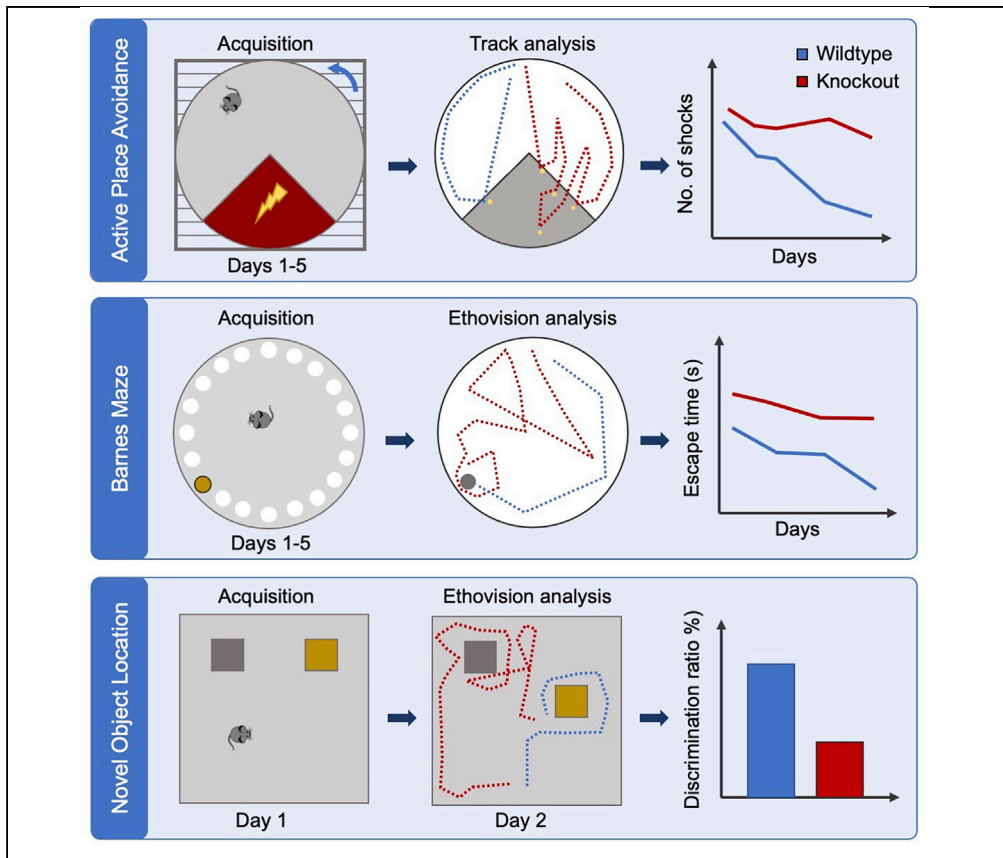


Protocol

Protocol for three alternative paradigms to test spatial learning and memory in mice



Here, we describe three alternative paradigms to overcome the limitations of the most widely used spatial learning paradigm for rodents: the Morris water maze. We outline the preparation of behavioral testing rooms and mouse handling/habituation prior to testing. We then detail three spatial learning and memory tasks: the Barnes maze, active place avoidance, and novel object location tasks. These tests have been successfully used across multiple ages (from 2 to 24 months) in both wild-type and transgenic animals.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Preparation of rooms for behavioral testing of mice

Handling and habituation of mice prior to behavioral testing

Experimental design for three spatial learning and memory tasks

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Protocol

Protocol for three alternative paradigms to test spatial learning and memory in mice

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<https://doi.org/10.1016/j.xpro.2022.101500>**SUMMARY**

Here, we describe three alternative paradigms to overcome the limitations of the most widely used spatial learning paradigm for rodents: the Morris water maze. We outline the preparation of behavioral testing rooms and mouse handling/habituation prior to testing. We then detail three spatial learning and memory tasks: the Barnes maze, active place avoidance, and novel object location tasks. These tests have been successfully used across multiple ages (from 2 to 24 months) in both wild-type and transgenic animals. For complete details on the use and execution of this protocol, please refer to Leiter et al. (2022).

BEFORE YOU BEGIN

The most widely used spatial learning paradigm for rodents, the Morris water maze, is stressful for mice and aged animals tend to float rather than actively seeking the hidden platform, thereby rendering the test invalid. This protocol describes three alternative paradigms to assess spatial learning and memory ability in mice: the Barnes maze, active place avoidance and novel object location tasks.

Institutional permissions

All experiments on live vertebrates or higher invertebrates must first be approved in accordance with the national guidelines by the appropriate institutional authority. Our experiments were performed under the authorization of the University of Queensland Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 8th edition, 2013 (updated 2021), and the Queensland Government Animal Care and Protection Act (2001).

Preparation of behavioral testing room and ante-room

⌚ Timing: weeks

1. The testing room should be approximately 2 m² in size, have minimal novel spatial cues and be of a consistent neutral color such as off-white or pale gray.
2. It should also contain a dimmable, diffuse overhead light source, computer, overhead video camera and spatial cues.

⚠ **CRITICAL:** Place a curtain or panel in such a way that it blocks the mouse being tested from being able to view its home cage and the experimenter.

3. Fit waste bins with a lid and keep closed to minimize odor cues.



4. Set the light intensity in both the testing room and ante-room prior to animals being brought in to habituate.
 - a. The ante-room light intensity should be low and range from 20–50 Lux.
 - b. The ante-room should be devoid of spatial cues and other animals, and be in a low traffic area.

