

Video Article

Analyzing Spatial Learning and Prosocial Behavior in Mice Using the Barnes Maze and Damsel-in-Distress Paradigms

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Abstract

The Barnes maze is a reliable measure of spatial learning and memory that does not require food restriction or exposure to extremely stressful stimuli. The Barnes maze can also assess other mouse behaviors, such as general motivation to escape from the maze platform and exploratory behavior. The Barnes maze can measure whether a genetic mutation or environmental variable can impact the acquisition and retention of spatial memories, as well as provide information about the search strategy employed by the mice. Here we use the Barnes maze to detect a memory deficit in adult mice following a single developmental ethanol exposure event. The newly described Damsel-in-Distress paradigm exposes a male mouse to a female mouse trapped in a chamber in the open center field of the arena. It provides an opportunity for the mouse to socially respond to the trapped female and exhibit prosocial behavior. The Damsel-in-Distress paradigm can also be used to examine mouse behavior in a novel arena and measure locomotor activity. Both the Barnes Maze and the Damsel-in-Distress protocols require minimal financial investment and most aspects of the tests can be constructed from common lab supplies. These flexible and accessible tools can also be used to detect behavioral changes over the course of development.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58008/>

Introduction

The purpose of this protocol is to measure spatial learning and memory in mice using the Barnes maze as well as social responsiveness and locomotion using the Damsel-in-Distress paradigm. Commonly used spatial learning and memory tests for rodents include the radial arm maze, which measures a mouse's ability to find hidden food in one arm of a multi-spoked apparatus, and the Morris water maze, which places a mouse in a large tub or pool of water and assesses how long it takes to find a hidden underwater platform. Typically, training for these paradigms spans multiple trials and allows the measuring of both learning acquisition rates and retention through short term and long-term memory trials.

Although the radial-arm maze and Morris water maze are reliable ways to test memory in rodents, they present complications for some researchers. Both mazes use deprivation or strong aversive stimuli as reinforcement¹. The radial arm maze uses food deprivation to ensure that rodents are properly motivated to find a food reward. In the Morris water maze, the effects of stress induced by a forced swim may alter results in the mice².

The Barnes maze is an alternative spatial awareness task that requires rodents to learn the position of a hole in order to escape the bright, open maze surface³. Weak aversive stimulation (light or sound) is then applied to increase the likelihood that mice will escape from the platform. The Barnes maze does not require food deprivation, so the amount of animal preparation is less than in the radial arm maze. It can be used without conflict by researchers who are investigating behaviors or molecules associated with eating, hormonal regulation, or hypothalamic pathways.

The Barnes maze also has advantages over the Morris water maze. It is less stressful than the Morris water maze, causing less elevated levels of corticosterone in mice⁴. In addition, it is much simpler to assemble and requires less dedicated space during the testing procedure and storage.

The Damsel-in-Distress assay is a simple two-part experiment that can assess locomotor activity followed by prosocial behavior. The Damsel-in-Distress assay is designed to assess exploratory behaviors and social responsiveness of a male rodent in response to an entrapped female rodent. A commonly used method to assess sociability (as well as preference for social novelty) is the use of Crawley's three-chamber sociability test, which assesses the free choice of a mouse to spend time near or away from other mice⁵.

Similar to Crawley's three-chamber sociability test, the Damsel-in-Distress assay also measures free choice regarding how to spend time in the presence of another mouse, but it also provides measurements for deeper aspects of social functioning: 1) In the Damsel-in-Distress assay, the trapped female mouse is held in the center of an open field, so the male's potential aversion to an open field is pitted against his drive to socially explore or investigate a distressed female conspecific. 2) The Damsel-in-Distress model also provides a way to assess prosocial and empathetic behavior in response to the trapped mouse, which has not been frequently explored in mice.

Animal empathy is definitely observable and measurable, though not many paradigms exist for this purpose. For example, in rats, a trapped cage mate can induce a state of prosocial motivation, where rats will work to free the trapped animal. In fact, rats will choose to help the trapped animal even before opening a similar container containing a chocolate snack, then access the chocolate and share it with the newly freed rat⁶.

