointed directly at that object. We do not count the following behaviors as exploration:

- **i.** When the mouse rears or jumps onto the object.
- **ii.** When the mouse bites the object.
- **iii.** When the mouse bumps into the object
- **iv.** When the mouse engages in grooming or other repetitive behavior near the object.

Scoring Considerations: Scoring is a skill that requires practice. We have provided a sample OUL test video scored by three trained observers to serve as a practice video. We recommend practicing with this video until your scores reliably match the provided scores before scoring videos from your experiment. Table 1 shows the raw object location scores for each the four animals in the provided test session. Table 2 shows these scores organized into categories (e.g. the original object (A_1), the updated object (A_3), etc.) to allow for DI calculations). Fig. 4 illustrates the object identities when the left object was moved during the update, the right object was moved, or neither object was moved (the No Update condition).

All scoring should be conducted by an experimenter blind to the experimental conditions. Objects should be scored as Top Left, Top Right, Bottom Left, and Bottom Right during scoring and then reorganized based on the object's identity (A_1, A_2, A_3, and A_4) when scoring is complete (to avoid biasing the results). Fig. 4 illustrates each object's identity based on whether the right object, left object, or neither object was moved during the update session.

All training, updating, and testing videos should be scored for object exploration. Exclude young animals that do not explore more than 3 seconds total for both objects or old animals that do not explore more than 2 seconds during training, updating, or testing. Additionally, exclude any animals that have a discrimination index (DI) ±20 during training as this indicates a location/object bias during training.

The discrimination index (DI) is calculated for locations A_1, A_2, and A_3 by the formula: (time exploring the novel location – time exploring the familiar location)/(time exploring novel + time exploring familiar) * 100 (e.g. $(A_4-A_1)/(A_4+A_1) * 100$). Separate DIs should be calculated for objects A_1, A_2, and A_3 by comparing exploration of each object location to the novel location A_4. For No Update mice, as locations A_3 and A_4 are equally novel, the experimenter should choose one location (A_3 or A_4) to serve as the novel location for calculating the DIs, making sure to counterbalance this choice across animals and conditions.

For the test session, each animal should therefore have three separate DIs: A_4-A_1, A_4-A_2, and A_4-A_3 to assess memory for the original (A_1 and A_2) and updated (A_3) locations.

A DI of approximately zero indicates an equal preference for the objects. Memory for an object location is indicated by a DI from ~20 to 45. Total exploration (objects $A_1 + A_2 + A_3 + A_4$) typically ranges from 3s-6s during the 5-min test.

COMMENTARY

BACKGROUND INFORMATION:

Memories are dynamic and amendable, not stable records of experience. For memory to be useful, it needs to be continuously updated and edited in response to new information, allowing an organism to anticipate future outcomes based on accurate information about the past. Understanding how memories are updated in response to new, relevant information is therefore a critically important but understudied problem in neuroscience. We have developed the Objects in Updated Locations (OUL) paradigm to better understand the molecular, cellular, and circuit-level mechanisms that support this process of memory updating in rodents.

Memories that have been stabilized through the process of consolidation are resistant to amnesic agents like protein synthesis inhibitors and were therefore initially believed to be fixed, unchangeable entities. More recent work, however, has demonstrated that memories can be modified through a process termed “reconsolidation” (Nader, Schafe, & Le Doux, 2000). During the putative reconsolidation process, retrieving a stored memory induces a transient period of lability, during which the memory is again sensitive to amnesic agents until it restabilizes. The initial empirical basis for this claim appeared when Misanin et al. (1968) reported that electroconvulsive shock (ECS) resulted in retrograde amnesia for fear memory immediately after a brief retrieval event, while ECS in the absence of retrieval had no effect on the memory. This idea became mainstream in 2000 when Nader and colleagues demonstrated that local blockade of protein synthesis in the basolateral amygdala could similarly disrupt a stored fear memory (Nader et al., 2000). Since this re-discovery, reconsolidation has received increasing attention as a possible way to disrupt or manipulate established memories that have become problematic, for example in post-traumatic stress disorder.

Recent work has indicated that the reconsolidation process functions, in part, to allow existing memories to be updated with new information. Consistent with this, exposure to new information is necessary for the reconsolidation process to be initiated; when retrieval contains only familiar information, the original memory remains stable (Diaz-Mataix, Ruiz Martinez, Schafe, LeDoux, & Doyere, 2013; Jarome, Ferrara, Kwapis, & Helmstetter, 2015; Kwapis et al., 2019; Kwapis, Jarome, Ferrara, & Helmstetter, 2017; Sevenster, Beckers, & Kindt, 2012). Thus, exposure to novel information may catalyze the reconsolidation process, making the original memory labile to allow the new information to be incorporated. A number of studies now show that the new information presented at retrieval can fundamentally alter the content of the original memory (Jarome et al., 2015; Kwapis et al., 2019; Kwapis et al., 2017; Lee, 2010) and this updating process has been leveraged to modify the emotional tone of a memory (Cogan, Shapses, Robinson, & Tronson, 2018; Goltseker, Levi, & Barak, 2016; Haubrich et al., 2015; Zeng et al., 2014), improve extinction (Clem & Huganir, 2010; Monfils, Cowansage, Klann, & LeDoux, 2009; Schiller et al.,

2010), or even alter the neural circuitry that supports a memory (Kwapis et al., 2017; Winters, Tucci, Jacklin, Reid, & Newsome, 2011).