Alternatives: If an ante room is unavailable due to space constraints, a housing carousel/ chamber separate from the testing space can be used. In this case the holding area needs to be dim and not visible from the testing apparatus.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Ethovision	Noldus	XT16
Microsoft Excel	Microsoft	16.53
Prism	GraphPad	9.0
APA analysis software	BioSignal	N/A
Experimental models: Organisms/strains		
Mice: C57Bl/6j, male and female from 2 to 24 months	Animal Resources Centre (Western Australia)	N/A
Other		
APA apparatus	BioSignal	N/A
Barnes maze	In house	N/A
Novel objects	In-house 3D print	N/A
Novel object boxes	In house	N/A
Paper towels	N/A	N/A
80% (v/v) ethanol in spray bottle	N/A	N/A
Clinical waste bin with lid	N/A	N/A
50 L clinical waste bin liner	N/A	N/A
Lux meter	N/A	N/A

STEP-BY-STEP METHOD DETAILS

Habituation and handling of animals

⌚ **Timing:** 2–3 days

This step is to habituate the mice to both the researcher conducting the experiments and the testing environments to which the mice will be exposed.

1. If purchasing the mice from an external supplier, ensure that they arrive at least one week prior to behavioral testing.
2. Transport mice, preferably using a trolley, to a quiet room. The light intensity for the room should be 30–70 Lux.
3. Leave animals to settle for 30 min.
4. Gently remove the first mouse by gently holding the base of its tail and place it on gloved hand. Allow the mouse some restricted movement on the hand and wrist.
5. After 2 min of handling, gently return the mouse to its home cage.
6. If the mouse has urinated or dropped scat on the glove, wipe off with tissue paper and spray with 80% (v/v) ethanol.
7. Once gloves are dry pick up the next mouse and repeat the procedure.
8. Repeat this procedure at the same time for the next two days.

△ **CRITICAL:** If both males and females are being tested, habituate the same sex at the same time. Once all animals of one sex have been habituated, thoroughly wipe down the area and return the animals to the holding rooms/ carousels. Depending on the air turnover rate, wait 30–60 min before habituating the other sex. This is to allow sufficient time for scents and pheromones to dissipate. The same approach should also be used during testing of animals.

△ **CRITICAL:** One experimenter should perform the entire procedure (both habituation and testing) and should wear the same laboratory coat to limit olfactory cues.

Note: Aged mice are generally used to being handled as they have gone through numerous cage changes during their lives. They should, however, still be handled prior to behavioral testing by the person who will perform the experiment, as it will habituate them to that person and reduce their anxiety levels.

Novel object location test (1–2 days)

⌚ **Timing:** 1–2 days

The novel object location (NOL) test is based on the spontaneous tendency of mice to spend more time exploring a novel object than a familiar one (Akirav and Maroun, 2006; Assini et al., 2009; Haetig et al., 2011; Leiter et al., 2022; Murai et al., 2007; Vogel-Ciernia and Wood, 2014). Mice are first allowed to explore an arena with two objects. In the second trial, one of the objects is moved and the amount of time the mouse spends exploring the object in the novel location is determined.

△ **CRITICAL:** Behavioral tests should always be performed during a fixed time period and remain constant for the entirety of the testing. Ideally, it is better to perform tests during the dark phase to minimize the disruption to the animals' sleep patterns. Animals should be maintained on a reverse light cycle and tested during their active dark cycle (Beeler et al., 2006). However, most animal houses use a 12:12 light: dark cycle with the light phase between 7 am and 7 pm. If it is not possible to reverse this cycle, aim to conduct behavioral testing during the initial hours of the light phase.

9. NOL test - day 1.

- a. Place boxes (ours are made of white Perspex with dimensions of 37 × 48 × 19 cm) on a 70 cm high pedestal.
- b. Align the boxes with markings on the pedestal to allow for the consistent placement of boxes during testing.

Note: Each box should have a piece of black tape along one edge to aid in spatial navigation.

- c. Set the light intensity over the box to 50–100 Lux.

△ **CRITICAL:** There should be minimal shadows in the boxes. If two boxes are used, ensure a gap between them to allow for post-test analysis.

- d. Secure two identical objects (Figure 1A) in each box 3 cm away from the long edge and 5 cm away from short edge of the box as shown in Figure 1B (left box).

△ **CRITICAL:** Significant variation can exist between the novel objects that are selected. Ideally the objects should be of similar size, durable, unable to be climbed, and easily cleaned. Many studies have relied on building blocks of various sizes to build novel and complex shapes. Extremely complex shapes using small building blocks are difficult to

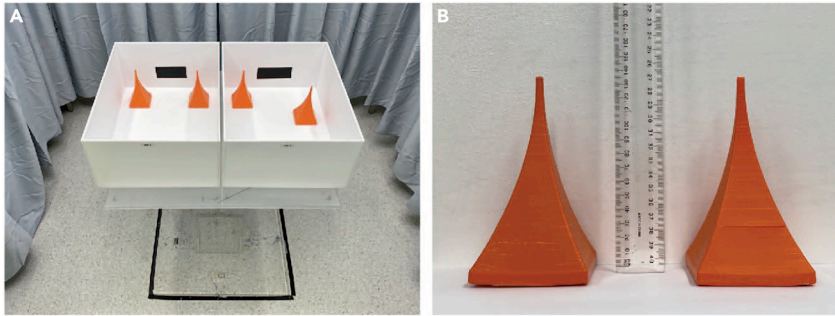


Figure 1. Novel object location test

(A) A mouse is placed in a white Perspex box containing two identical objects and allowed to explore (left box). In a second trial one of the objects is moved to a new location (right box) and the animal is retested. (B) Two identical objects approximately 10–15 cm in height are required.