Measures of empathy in mice typically involve the infliction of pain; indeed, mice who watch other mice in pain are more sensitive to pain themselves^{7,8}. Restraint stress is a characterized phenomenon in rodents and has been coupled with shock to examine stress responses, as one measure of empathy in mice^{9,10}.

There are ethical considerations associated with induced pain or shock, so stress-inducing alternatives are needed. We developed the Damsel-in-Distress paradigm as a measure of empathetic behavior in the absence of either pain or shock treatment. The trapped mice in our Damsel-in-Distress protocol show overt signs of distress after only several moments as they are unable to turn around within the small container, yet they are unharmed while providing an opportunity for other mice to respond to their distress.

To begin the Damsel-in-Distress assessment, a male mouse is placed in a large novel arena containing a small empty central cylinder. Exploratory behaviors are recorded for several minutes, including how many sections of the arena are crossed and how much time is spent in the central open area. This method provides a quick and easy way to rule out locomotor deficits as a potential confounder in a learning situation that requires coordinated movement for successful completion. It also provides a basic measure of how much aversion to the open center field is present. Both measures could influence Barnes maze performance.

Following the initial exploration, the male is removed from the arena and a female mouse is placed in an enclosed, small clear central cylinder (similar to that used to collect blood from mice). Then, the original male mouse is reintroduced into the arena, and exploratory behavior is again scored. The Damsel-in-Distress paradigm assesses whether a mouse is interested in a social interaction based on changes in behavioral patterns when a trapped female is present (scored by time spent in the center square and the number of digging episodes), and whether the mouse exhibits prosocial behavior towards the trapped female (scored by the amount of cylinder investigations and contact events with the trapped female). The Damsel-in-Distress assay can be used to measure a propensity for social novelty (similar to Crawley's three-chamber sociability test) depending on whether researchers trap a familiar or novel mouse.

Together, the Barnes maze and Damsel-in-Distress experiments allow the accurate evaluation of mouse learning capabilities and social responsiveness in the absence of extremely stressful stimuli. As with all behavioral assays, these experiments should be performed with great sensitivity towards the animal experience, minimizing the discomfort experienced by the animal.

Like most mazes, differences in locomotor activity may influence performance on the Barnes maze, so researchers should also assess locomotor activity, especially when using the Barnes maze to assess learning in mice with mutations that may impair movement (such as those found in Huntington's disease mouse models, or those exposed to toxins that may induce hyperactivity or retard movement, such as ethanol). In addition, maze and chamber surfaces should be thoroughly cleaned and bedding changed between each animal to avoid scent cue confounds.

Importantly, all materials can be fabricated on site with minimal financial investment and the small physical footprint of these assays means that these experiments can be replicated in almost any setting, allowing for great flexibility. This type of accessibility allows good science to be performed at smaller institutions with limited resources or in situations where pilot data needs to be collected quickly in the absence of substantial support.

Protocol

All methods described here were approved by the Institutional Animal Care and Use Committee (IACUC) of Hampden-Sydney College or Randolph-Macon College (where some work was previously performed).

1. Basic Housing of Mice

1. House the mice in plastic cages with solid bottoms and sides, and a layer of soft bedding and nesting material such as paper shredding. Use bedding that is composed of shredded corn cobs or wood shavings, and ensure that the bedding is changed regularly for sanitation.
2. Provide access to food and water *ad libitum*. The top of the plastic cage houses a food hopper. Dispense properly formulated mouse food pellets from the food hopper and provide a water dispenser. Ensure that the cage top features an air filter to protect mice from outside contaminants.
3. Maintain the natural circadian rhythms of the mice by following a 12-hour light-dark cycle in the housing facility. Conduct behavioral testing at the same time of day, ideally during the animal's dark cycle, such as during the evening hours.
NOTE: Care should be taken to distinguish mice in the housing. Various methods for distinguishing mice are available, such as ear punches, ear tags, and tail markings.