Although it has become increasingly clear that reconsolidation allows memories to be updated, the molecular mechanisms that support this process remain largely uncharacterized. In particular, it is critical to understand how the mechanisms that support memory updating are unique from those underlying initial memory formations. Most of the research on the reconsolidation process has relied on fear memories, which are rapidly acquired, robust, and persistent. However, fear memories are disadvantageous for understanding memory updating for several reasons. First, fear memories are extremely strong and often resist modification, resulting in boundary conditions (Suzuki et al., 2004; Wang, de Oliveira Alvares, & Nader, 2009; Winters, Tucci, & DaCosta-Furtado, 2009), in which the original memory resists destabilization. Second, fear conditioning does not allow for simultaneous assessment of both the original and the updated information at test. Typically, rodents will freeze for the duration of the conditional stimulus (CS), making it difficult to behaviorally distinguish between freezing to the original information and freezing to the updated information; the freezing can reflect either the original memory or the updated information. Finally, as fear conditioning is an aversive and stressful task, it may not reflect the type of memories that occur in typical, everyday life and are often affected during the normal aging process. Thus, we developed the OUL task to address these shortcomings. OUL is a novel hippocampus-dependent task that is non-stressful and can assess both the original and updated information in a single test session. This task is therefore ideal for characterizing the molecular, cellular, and circuit-level mechanisms that support memory updating in both young and old mice. As the only difference between the initial training and the update session is the placement of one of the objects, OUL also allows the researcher to directly compare the mechanisms necessary for memory updating to those required for initial memory formation.

We have shown that OUL is hippocampus-dependent and, like object location memory, is acutely sensitive to manipulations in dorsal CA1 (Kwapis et al., 2019). We have also confirmed that the update session in OUL engages the original memory, rather than forming a new, discrete memory (Kwapis et al., 2019). First, we demonstrated that post-update anisomycin infusion into the dorsal hippocampus impairs memory for the both the update location and the original training object locations, indicating that the original memory is destabilized in response to the update session. Second, we used *Arc* catFISH (cellular compartment analysis of temporal activity by fluorescence in situ hybridization) to show that the original memory and the updated information activate a largely overlapping neuronal population in area CA1 of the hippocampus, suggesting that the neurons supporting the original memory are re-activated in response to the updated information. Finally, we have also used OUL to demonstrate that memory updating is impaired with age; 18-month-old mice show severe deficits in learning the update despite showing intact memory for the original training locations (Kwapis et al., 2019). OUL is therefore a simple but powerful and sensitive task that is can be used to better understand the mechanisms that underlie memory updating.

CRITICAL PARAMETERS:

General guidelines for behavior

As OUL relies on incidental learning (rather than motivated learning), any manipulation that affects the health, well-being, or comfort of the animals can drastically impair their ability to learn or update the memory. Rodents that are not well-habituated to the experimenter, the handling procedure, or the context itself often fail to sufficiently explore the objects, confounding any results. As unnecessary stress can drastically impact exploration, consistency is critical and mice should be familiar with all procedures (e.g. transportation, handling, injections, etc.) before training. The critical parameters that exist for object location memory, which have been thoroughly reviewed (Vogel-Ciernia & Wood, 2014), also apply to OUL. Briefly, in addition to reducing stress levels, the researcher should also ensure the animals are well-habituated, assess potential performance confounds like changes in anxiety, and should carefully consider the age, sex and strain of mice used in the task. While we have not observed sex difference in OUL performance to date, future experiments using this paradigm should continue to use both male and female mice to determine whether the neurobiological underpinnings of memory updating are different in males and females.

As the OUL box is larger than a chamber typically used for object location memory, it is important to track how each group's movement changes across days to ensure that the animals sufficiently habituated to the context before training. Well-habituated mice will show decreased locomotion across habituation sessions, reaching a stable, low level of movement over the final few days of habituation. If the animals do not show this consistently reduced movement by the end of the planned habituation period, the number of habituation days can be increased for all groups to ensure that all animals have sufficiently habituated to the context before training.

Performance confounds should also be ruled out. Performance confounds include manipulations or genotypes that affect the animals' activity or anxiety levels in the task. Researchers should rule out differences in movement by assessing the speed and distance traveled during habituation to ensure that general movement is similar across groups. Similarly, the researcher should also test performance in a task such as the elevated plus maze to measure anxiety-like behavior, as group differences in anxiety might confound any observed effects in OUL. Finally, different strains of rodents might show different performance levels on OUL. Although OUL has only been tested on C57Bl/6J mice to date, strain differences have been observed on the similar object location memory task (see Vogel-Ciernia & Wood, 2014). It is therefore important to adjust the parameters of the training and update sessions to ensure that young wildtype animals of the desired strain can learn both the original and the updated information.