replicate and clean sufficiently, especially to remove the scent of previous mice. Although larger blocks are easier to clean, this requires dismantling the shape. To circumvent these issues, we 3D print objects and have included the files required to produce them (see [Data S1, S2, S3, and S4](#) and [Figures S1–S4](#)). Our objects are all printed on a Ultimaker S5 FDM printer with orange ABS filament, 0.1 mm slice thickness and 20% fill.

Optional: Lightly cover the bottom of the box with bedding. Some researchers believe that having bedding covering the arena floor lowers the anxiety of the mice during testing. Appropriate handling of animals prior to any testing will also minimize this risk. However, some strains are inherently anxious. If bedding is not used, the base of the open field should be a matte finish to minimize reflections during the video tracking.

- e. Bring animals into the ante-room or testing room for habituation.
- f. Leave animals to habituate for a minimum of 30 min.
- g. Record 1–3 s of video showing empty boxes with the objects to be used for mask creation during the analysis phase. A “mask” is a region of interest that defines the location of the objects that the animals will be examining.
- h. Start video recording.

Optional: Hold a card identifying the run number or animal ID for record keeping/analysis purposes or place it on the edge of the pedestal at the start of recording.

Note: We recommend a Firewire video camera, suitable for use in low-light settings, that can capture 15–60 frames per second, e.g., a Point-grey FL2 or FL3 video camera, or similar.

⚠ **CRITICAL:** Do NOT leave the card on top of the box during testing.

- i. Gently pick up the first mouse from the home box and place it in the middle of the NOL box facing away from the objects.
- j. If a second box and mouse is being tested, simultaneously place the second mouse in the second box.
- k. Retreat behind the curtain during recording and ensure that the lid to the mouse home cage is secured to minimize any scent.
- l. After a pre-determined duration, stop the video recording. Re-testing can be conducted either 90 min following the first test or after 24 h (long-term memory). For shorter re-test periods, a 5 min exploratory period can be used. For a long-term re-test, a 10 min exploratory time is recommended.

- m. Retrieve mouse and place it back in home cage.
 - n. Empty bedding into a clinical waste bin. Clean down the box and objects with 80% (v/v) ethanol.
 - o. Refill the box with bedding and ensure that it is placed back in the correct spot.
 - p. Repeat steps h-o until all animals are tested.
10. NOL test - day 2.
- a. Set up the testing room exactly as on day 1.
 - b. Move one of the two identical objects to a novel location 3 cm from the long edge and 14 cm from the short edge of the box (Figure 1B; right box).

△ **CRITICAL:** The novel location should be alternated during testing to minimize the chance of nonspecific cues affecting the mouse's exploration.

△ **CRITICAL:** Ensure the novel location is noted in a template, i.e., A or B for later accurate analysis.

- c. Repeat steps 9 e-o until all animals are tested.
11. Analysis.

Note: Several parameters are included within Ethovision XT to aid in the analysis of the NOL test.

△ **CRITICAL:** Setting masks appropriately in Ethovision is critical to ensure accurate data collection.

- a. Create masks both immediately around the objects and a second "zone of interest" 0.5–1 cm bordering the objects.

△ **CRITICAL:** This step is required to determine if the animals are paying interest to the objects.

- b. For this test it is recommended to use the 3-point body analysis option so that the direction of the head can be automatically determined during analysis.

Note: Alternatives to the Ethovision software are also available, including Deep Lab Cut and ezTrack (Pennington et al., 2019).

Note: See the following manuscript for a review of free animal-tracking software applications (Panadeiro et al., 2021).

Barnes maze

⌚ **Timing:** 4–5 days

In this task, mice are placed in the center of a brightly lit table with 32 evenly spaced holes around its circumference. They are then tested for their ability to use the visual cues placed around the room to locate the one hole with an escape chamber located beneath it (Barnes, 1979; Harrison et al., 2009; Leiter et al., 2022; Reiserer et al., 2007).

12. Prepare room for Barnes maze testing.
- a. Our Barnes maze consists of a circular white piece of acrylic plastic (100 cm diameter) with 32 holes each measuring 5 cm in diameter evenly spaced around the circumference 7.5 cm from