2. Barnes Maze Testing: Construction

1. Obtain a circular wooden board that is 120 cm in diameter.
2. Cut 20 circular holes (4.5 cm in diameter) around the perimeter of the circle. Position each hole to be 2.5 cm away from the maze edge and 13 cm apart from neighboring holes.

3. Smooth the surfaces and paint them glossy white (a bright color is recommended).
 4. Designate one side of the maze board for insertion of small cup hooks roughly 2-3 cm away from each of the 20 holes and place two hooks for each hole (one on either side of each hole).
 5. Use shallow black (plastic) disks for covering holes on side of maze with hooks. Use hooks to secure thick rubber bands in order to hold the disks onto the bottom of the maze.
 6. Securely mount the Barnes maze 120 cm above the ground and away from other similarly tall objects like tables or chairs. A box or stool can be used to support the middle of the maze.
 7. Place large white posters with one shape on each wall (triangle, circle, and cross) as extra-maze cues on 3 sides of the maze. Maintain the posters on the walls around the maze for each trial.
 8. On last side of maze, set up a solid black curtain to hide observers so that data can be recorded accurately without the researchers being visible to mice on the maze.
 9. Suspend a video camera over the arena with a bird's-eye view of the entire maze surface.
 10. Clean every surface of the maze (black disks and target box included) with water then a 70% ethanol solution before and after each mouse trial.
 11. Place a 100 W light source 25 cm over center of maze (turn on/off at the start/finish of trial) and be sure to have the disks and hooks facing the floor.
- NOTE: All other overhead lights in the room should be turned off during testing. The addition of an ultrasonic noise maker hung next to 100 W light is recommended. Turn it on/off at the start/finish of each trial.

3. Barnes Maze Testing: Procedure

NOTE: Ensure that all the maze components are cleaned with water and 70% ethanol solution before and after each trial, allowing for time to dry completely before testing resumes. Be sure to have cleaning supplies ready as well as timers for the trials.

1. House all the mice in groups while using a reliable identification method. Keep mice outside of the testing room when they are not actively running on the maze. This is to ensure they are not subjected to the ultrasonic noisemaker prematurely.
2. Handle mice by gently picking up and holding them from the base of the tail, with the paws kept on one's hand.
3. For training, have the mice run on the maze daily once per day for 7 days in a row to assess learning/acquisition.
NOTE: A single long-term trial can be done on a later date to assess memory/retention. The current protocol trained adolescent mice beginning at postnatal day 32 and a long-term trial was performed in adults at 4 months of age.
4. Randomly assign each mouse to a target hole to use throughout the testing period. The holes can be labeled 1-20 on the bottom of the maze or on the outside of the maze perimeter, where they are not visible to the mouse in the maze.
5. Replace the assigned hole disk with a small black box (23 x 11 cm). Firmly attach it to the Barnes maze using rubber bands connected to the nearest hooks.
NOTE: The target box is shallow enough so that the mouse can easily step down into it or contains a step to ensure that the mouse does not have to jump down into it.
6. Place the mouse onto the center of the maze beneath a cup to acclimate for 30 seconds until the test begins. Begin video recording.
7. Turn on the light and ultrasonic noisemaker. Lift the cup via a string mechanism to avoid biasing the initial heading of the animal. Start a timer and sit behind the curtain to observe.
8. After the mouse enters the target hole, cover the hole with the same opaque cup used at the start of the trial or a binder and turn off the ultrasonic noisemaker. If the mouse has not entered the target after 5 minutes have elapsed, corral the mouse into the target hole.
 1. Ensure the mouse enters the hole. When mouse is inside the target box, cover the hole and turn off the noisemaker. Allow the mouse to remain in the target box for 1 minute undisturbed.
9. Although the target hole will remain constant for each mouse throughout the training period, run mice in a random order each day to ensure that they are not following any cues/scents left by the previous mouse.