In addition to the critical parameters identified for object location memory (Vogel-Ciernia & Wood, 2014), OUL has an additional consideration: in order to make conclusions about memory updating, it is essential that the animals successfully acquire the original object location memory. Without an intact original memory, the memory update itself cannot be interpreted. The researcher can assess whether the original memory has been acquired by measuring object preference during the update session, in which one object is moved to a

new location. If mice remember the original training locations, they should preferentially explore the moved object. Although young C57BL/6J mice reliably show robust long-term memory following a 10-minute object location memory training session (Kwapis et al., 2019; McQuown & Wood, 2011; Vogel-Ciernia et al., 2013; Vogel-Ciernia & Wood, 2014), other strains and ages may need more training. For example, we have previously shown that object location memory is impaired in 18-month-old C57BL/6J mice (Kwapis et al., 2018). In order to study memory updating in these mice, we increased the amount of training from one 10-min session to three 10-min sessions (one session per day for 3d) and verified that old mice successfully learn the original object locations under this increased training protocol (Kwapis et al., 2019). If necessary for a particular age or strain, the training protocol could be additionally modified by lengthening the training session or increasing the number of training sessions to ensure acquisition of the training information.

Object choice

As OUL relies on rodents' innate preference for novelty, it is important to choose objects that are neutral but identical and easily replaced. The researcher should avoid objects that evoke a fear response or are particularly salient to the organism. Objects should be screened before use to ensure that animals show adequate exploration of the object (at least 3s per object) over a 10-minute session. We recommend using 200mL tall form glass beakers filled with cement (to prevent rodents from moving the objects during the session). These beakers are tall, which discourages mounting, are easy to clean, and can be ordered from numerous general lab suppliers. It is important that the object chosen in cheap and readily available, as you will need four copies of the object for each box (16 total copies for a typical 4-chamber experiment), in addition to several backup objects, in case one of the objects is damaged or broken during the experiment.

Session length and number

In OUL, there are three phases (training, updating, and testing) that can be adjusted in length or number to produce optimal performance for the desired experiment. The session parameters presented here were chosen based on the performance of 3-month-old and 18-month-old C57BL/6J mice and may need to be adjusted to be compatible with other strains and ages. Additionally, parameters should be chosen based on experimental demands, including the timing requirements necessary for the intended manipulation.

For training, we typically use three 10-minute sessions (one per day across three days) to ensure that all animals learn the original object locations. Young mice readily learn the object locations with a single 10-minute training session, but old mice (18-m.o.) need additional training (3 sessions, 10-min each) to form long-term memory for the original locations, as discussed above. Importantly, young mice show successful memory updating regardless of whether one or three training sessions is used (Kwapis et al., 2019). Thus, if the experiment aims to directly compare young and old mice, the stronger 3d training protocol should be used for both ages. If the intended strain or age fails to show long-term memory for the original training locations during either the update session (mice should prefer the moved object) or the test session, the researcher can increase the amount of training (lengthen each session or provide more sessions) to ensure the animals learn the

original configuration. Additionally, if using a manipulation with a slow onset (for example, an AAV or other virus that takes 2 weeks to fully express), the researcher may need to significantly increase the amount of training to ensure that the original memory lasts long enough to be updated in the presence of the manipulation. Preliminary work from our laboratory has indicated that 3 days of training with 10-minute sessions produces an object location memory that lasts at least 5 days in both young and old C57BL/6J mice (unpublished observations), but we have not tested more remote timepoints. Whenever possible, we recommend using fast-onset manipulations, including fast-acting viruses (e.g. HSV) and pharmaceutical manipulations to target the update session.

For updating, we chose a 5-minute session, as we reasoned that updating should happen more rapidly than initial training. To encourage updating of the original memory (and to avoid re-training the animals to learn the two objects as a *de novo* memory), we use a shorter, single 5-minute update session. This duration is sufficient to drive memory updating in a young mouse and we have confirmed that updating with these parameters involves retrieval of the original memory.

Finally, for testing, we use a five-minute session and have not yet tested other durations. This test duration provides sufficient time for the mice to explore all of the objects and typically produces robust preference scores (Kwapis et al., 2019). A shorter test session is not recommended, as the animals need sufficient time to explore all four objects in this larger chamber.

Manipulations: Injections, cannulations, viral surgeries, etc.

Numerous systemic or site-specific manipulations can be used to investigate the mechanisms underlying memory updating in OUL. Timing is the single most important factor to consider when planning an updating manipulation. In order to selectively investigate the mechanisms required for memory updating, it is critical that the manipulation is restricted to the update session without affecting the training sessions so that the original memory is formed normally. Fast-onset manipulations (e.g. pharmaceutical compounds or rapidly expressed viral manipulations) are therefore ideal for the OUL paradigm, as they can be administered after the training is complete but before the original memory begins to fade.

The delivery of the compound should be timed so that the peak effectiveness occurs during either the update session itself or immediately after the update session during the reconsolidation window, an approximately 6h period during which the memory first destabilizes and then restabilizes (Jarome & Lubin, 2014; Lee, Nader, & Schiller, 2017). Ideally, the compound can be delivered immediately after the update session to avoid potential disruptions in learning the update, performance during updating, or state-dependent effects. This may not be possible for viral manipulations, which typically peak days (HSV) or weeks (AAV, lentiviruses) after infusion (Sarno & Robison, 2018). Viruses should be delivered 24h after the final training session to allow the original memory to consolidate before the manipulation is induced and the update session should be performed when the virus expression peaks.