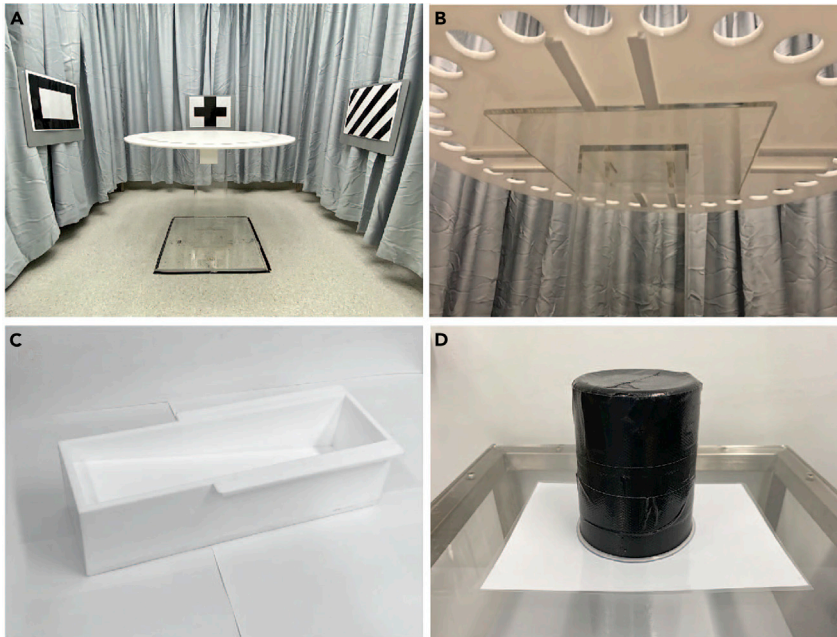


Figure 2. Barnes maze test

- (A) A circular table with 32 evenly spaced holes around its circumference is placed on a pedestal in the center of a room with a different cue placed on each of the four walls.
- (B) Tracking rails for the escape tunnel are located under the Barnes maze to ensure that the maze remains in the same location throughout the experiment and to allow for placement of the escape tunnel.
- (C) The escape tunnel can be fitted with a ramp to aid aged animals to enter.
- (D) A start chamber consisting of a laminated square of paper, and a darkened beaker is used to place the mouse in the starting location.

the outer edge. The Barnes maze is placed on a 70 cm high pedestal located in the middle of the behavioral room equidistant from all walls (Figure 2A). There is one removable escape tunnel underneath the Barnes maze. The tracking rails for this escape tunnel ensure the maze remains in the same location throughout the experiment (Figure 2B).

- b. Place a clean tissue in the end of the escape tunnel.

Note: For aged animals or those animals with mild motor deficits, use an acrylic plastic ramp inside the escape tunnel at an angle of less than 45° as shown in Figure 2C. This is to encourage these animals to enter the escape tunnel. The ramp should be the same color as the escape tunnel so that it does not provide an additional spatial cue.

- c. Place spatial cues equally around the walls level with the Barnes maze surface.
 - d. Set light intensity to 1000 Lux.
 - e. Bring mice into the testing or ante-room to habituate for at least 30 min.
13. Habituation of mice to the Barnes maze.
 - a. Attach the escape chamber and place clean paper towel in the end of the chamber (this will act as bedding and should help to reduce stress).
 - b. Place the mouse into the escape tunnel.
 - c. After 1 min gently remove the mouse from the chamber and place it in the center of the apparatus.
 - d. Allow the mouse to explore until it enters the escape tunnel.
 - e. If 5 min have elapsed without the animal locating the escape tunnel, gently move it to the tunnel.
 - f. After 30 s in the escape tunnel place the animal back in its home box.

- g. Clean the escape tunnel and Barnes maze with 80% (v/v) ethanol.
- h. Repeat with the next animal.

14. Acquisition of Barnes maze data.

Three or four days of acquisition with three trials per day are commonly used. There should be at least 1 h between each daily trial.

△ CRITICAL: Ensure that the escape tunnel is in a different location to that used during habituation (move the tunnel 90 °).

- a. Click the start record button for the camera.
- b. Gently remove the mouse from its home cage and place it onto a laminated square of paper – do not let go of its tail.
- c. Continuing to hold the tail, lower the start chamber (consisting of a darkened beaker) down, ensuring that the beaker spout is aligned with the tail (to prevent injuring it).
- d. Set down the laminated square, mouse and chamber down in the starting location (Figure 2D).
- e. Carefully pull the laminated square out without releasing the mouse.
- f. Wait for 15 s.
- g. Lift the start chamber and retreat behind the curtain.
- h. Stop the trial when the mouse enters the escape tunnel, or once 3 min have elapsed. If a mouse fails to enter the escape tunnel place it gently in the escape tunnel for 15 s.
- i. Click the stop record button for the camera.
- j. Clean the escape tunnel, Barnes maze and start chamber and laminated square with 80% (v/v) ethanol. Add fresh tissue to the escape tunnel.
- k. Repeat with the next animal.

Note: Each mouse should be placed in the center of the appropriate quadrant of the apparatus. Each daily trial of the mouse should be initiated from a different quadrant. A template of running order will aid in ensuring that this is done accurately.

15. Probe trial (Optional).

The probe trial is to test the memory consolidation of the mice following the acquisition phase. Typically, a well-trained mouse will move directly to the escape hole where the tunnel was located, thereby showing spatial bias and evidence of spatial learning.

- a. Do not add the escape tunnel to the maze.
- b. Click the start record button for the camera.
- c. As described above, move the mouse over to the maze with the laminated square and start chamber. Place it in the center of the appropriate quadrant on the apparatus.
- d. Wait for 15 s and then lift the start chamber.
- e. Stop the trial after 1 min.
- f. Click the stop record button for the camera.
- g. Collect the mouse and return it to its home cage.
- h. Clean the Barnes maze and start chamber with 80% (v/v) ethanol.
- i. Repeat with the next mouse.

Active place avoidance

⌚ Timing: 1–6 days

This task involves the mice using visual cues to orient themselves within a rotating arena in order to actively avoid a stationary shock zone (Cimadevilla et al., 2000a, 2000b; Leiter et al., 2022; Vukovic et al., 2013; Willis et al., 2017, 2020).