4. Barnes Maze Testing: Data Analysis

1. During the Barnes maze trials, record the following timed data: total time spent on maze, time to find target, and time to enter target hole if the target is entered.
2. Track the overall movement of the mouse to determine the number of errors (number of incorrect holes explored) before and after the target is found. Also record the distance from the target hole to the first hole explored (distance is measured in number of holes away), along with any notable grooming behavior.
3. Track the movements of each animal on a piece of paper with a diagram of the maze during each trial and use it to analyze search strategies, as well as determine the number of holes that were explored in the quadrant opposite the target. Use video analysis to confirm the paths.

5. Damsel-in-Distress Testing: Constructing the Restraint Chamber

1. Cut a clear cylinder to a length equal to $\frac{3}{4}$ of the length of the female mouse from the base of the tail to tip of the nose. Ensure that the dimensions of the cylinder are such that the mouse placed inside cannot turn around.
NOTE: We cut the cone end of a 50 mL conical tube down to make a 4 cm long tube for 1-month-old mice and an 8 cm long tube for 4-month-old mice.
2. Cap both ends of the cylinder such that one of the caps can be removed and reattached. Punch 3-4 holes, approximately 0.5 cm each, in each cap. Ensure that the holes are large enough to allow nose-touching between mice and breathing¹².
NOTE: Alternatively, a device for mouse restraint during blood collection can be used.

6. Damsel-in-Distress Testing: Preparing the Arena

1. Ensure that the arena is an opaque topless plastic square box of 38 x 38 cm with 19 cm walls. Fill it uniformly with corn bedding to a height of approximately 2.5 cm.
NOTE: The corncob bedding makes digging events easier to detect.
2. Suspend a video camera over the arena so that the entirety of the arena is in view.

7. Damsel-in-Distress Testing: Exploratory Behavior Measure

1. Place the closed and empty stress chamber into the center of the arena.
2. Begin recording with the suspended camera.
3. Select a male mouse. If the mice are marked for identification, note this male's identity; if not, mark this mouse so that it can be distinguished after returning to the cage.
4. Gently place the male mouse beneath a cup at the lower left corner in the arena. After 30 seconds, remove the cup via a string mechanism.
5. Allow the mouse to explore for 10 min, taking care to stay out of its field of view during recording. At the end of the 10 min, remove the mouse from the arena and return it to a holding cage for the next 5 min¹².
6. Stop recording and save the video file with an appropriate identifier.

8. Damsel-in-Distress Testing: Social Responsiveness Measure

1. Use the same arena and recording setup as in the exploratory behavior measure.
2. Select a female mouse. If the mice are marked for identification, note this female's identity; if not, mark this mouse so that it can be distinguished after it is returned to the cage.
NOTE: A littermate, cage mate, or novel mouse can be used, depending on the research question. If multiple trials are being performed, maintain the type of relationship across all trials.
3. Hold the female mouse gently by the base of the tail, or by neck restraint, if necessary, and lower it into the restraint chamber. Close the open end behind it and ensure that it is unable to turn around.
NOTE: Consider wearing bite-resistant gloves, as the female mouse will be resistant to entering the restraint chamber.
4. Begin recording with the suspended camera.
5. Place the restraint chamber with the trapped female mouse inside in the center of the empathy arena and allow the female to acclimate for 10 min. Take care to remain out of the female's field of view.
6. After the female mouse has been in the restraint chamber for 5 min, place the marked male mouse back into the empathy arena using the same process as before. Allow the male mouse an additional 5 min to explore the arena, again taking care to remain out of the field of view of the mice.
7. Stop recording and save the video file with an appropriate identifier.
8. At the end of the 5 min, remove both mice from the arena and place them into their cages. Replace the corn bedding and sanitize both the arena and the stress chamber with 70% ethanol¹².

9. Damsel-in-Distress Testing: Data Analysis

1. Tracking software can be used, but the video can also be analyzed manually. Once the video file is visible on the computer, overlay a transparent sheet over the screen and outline the square of the arena using a marker. Divide the arena square into nine equal compartments.
2. Review the video data for the first 5 min of the initial exploratory measure. Record the number of compartments crossed (locomotor activity/exploratory behavior), time spent in the center square of the arena (open field aversion), number of digging and grooming episodes, and number of times the mouse touched the empty center restraint chamber.
NOTE: Scoring is not done for the second 5 min of the male's 10-minute initial exploration, nor for the 10 min the female is trapped in the central chamber in the absence of the male.
3. Review the video data for the 5 min of the social responsiveness measure immediately after the male is reintroduced into the arena with the trapped female. Record the same data as for the initial exploratory measure, but additionally record the number of times the male mouse touched noses with the trapped female mouse.