To investigate the role of a specific brain region in memory updating, mice will need to undergo stereotaxic surgery to either implant cannulae or locally inject a virus. Cannulae can be implanted before habituation begins to allow for site-specific delivery of a drug or virus just before or after the update session, depending on the temporal dynamics of the manipulations' actions. If a mouse needs to be placed under anesthesia to deliver the virus, siRNA, or other compound, this should occur at least 24h after the final training session is complete to avoid disrupting the original memory.

TROUBLESHOOTING:

Animals fail to learn original memory

The original memory must be successfully acquired in order to make any conclusions about memory updating. To confirm that the initial information is learned, the researcher should carefully assess behavior during the update session itself to ensure that mice show a significant preference for the moved object. Mice that show no preference for the moved object during the update session (or show a low DI) may not have learned the original training information, making it nearly impossible to interpret the test session data. We encourage researchers to replicate the findings presented here using young (3-6 month-old) C57BL/6J mice to rule out the possibility that poor initial learning is due to a strain difference. Additionally, the researchers should ensure that the mice are not exposed to unnecessary stress or anxiety. Mice should be thoroughly handled and habituated (described above), objects should be wiped with 10% ethanol (higher concentrations can cause avoidance), and loud noises and scents should be avoided. As mentioned previously (Vogel-Ciernia & Wood, 2014), transporting the mice into the behavior room an hour before behavior can give animals time to acclimate and reduce stress. Habituation curves should be assessed to ensure the animals have achieved a stable, low level of movement before training occurs.

Young control animals fail to learn the update

Similarly, in order to test whether a manipulation affects updating, control animals must show a preference for the novel location (A_4) over the update location (A_3), with a DI of approximately 25 or higher. There are numerous reasons why the control animals would fail to learn the update. First, the researcher should ensure that the animals showed normal levels of exploration during the update session. Young mice should spend at least 3s exploring objects during each session and old mice should show at least 2s of total exploration. Exploration levels lower than this are often due to high anxiety levels, so the experimenter should assess the experimental setup for the source of anxiety. To lower anxiety, the researcher should ensure that the animals are well-trained and habituated, check that the lighting is within the suggested range (~16 to 20 lux), ensure the behavior room is protected from outsides noises, and check that the animals are being handled properly by familiar experimenters. If the animals are exploring normally but still fail to learn the update, the researcher should consider the strain and age of the mice used to determine whether a strain difference may cause this memory updating failure. If the mice show good memory for the original locations but fail to learn the update, the researcher can consider increasing the length of the update session to improve updating. If the length of the update session is

altered, however, the researcher should ensure that this longer update session still engages the original memory (instead of forming a new, separate memory) using anisomycin injections, catFISH ensemble monitoring, or another method.

Anticipated Results

Expected OUL results for young and old mice are shown in Figures 2 and 3, respectively. For training (Fig. 2A), all animals should show approximately equal exploration of both object locations, as both are equally novel at this point. Mice should show at least 3s of total object exploration during training (at least 2s for 18-month-old and older animals). If doing the 3-day training protocol, the amount of exploration should be at least 3s on the first day, but this exploration typically decreases across training days, as mice become familiar with the objects.

For the updating day (Fig. 2B), rodents in the No Update condition should continue to show approximately equal amounts of exploration for the two objects in the familiar locations. Mice in the Update condition, in comparison, should show significantly more exploration of the moved object compared to the unmoved object, demonstrating memory for the original training locations. Mice in the No Update condition should therefore show a low DI near zero whereas mice in the Update condition should show a DI of 25 or greater, indicating preference for the moved object.

Finally, for the test session (Fig. 2C), mice in the No Update condition should preferentially explore the two objects in the new locations (A_3 and A_4) compared to the objects in the familiar locations (A_1 and A_2). No Update mice should show significantly more exploration of location A_4 (randomly chosen between the two novel locations for each mouse) compared to location A_1 or A_2 and should therefore show a high DI (>25) for both A_1 and A_2. As locations A_3 and A_4 are equally novel in the No Update condition, mice should show similar levels of exploration of these locations, reflected in a DI near zero. For the Update condition, mice should preferentially explore the novel location (A_4) compared all three other locations (A_1, A_2, and A_3). These mice should remember the original training session and therefore explore the new location A_4 more than locations A_1 and A_2, reflected as a high DI (>25) for locations A_1 and A_2. As location A_2 was not presented on the update day for these animals, it is important to note that the DI for A_2 is often lower than that for A_1 (which was presented during updating the previous day) and the DI for A_2 may provide information about the persistence of the original memory (see Kwapis et al., 2019). Finally, Update mice should also show memory for the updated location A_3 and preferentially explore the novel location A_4 over the update location A_3, again with a DI of ~25 or greater.