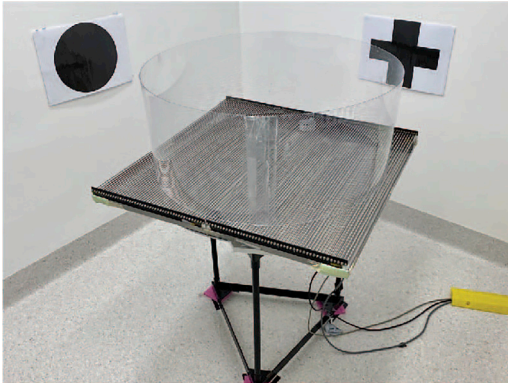


Figure 3. APA test

Animals are placed on a rotating arena and must use visual cues placed around the room to actively avoid a stationary shock zone.

16. Active place avoidance (APA) apparatus and tracking setup.
 - a. Our APA apparatus (BioSignal Group) consists of a square elevated arena under evenly spaced metal bars (0.5 cm apart) enclosed by a 32 cm high transparent circular boundary (Figure 3).

Note: The APA apparatus can also be purchased from Conduct Science. The original developers of the APA paradigm also included detailed manufacturing instructions in their initial publications (Cimadevilla et al., 2000b, 2001).

- b. The APA apparatus should be placed directly under a ceiling-mounted camera, with the position of the mouse being tracked using Tracker software (BioSignal Group).
- c. The arena rotates (typically counterclockwise at a speed of 1 rpm) and a foot shock (500 ms, 60 Hz, 0.5 mA) is delivered when the mouse enters a pre-designated 60° shock zone.
- d. Place four cues equally around the room at the same height as the rotating platform. Cues should typically be 30–50 cm from the platform.

Note: Cues need to be different from each other, and similar cues (e.g., a filled circle and a filled oval) should be avoided. Cues should be of A3 size or larger and typically black and white in nature. Laminating the cues increases durability and allows for easy cleaning.

- e. Light levels should be 30–70 Lux.
- f. Before starting, create a folder in the Tracker program and select the APA task. This will populate the tabs required to configure the parameters required to run the test. This can then be saved as a configuration file and modified as required.
- g. In the “Experiment” tab select the duration of the experiment. This is represented as seconds, i.e., 10 min is 600 s.
- h. Ensure that the “Enable Timer” is ticked. Within this it is possible to alter various shock parameters. Common settings are: shock initiation latency = 500; shock duration = 500; outside refractory = 1500; and inter-shock latency = 1500. All shock timings are in milliseconds.
- i. Select the “Target” tab and then ensure that the circular mask includes the entire area of interest. The mask is to define the area in which the mouse is tracked during the APA task.
- j. Select the angle and size of the aversive zone, represented by a shaded wedge. A typical shock zone is 60 degrees and extends from the center point to the edge of the arena. These settings can be altered using the “Target” tab by clicking on the edit button.
- k. Using the “Tracking” tab you can select if the animal is darker or lighter than the rig (e.g., darker is used for C57BL/6 animals).
- l. Clicking the “From Calibrator” tab allows appropriate thresholds for effective tracking of the animal to be set. This includes the size of the animals.

- m. The “Mask” tab will ensure that only the arena is included in the tracking of the animal.
 - n. The “Device” tab allows the rotation direction and speed of the arena to be set using the velocity button.
 - o. The current source section allows for the shock intensity to be set. The most common setting for mice is 0.5 mA. The mode of current is typically set to Track dependent; however, shocks can be set to be delivered at a constant time. Previous tracks can also be used to shock an animal. This is typically done to provide a yoked control animal that received the same number of shocks but not in a spatially dependent manner.
17. Habituation to the APA arena (1 day).
- a. Bring animals into the ante-room or testing room for habituation. Leave the animals to habituate for a minimum of 30 min.
 - b. Set up the Tracker software.
 - c. Habituation to the APA apparatus typically involves a 5 min exposure where the apparatus is rotating but no shocks are delivered.
 - d. Place the animal on the opposite side to the aversive shock zone, facing the arena.
 - e. Retreat behind the curtain and begin the trial.
 - f. Ensure the animal is being tracked efficiently.
 - g. At the end of the trial gently pick up the animal and return it to its home cage.
 - h. Remove any scat from the arena and grid. Wipe down the grid and arena with 80% (v/v) ethanol.
 - i. Repeat steps 17 d–h for all animals.
18. Acquisition training using APA (1–6 days).
- a. Set the room light to same intensity as on the habituation day.
 - b. Bring animals into the ante-room or testing room for habituation. Leave them to habituate for a minimum of 30 min.
 - c. Set up the Tracker software.
 - d. Set the duration of the trial.

Note: Common times are 10 min for up to 6 days or a single trial of 20 or 30 min.

- e. Ensure that the current source is on and set (i.e., 0.5 mA).
- f. Repeat steps 17 d–h for all animals.

Note: In some experimental paradigms researchers test animals prior to an intervention in order to obtain baseline spatial learning ability. Following treatment, animals need to be re-tested to examine changes in cognitive ability. The APA testing paradigm can accommodate this by changing several components of the test. This includes changing the rotation of the APA apparatus from clockwise to anti-clockwise. The aversive zone can also be moved to an alternative location and novel cues can be used to alter the parameters of the test. These changes will provide a novel spatial task for the animals to learn. There is no need to conduct a second habituation session in this instance.