Representative Results

The Barnes Maze

To illustrate how the Barnes maze can be used, we investigated whether a single early ethanol exposure caused a difference in learning over the course of mouse development. C57Bl6/J mice were either injected with a 2.5 g/kg ethanol solution (n = 8) or with saline (n = 6) twice, two hours apart, at postnatal day 6. We trained the animals on the Barnes maze during adolescence (P30), then performed a single long-term trial in adulthood (4 months). Our collected data can be divided into several main categories: 1) accuracy by time and error rate, 2) target entry, and 3) mapping the exploration path.

Accuracy by time and error rate:

Time measurements encompass several important aspects: The latency to reach the first hole (the time it takes for the mouse to investigate any first hole after starting the trial) will illustrate if the mouse is learning that it is supposed to go find the escape hole (**Figure 1A**). The latency to find the target hole (scored by an initial lowering of the nose across the plane of the target hole) is able to find the correct hole (**Figure 1B**). With each session, the time to run the training session should decrease, indicating that the animals are learning the maze.

The number of wrong hole investigations (termed errors) the mouse performs before locating the correct hole (**Figure 1C**) indicates if the animal is able to go relatively straight to the target escape hole. The total number of times a mouse explores an incorrect hole over the course of the trial gives a rudimentary measure of locomotor activity and exploratory behavior. (**Figure 1D**). All mice were successfully trained on the Barnes maze over a 7-day testing period during adolescence (represented here by daily sessions on postnatal days 32-39), indicated by a decrease in error rate and time to find the target hole. There was no statistical difference in learning between the ethanol and control animals over the course of the training period.

Target entry rate:

The latency to actually enter the target hole is commonly used as the criteria for successful maze completion, but likely does not give as much information as other aspects of the testing. Entry into the target can also be scored as a 0 or 1. If the mouse enters the target before the 5 min elapse, the testing is considered successful, but if the mouse does not enter the target, a binder or book should be used to corral the animal into the target so it experiences the safety of the chamber beneath. On the first training days, it should be expected to see low rates of entry into the target and animals that take the full 5 minutes on the maze. In subsequent days, the testing should take less time.

Making multiple errors after finding the target indicates low motivation to enter the target box and may also detect abnormal comfort with a situation that is typically aversive to mice (elevated platform, bright light, open field, ultrasonic noise). A large difference between errors made before finding the target and the total number of hole explorations during a trial indicates poor motivation to enter the target escape chamber (**Figure 2A**). If mice are not entering the target escape hole after discovery, bright lights or noise can be added to the testing situation as mildly noxious stimuli.

In our experiment, an ultrasonic noise emitter was added above the maze on test day 5 to introduce extra motivation to enter the target. Prior to the introduction of the ultrasonic noise, no saline treated mice had ever entered the target, but entrances spiked after the introduction of the noise. Significantly more mice (saline and ethanol combined) entered the target on Trial 6 compared to Trial 4 (*t*-test, $p = 0.0014$) (**Figure 2B**).

Path mapping:

Mapping the path of the mouse can help assess information retention and learning strategies, as well. The distance between the first explored hole and the target hole is a good indicator of memory for target location, as this tracks where the mouse goes first and how close they are to the target (**Figure 3A**). Expect this measure to vary widely at the beginning of training, but the first hole visited should move closer to the target as the mice learn the target location.

Although no statistical difference in learning was found between the ethanol and control animals over the course of the training period, when these mice were re-run as adults using a single long-term trial (at 4 months), the ethanol-injected animals had a harder time remembering where the target hole was located (**Figure 3B**)¹². This difference in memory retention was not found in the initial Barnes maze training, so using the maze again at a later time allowed us to pick up subtle differences in learning/memory, such as those found with a single developmental ethanol intoxication event. A representative diagram of the Barnes maze surface allows researchers to map the path the mouse took, including the number of errors and a record of the first hole visited (**Figure 3C**).