For old mice, the test session results should reflect an intact original memory but impaired memory for the updated location (Fig. 3). Thus, old mice in both the No Update and Update conditions should show a high DI for objects A_1 and A_2, indicating intact memory for the original locations. For location A_3, on the other hand, both the No Update and Update groups should show a DI near zero, indicating impaired memory updating. Again, it is common for aging mice in the Update group to show weaker memory for location A_2 at test compared to the No Update group, as location A_2 was not presented the previous day for Update mice (Fig. 3B). As before, there should be no differences in the amount of total

exploration between conditions during the test session for either young (Fig. 2C) or old (Fig. 3C) mice.

TIME CONSIDERATIONS:

The OUL task takes a total of 11 days when exclusively using young mice with a single training session. Running OUL with old mice (or when directly comparing young and old mice) requires 3 successive days of training and takes a total of 14 days. To make the task more high-throughput, multiple animals can be tested at once using multiple identical contexts. For our task, we regularly run four animals at once placing four chambers in a square under the camera (see video 1). When running multiple contexts, be certain to place each animal in the same chamber for each habituation, training, updating, and test session. Additionally, when running multiple animals during the same session, ensure that the experimenter is able to place and remove all animals from the chambers within approximately 15s. Expect the animal removal and cleaning process to take approximately five minutes between each round of animals. Due to the size of the OUL chambers, removing the animals may take slightly longer than in standard chambers and it is therefore recommended that this task be performed by two experimenters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS: (mandatory for NIH, optional for all others)

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SIGNIFICANCE STATEMENT:

Memories are not permanent, fixed records of experience. Instead, memories are malleable, easily modified by new information. Although this updating process is critically important for an organism's survival, we understand relatively little about how existing memories are modified. Here, we describe a novel but simple behavioral task called the Objects in Updated Locations (OUL) task that can be used to understand the neural mechanisms that underlie memory updating. OUL is hippocampus-dependent and non-stressful, making it appropriate for both young and old rodents. Further, OUL allows both the original memory and the updated information to be assessed in a single test session. OUL is therefore a powerful task that is well-positioned to elucidate the neural underpinnings of the memory updating process.

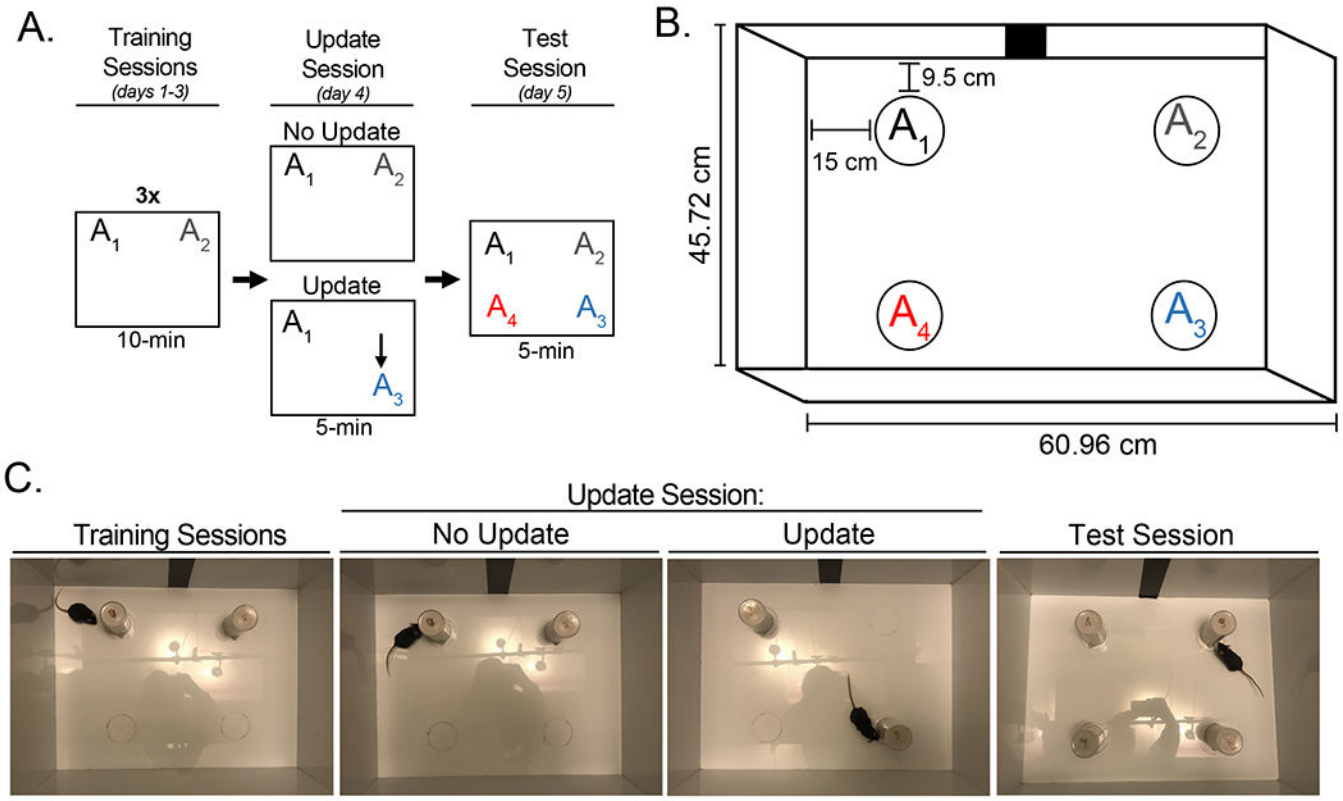

Figure 1.