△ CRITICAL: The bedding in the home cage should not be changed during the behavioral testing as this could provide stimulation that affects performance.

△ CRITICAL: It is important to remain quiet while the animals are performing the task, as any noise may result in additional cues that affect performance.

19. Reversal acquisition training (Optional, 1–6 days).
- a. In the Reversal test, the shock zone is re-located to a new location, typically 180 degrees from the original shock zone and the animals are tested for their ability to flexibly learn a novel location.
 - b. Repeat steps 18 a–f for all animals.
20. Probe trial (Optional, 1 day).

- a. Set room light intensity.
- b. Bring animals into the ante-room or testing room for habituation. Leave animals to habituate for a minimum of 30 min.
- c. Set up the Tracker software.
- d. Set the duration of the trial to 10 min.
- e. For this trial do not turn the current source on.
- f. Place the animal on the opposite side to the aversive shock zone, facing the arena.
- g. Retreat behind the curtain and begin trial.
- h. Ensure the animal is being tracked efficiently.
- i. Carefully observe the animal. When it enters the aversive shock zone area stop the trial.
- j. Gently pick up the animal and return to home cage.
- k. Remove any scat from arena and grid. Wipe down grid and arena with 80% (v/v) ethanol.
- l. Repeat steps 20 f–k for all animals.

EXPECTED OUTCOMES

Novel object location task

If the animals remember the original location of an object, it is expected that the novel location will receive a higher level of interest. Several parameters should be considered. For example, a simple comparison between groups for the time spent investigating the object at the novel location may mask other, subtle differences.

Parameters typically reported include:

Distance traveled: This ensures animals are active within the box and not stationary within a corner.

Time spent investigating objects: Mice with intact memory typically spend more time exploring the object in the novel location.

Head directed to zone: This indicates if the object is being investigated by the mouse.

Distance from object: This may indicate if the animal is preferentially remaining closer to the novel location.

Percentage of time spent investigating at novel location: Determining the amount of time the animal spends investigating each object and converting this to a percentage is an effective and accurate way to standardize analysis. For example, if control animals spend less time investigating the object but spend most of their time observing the novel location this may skew comparison against the treatment group if only total time is considered. The percentage of total investigation time of the novel object is calculated as: $(\text{time at novel location}) / (\text{time at familiar location} + \text{time at novel location}) \times 100$. A result of more than 50% indicates higher investigation of the novel location.

Discrimination index: This can be used to calculate a standardized index of investigation. It is calculated as: $(\text{time at novel location} - \text{time at familiar location}) / (\text{time at novel location} + \text{time at familiar location})$. A positive value indicates a higher level of investigation of the novel location.

Barnes maze

Note: Ethovision software has templates for Barnes maze analysis.

Note: During testing the escape latency can be directly recorded on a spreadsheet.

Parameters typically reported include:

Primary latency: latency to locate the escape tunnel.

Escape latency: latency to enter the escape tunnel.

Primary errors: number of incorrect holes checked.

Distance traveled: distance traveled prior to locating the escape tunnel and total distance traveled for each trial.

Primary hole distance: first location checked relative to the escape tunnel. Ranges from 0 - 16 (directly opposite the escape tunnel). This is an arbitrary unit which describes how close to the escape tunnel the mouse initially investigates. The lower the number, the better the mouse is performing.

Percentage of holes searched.

Search strategy: spatial, serial or random. Trials where the mouse spends the majority of its time on the periphery systematically checking the holes reflect a serial strategy. Mice using a spatial strategy use the spatial cues as evidenced by movement to the quadrant that contains the escape tunnel. Random searching consists of searching non-adjacent holes.

APA task

Several parameters are measured automatically during the APA test. The Tracker software provides modifiable spreadsheets that can be opened in Excel for grouping and analysis. Trace maps for each animal are also provided. The duration of analysis is modifiable whereby data can be “binned” in order to examine the performance at specific times and for specific durations. For example, the first 2 min for each day can be compared, as opposed to the daily total time. Binning the data in this way allows researchers to examine the performance of animals during discrete periods such as working memory at the start and end of each trial.

Parameters typically reported include:

Entries into the aversive zone: the number of entrances into the shock zone indicates if the mouse remembers the location of the shock zone. In some instances, the mouse will receive multiple shocks before successfully exiting the shock zone. This indicates that it does not understand how to escape the aversive zone.

Number of shocks received: during acquisition training the number of shocks should decrease if the mouse is learning the task. Upon receiving a shock, it is common for the animal to vocalize, rear/react and then leave the aversive zone. These are important indicators to ensure that it is receiving the shock.

Time and distance to first entrance of the shock zone: these measures will indicate if the mouse is learning during the multiday acquisition phase of the test. A longer time (and distance) until the first entrance into the shock zone indicates that it remembers the location of the aversive zone and is actively avoiding it. There should be an increase in time and distance to first entrance as the number of acquisition trials increases.

Maximum avoidance time: the maximum time spent avoiding the shock zone is a very important readout and demonstrates if the mouse is effectively learning. The maximum avoidance time should increase even during a single acquisition test if the mouse has intact spatial learning capabilities.

Percentage learning: converting the learning ability by comparing the performance from the start of the acquisition phase to the end is an effective way to standardize the result and compare between groups. This is calculated as: $(d5 \text{ value} - d1 \text{ value}) / d1 \text{ value} \times 100$. This can be used for most parameters including shock number, maximum avoidance time and time to second entrance of the shock zone.

Distance traveled: this measure is required when comparing treatment groups to control animals to ensure that animals are traveling similar distances during the test.

Speed: similar to distance traveled, it is important to know that all animals are traveling at similar speeds.

Time and distance to second entrance of the shock zone: some animals appear to require an initial shock to focus their attention on the task. For this reason, the time to second entrance of the shock zone is an important measure and, like maximum time avoidance, reflects the working memory of the mice during testing.

Entries per distance traveled: the software automatically calculates the number of entries to the shock zone per distance traveled as a way to standardize the performance of the animals.

Time spent in the quadrant opposite the aversive zone: this can be used as a measure of how well the animal has learnt the location of the shock zone.

Note: Time measurements are generally more effective than distance measurements as animals can use different avoidance strategies. For example, some mice stay in the middle of the grid which results in a very short distance measurement whereas others get close to the aversive zone before moving to the opposite side of the shock zone. Although both strategies are effective, there is a large difference in distance reported.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are typically analyzed using either a one-way repeated measures ANOVA (to examine differences for one group during the acquisition phase) or a two-way repeated measures ANOVA with Bonferroni post hoc test (comparing between groups during the acquisition phase). Data are typically expressed as mean \pm SEM and differences are considered significant when $p < 0.05$.

LIMITATIONS

The NOL is a very simple task and may not reliably measure subtle changes in spatial learning and memory. This can be overcome to some degree by the more challenging Barnes maze and APA tasks. The Barnes maze and APA test are relatively good at determining an impairment in learning ability (i.e., in a particular knockout strain or following a brain injury such as stroke). However, it is much more difficult to determine whether an intervention improves memory in a young mouse with intact learning and memory function.

TROUBLESHOOTING

Problem 1

Poor performance/discrimination of the novel location by wild-type animals (step 11).

Potential solution

Poor performance can occur if no additional spatial cues are included in the NOL box to aid in referencing the novel spatial location of the object. We overcome this by placing a piece of black tape on one wall of the novel object box. The novel location should also be sufficiently different from the original location.

Problem 2

Animal not moving in the Barnes maze (steps 14a–k).

Potential solution

Ensure that the habituation has been appropriately completed. Albino mice, such as Swiss or Balb/c, may struggle with very bright light, which is a consideration if these strains are to be tested. For these strains a different colored Barnes maze (gray or black) will be required to allow for appropriate contrast.

Problem 3

Poor tracking of animals in NOL (steps 9 and 10).

Potential solution

Optimizing the tracking of the mice in Ethovision is a critical component to ensure the accuracy of the test. Within Ethovision there are several different tracking options, including differencing, gray scaling and dynamic subtraction. The selection of different tracking options is dependent on the difference in contrast between the animal, the background and the objects. Another critical component that will influence tracking choice is whether the lighting is even during the trial. Shadows from the walls of boxes commonly require a tracking or detection setting of differencing or gray scaling. It is also important to ensure that there is a high level of contrast between the animal and the objects to allow for good discrimination between the two.

Problem 4

Animals not responding to the shock (step 18).

Potential solution

Careful attention to the shock intensity is required. The shock is delivered by a large rechargeable battery. After a full day of testing, it is critical that the battery be charged to ensure that animals receive the appropriate shock level. If the shock intensity does not reach the appropriate level, then the trials should cease until the battery has been charged.

Problem 5

Animals not receiving an appropriate shock (step 18).

Potential solution

Scat can get stuck between the metal bars that create the grid. This may alter the resistance and hence lower the shock being delivered to the animal. It is therefore critical that the grid remain clean and devoid of scat and urine, and it must be cleaned after each animal. Minimizing aversive cues such as urine and scat scent is required to minimize the stress of the mice so that they can focus on learning the task.

Problem 6

Tracking showing bad or lost frames in APA (step 18).

Potential solution

Accurate tracking of the mouse is essential. Poor tracking will result in inaccurate parameters being obtained. If the tracking is intermittent the mouse may also enter the aversive zone and not receive the shock immediately, thereby preventing effective learning. Very aged mice that have thin fur can be difficult to track. Habituation is therefore critical to ensure that effective tracking settings are established. Having the room too bright can cause anxiety for the animals and may also cause reflections from the clear plastic arena, resulting in inaccurate tracking. Lost frames refer to an issue when the computer is unable to collect the video efficiently. This can occur if the computer memory is too

full or if multiple tasks are being undertaken on the computer during acquisition of the video. For a 10 min trial anything over 600 bad or lost frames is considered poor tracking.

Problem 7

Ineffective learning strategies (step 18).

Potential solution

Visual cues must be easily discriminated. Having cues that are too similar may prevent effective learning strategies.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr Tara Walker (t.walker1@uq.edu.au).

Materials availability

This study did not generate any unique reagents.

Data and code availability

This study did not generate any new datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101500>.