OUL task design. **(A)** Experimental timeline for OUL. When comparing young and old mice, 3 training sessions (10-minutes each) should be used. If only assessing young mice, a single 10-minute training session is sufficient. For OUL, the update session is shown with the right object displaced. In an actual experiment, the moved object should be counterbalanced across groups. **(B)** Diagrams of context and object placement for OUL. The height of the arena is 26.67 cm. **(C)** Images of the actual experimental setup for OUL for all sessions: training, updating and testing. Mice shown are 3-6-month-old C57Bl/6J males. Note that the objects, beakers, are filled with gray cement.

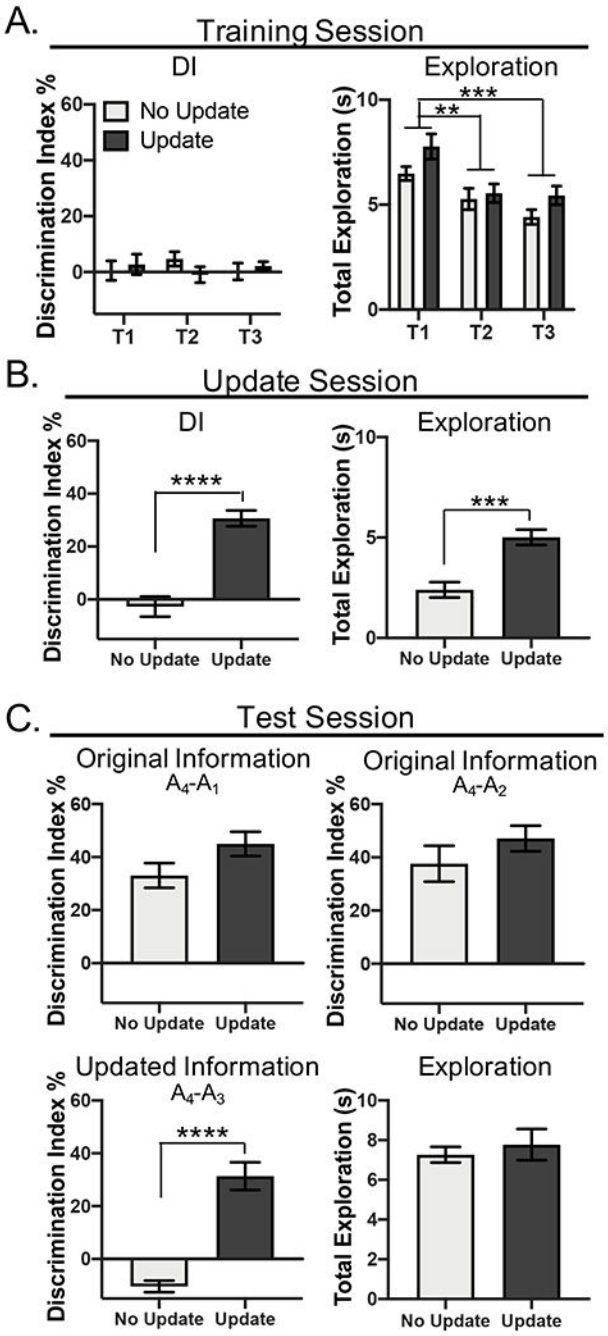

Figure 2.

Expected results from OUL with young mice (3-6-month-old, n=9/group). **(A)** Discrimination index (DI) and total exploration time across the 3 10-min training sessions for two experimental groups (No Update and Update). Mice show significantly reduced exploration across the 3 training sessions ($F_{(2,32)}$=19.92, p<0.0001; Tukey's *post hoc* tests, **p<0.01, ****p<0.0001.) but there are no group differences within each session. **(B)** DI and total exploration for both groups during the update session. Update mice show a significantly higher DI than No Update mice ($t_{(16)}$=6.844, p<0.0001) indicating they

remember the training locations. Update mice also show significantly more exploration than No Update during the Update session ($t_{(16)}$=4.808, p<0.001). **(C)** Results from the test session. Exploration of each of the 3 familiar locations (A_1, A_2, and A_3) is compared to exploration of the novel location A_4. Both No Update and Update groups prefer the novel location A_4 over the original training locations A_1 and A_2, indicating intact memory for the training session. For the Update location A_3, mice given the Update show a strong preference for the novel location A_4, indicating intact memory for the update. No Update mice (which were not previously exposed to location A_3) show equal preference for locations A_3 and A_4, resulting in an Update DI of approximately zero. Update mice show a significantly higher A_3 DI than No Update mice ($t_{(16)}$=7.333, p<0.0001). The total exploration time for both groups was similar during the test session, however. Part of this data set was previously published (Kwapis et al., 2019) and is reprinted with the authors' permission.

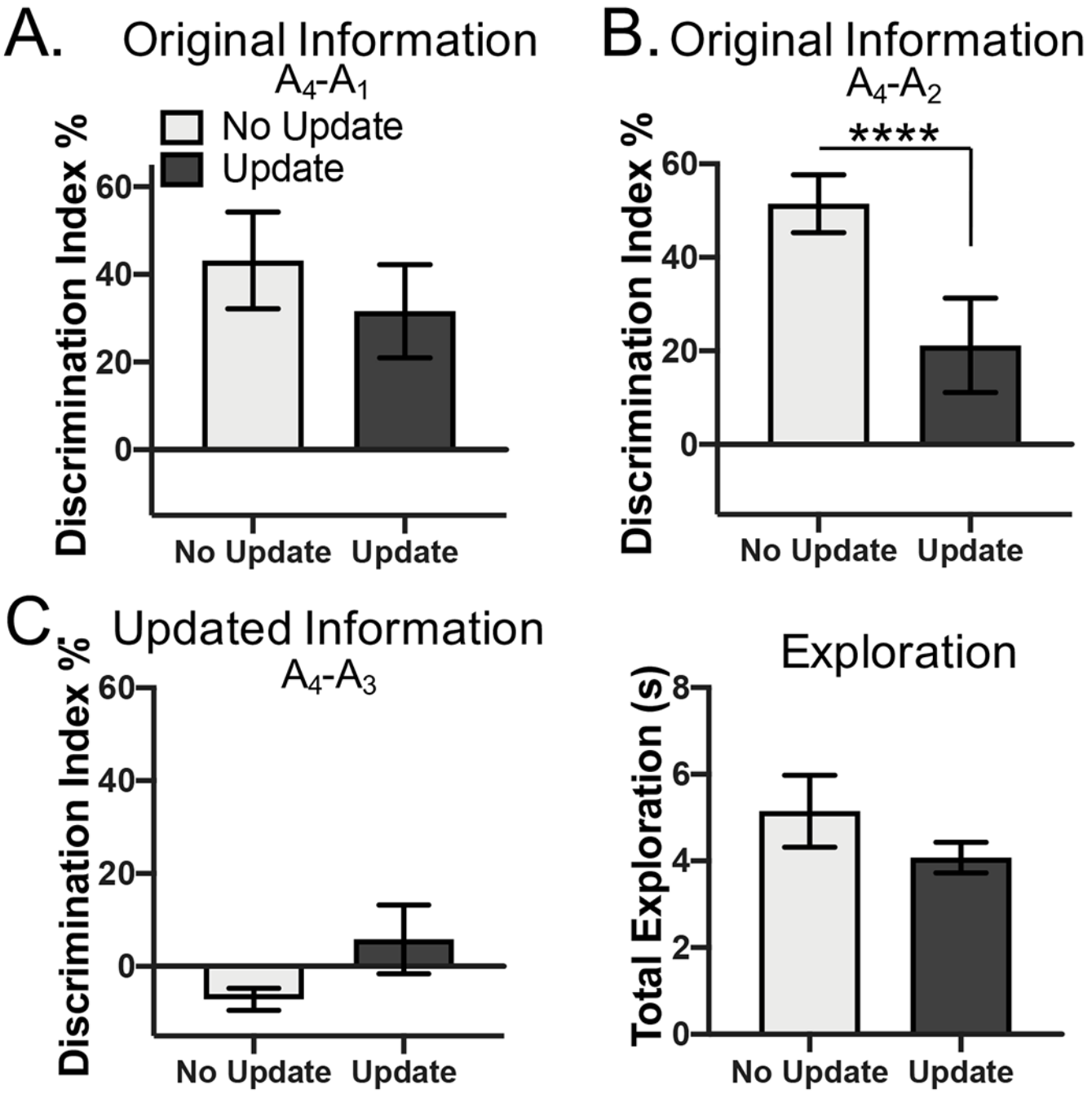

Figure 3.

Expected OUL results in old (18-20-month-old, n=7-8/group) mice during the OUL test session. **(A)** DI comparing the original location (A_1) to the novel location (A_4). Both Update and No Update animals show memory for the original location A_1. **(B)** DI comparing the original location (A_2) to the novel location (A_4). Old mice given the Update show reduced memory for A_2 ($t_{(13)}$=2.464, p=0.028), which may be due to the longer retention interval (Update mice have not seen object A_2 for 48h). **(C)** DI comparing the updated location A_3 to novel location A_4. Both groups show similar exploration of objects A_3 and A_4 (resulting in a

DI near zero), indicating they have little memory of the updated location. **(D)** There were no group differences in exploration during the test session.