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AUTHOR CONTRIBUTIONS

D.G.B. and T.L.W. wrote the manuscript. D.G.B., D.B., and T.L.W. prepared the figures. All authors edited and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Akirav, I., and Maroun, M. (2006). Ventromedial prefrontal cortex is obligatory for consolidation and reconsolidation of object recognition memory. *Cereb. Cortex* 16, 1759–1765. <https://doi.org/10.1093/cercor/bhj114>.
- Assini, F.L., Duzzioni, M., and Takahashi, R.N. (2009). Object location memory in mice: pharmacological validation and further evidence of hippocampal CA1 participation. *Behav. Brain Res.* 204, 206–211. <https://doi.org/10.1016/j.bbr.2009.06.005>.
- Barnes, C.A. (1979). Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. *J. Comp. Physiol. Psychol.* 93, 74–104. <https://doi.org/10.1037/h0077579>.
- Beeler, J.A., Prendergast, B., and Zhuang, X. (2006). Low amplitude entrainment of mice and the impact of circadian phase on behavior tests. *Physiol. Behav.* 87, 870–880. <https://doi.org/10.1016/j.physbeh.2006.01.037>.
- Cimadevilla, J.M., Fenton, A.A., and Bures, J. (2000a). Functional inactivation of dorsal hippocampus impairs active place avoidance in rats. *Neurosci. Lett.* 285, 53–56. [https://doi.org/10.1016/s0304-3940\(00\)01019-3](https://doi.org/10.1016/s0304-3940(00)01019-3).
- Cimadevilla, J.M., Fenton, A.A., and Bures, J. (2001). New spatial cognition tests for mice: passive place avoidance on stable and active place avoidance on rotating arenas. *Brain Res. Bull.* 54, 559–563. [https://doi.org/10.1016/s0361-9230\(01\)00448-8](https://doi.org/10.1016/s0361-9230(01)00448-8).
- Cimadevilla, J.M., Kaminsky, Y., Fenton, A., and Bures, J. (2000b). Passive and active place avoidance as a tool of spatial memory research in rats. *J. Neurosci. Methods* 102, 155–164. [https://doi.org/10.1016/s0165-0270\(00\)00288-0](https://doi.org/10.1016/s0165-0270(00)00288-0).
- Haettig, J., Stefanko, D.P., Multani, M.L., Figueroa, D.X., McQuown, S.C., and Wood, M.A. (2011). HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. *Learn. Mem.* 18, 71–79. <https://doi.org/10.1101/lm.1986911>.
- Harrison, F.E., Hosseini, A.H., and McDonald, M.P. (2009). Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks. *Behav. Brain Res.* 198, 247–251. <https://doi.org/10.1016/j.bbr.2008.10.015>.

Leiter, O., Zhuo, Z., Rust, R., Wasielewska, J.M., Grönnert, L., Kowal, S., Overall, R.W., Adusumilli, V.S., Blackmore, D.G., Southon, A., et al. (2022). Selenium mediates exercise-induced adult neurogenesis and reverses learning deficits induced by hippocampal injury and aging. *Cell Metabol.* 34, 408–423.e8. <https://doi.org/10.1016/j.cmet.2022.01.005>.

Murai, T., Okuda, S., Tanaka, T., and Ohta, H. (2007). Characteristics of object location memory in mice: behavioral and pharmacological studies. *Physiol. Behav.* 90, 116–124. <https://doi.org/10.1016/j.physbeh.2006.09.013>.

Panadeiro, V., Rodriguez, A., Henry, J., Wlodkowic, D., and Andersson, M. (2021). A review of 28 free animal-tracking software applications: current features and limitations. *Lab. Anim.* 50, 246–254. <https://doi.org/10.1038/s41684-021-00811-1>.

Pennington, Z.T., Dong, Z., Feng, Y., Vetere, L.M., Page-Harley, L., Shuman, T., and Cai, D.J. (2019). ezTrack: an open-source video analysis pipeline for the investigation of animal behavior. *Sci. Rep.* 9, 19979. <https://doi.org/10.1038/s41598-019-56408-9>.

Reiserer, R.S., Harrison, F.E., Syverud, D.C., and McDonald, M.P. (2007). Impaired spatial learning in the APPSwe + PSEN1DeltaE9 bigenic mouse model of Alzheimer's disease. *Gene Brain Behav.* 6, 54–65. <https://doi.org/10.1111/j.1601-183X.2006.00221.x>.

Vogel-Ciernia, A., and Wood, M.A. (2014). Examining object location and object recognition memory in mice. *Curr. Protoc. Neurosci.* 69, 8–31. <https://doi.org/10.1002/0471142301.ns0831s69>.

Vukovic, J., Borlikova, G.G., Ruitenberg, M.J., Robinson, G.J., Sullivan, R.K.P., Walker, T.L., and Bartlett, P.F. (2013). Immature doublecortin-positive hippocampal neurons are important for learning but not for remembering. *J. Neurosci.* 33, 6603–6613. <https://doi.org/10.1523/JNEUROSCI.3064-12.2013>.

Willis, E.F., Bartlett, P.F., and Vukovic, J. (2017). Protocol for short- and longer-term spatial learning and memory in mice. *Front. Behav. Neurosci.* 11, 197. <https://doi.org/10.3389/fnbeh.2017.00197>.

Willis, E.F., MacDonald, K.P.A., Nguyen, Q.H., Garrido, A.L., Gillespie, E.R., Harley, S.B.R., Bartlett, P.F., Schroder, W.A., Yates, A.G., Anthony, D.C., et al. (2020). Repopulating microglia promote brain repair in an IL-6-dependent manner. *Cell* 180, 833–846.e16. <https://doi.org/10.1016/j.cell.2020.02.013>.