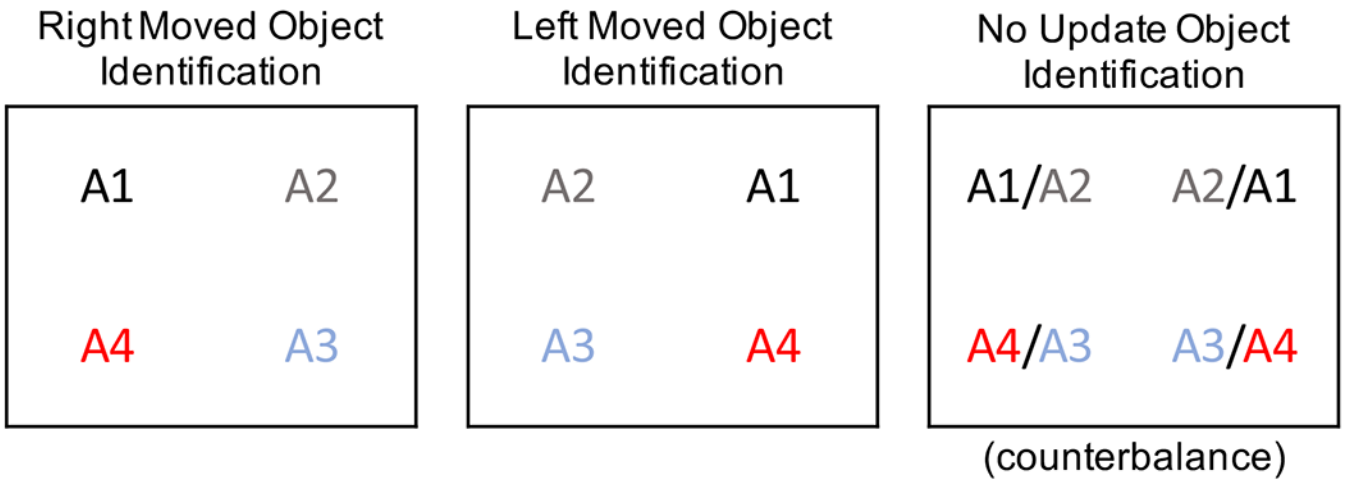

Figure 4.

Schematic illustrating each object's identity (A_1, A_2, A_3, A_4) based on whether the left object was moved during the update session, the right object was moved during the update session, or neither object was moved during the update session (No Update condition).

Table 1.

Raw OUL test scores from three different observers from Video 1, indicating the number of seconds each animal explored each of the four object locations (in seconds).

Scorer 1						
Box	**Moved**	**Top Left**	**Top Right**	**Bottom Left**	**Bottom Right**	**Total**
Top left	Right moved	1.649	0.881	1.987	1.081	5.598
Top right	No update	0.339	1.06	2.82	2.92	7.139
Bottom left	Left moved	1.616	1.148	1.003	2.127	5.984
Bottom right	No update	1.067	1.403	1.938	1.542	5.95
Scorer 2						
Box	**Moved**	**Top Left**	**Top Right**	**Bottom Left**	**Bottom Right**	**Total**
Top left	Right moved	1.263	0.941	1.707	1.01	4.921
Top right	No update	0.362	0.719	2.134	2.357	5.572
Bottom left	Left moved	1.208	1.082	0.953	1.906	5.149
Bottom right	No update	0.951	1.065	1.797	1.27	5.083
Scorer 3						
Box	**Moved**	**Top Left**	**Top Right**	**Bottom Left**	**Bottom Right**	**Total**
Top left	Right moved	1.372	1.075	1.69	1.246	4.949
Top right	No update	0.489	0.753	2.558	2.647	5.453
Bottom left	Left moved	1.685	1.3739	1.029	2.484	4.792
Bottom right	No update	0.905	0.901	1.375	1.184	4.365

Table 2.

OUL test scores from Table 1 reorganized to allow for DI calculations.[1]

Reorganized data for Scorer 1							
A1 (Original)	A2 (Original)	A3 (Updated)	A4 (Novel)	Total Exploration	DI Calculations		
					A1 vs A4	A2 vs A4	A3 vs A4
1.649	0.881	1.081	1.987	5.598	9.3	38.6	29.5
1.06	0.339	2.82	2.92	7.139	46.7	79.2	1.7
1.148	1.616	1.003	2.127	5.984	31.8	15.7	37.7
1.067	1.403	1.542	1.938	5.95	28.98	16.0	11.4
Reorganized data for Scorer 2							
A1 (Original)	A2 (Original)	A3 (Updated)	A4 (Novel)	Total Exploration	DI Calculations		
					A1 vs A4	A2 vs A4	A3 vs A4
1.263	0.941	1.01	1.707	4.921	14.9	28.9	25.7
0.719	0.362	2.134	2.357	5.572	53.3	73.4	4.97
1.082	1.208	0.953	1.906	5.149	27.6	22.4	33.3
0.951	1.065	1.27	1.797	5.083	30.8	25.6	17.2
Reorganized data for Scorer 3							
A1 (Original)	A2 (Original)	A3 (Updated)	A4 (Novel)	Total Exploration	DI Calculations		
					A1 vs A4	A2 vs A4	A3 vs A4
1.372	1.075	1.246	1.69	4.949	10.4	22.2	15.1
0.753	0.489	2.558	2.647	5.453	55.7	68.8	1.7
1.3739	1.685	1.029	2.4839	4.792	28.8	19.2	41.4
1.166	1.075	1.345	1.919	4.365	20.6	20.8	7.5

[1] DI calculated for each object as: $(A_4 - A_{1/2/3})/(A_4 + A_{1/2/3})$ x100