Time spent in the opposite quadrant corresponds to the amount of time a mouse explores one section of the maze over others, specifically the one opposite of the target. It is possible for a researcher to obtain and calculate all other measures as the mouse is running the maze, but this measure requires analysis at a later time. A mouse who is learning should decrease time spent exploring holes in the opposite quadrant. On the long-term adult trial, ethanol treated mice explored significantly more holes in the opposite quadrant from the target, indicating a failure to remember the correct general spatial location ($p = 0.03$) (**Figure 4A**).

It is important to break up this mapping measurements into errors made before and after actually finding the target. The number of errors before finding the target can reveal if learning is occurring, but it also can tell us about the mouse's search strategy. Instead of randomly visiting holes, the mouse may adopt a strategy to quickly find the target. During the long-term trial, all of the ethanol-treated animals used a sequential hole-to-hole search in a ring-shaped fashion until the hole was found, whereas significantly less saline treated mice employed this technique ($p = 0.004$) (**Figure 4B** and **4D**). Alternatively, mice can target a general direction of the maze (**Figure 4C**), which indicates that spatial learning is occurring and the animal is likely using external cues to head for the vicinity of the target hole.

Damsel-in-Distress

The Damsel-in-Distress arena was constructed and used to test the exploratory and social behavior of C57Bl6/J mice at both 1M ($n = 15$) and 4M ($n = 12$) of age. An empathy arena was utilized where the male mouse placed in a corner (**Figure 5A**). Mouse exploratory behavior is quantified as the number of squares crossed in 5 min in the novel arena. Our results show that older mice (4 month) cross more squares than younger mice (1 month) ($p < 0.0001$) (**Figure 5B**).

Next, the trapped female was placed in a cylinder in the middle of the arena (**Figure 5A**). Once the female is trapped in the chamber, exploratory behavior can again be quantified as the number of squares crossed. The presence of the trapped female impacts male exploratory behavior. Regardless of age, male mice explore less in the presence of a trapped female. Juvenile males explored the arena less (as measured by the number of squares crossed in the presence of a trapped female mouse than when alone (at 1 month: *t*-test, $p = 0.001$; mean male alone 46 ± 4.34 squares vs. with female 26.13 ± 3.42 squares). This difference was also apparent in adults (at 4 months: *t*-test, $p < 0.000001$; mean male alone 151.5 ± 5.31 squares vs. with female $103.08 \pm 0.3.59$ squares) (**Figure 5B**).

In addition, the amount of time a mouse spent in the middle square over a 5-minute period should also be assessed. Time spent in the center square should be compared in the presence and the absence of the female as a measure of social interest. Our results show that males spend more time in the center square in the presence of the trapped female. Juvenile males spent more time exploring the center of the arena in the presence of a trapped female mouse than when alone (at 1 month: $p = 0.05$; mean male alone 13.14 ± 2.79 s vs. with female 33.42 ± 9.32 s). This difference was also seen in adults (at 4 months: $p = 0.001$; mean male alone 14.80 ± 1.89 s vs. with female 52.87 ± 8.9 s) (**Figure 5C**).

In addition, researchers should also quantify the number of digging events in the arena, which could be a measure of anxiety or prosocial behavior towards the trapped female. This measure was significantly different in the presence of the female for 1 month (1M) mice but not 4 month (4M) mice. Juvenile males spent more time digging during their time in the arena in the presence of a trapped female mouse than when alone (at 1 month: $p = 0.027$; mean male alone 0.4 ± 0.27 times vs. with female 3.2 ± 1.09 times). This difference was not seen in adults (at 4 months: $p = 0.65$; mean male alone 1.33 ± 0.54 times vs. with female 1 ± 0.49 times) (**Figure 5D**).

The number of times a male touches the restraint chamber containing the trapped female and the number of times the animals touched noses through the air holes are measures of prosocial behavior. In our results, male mice touched the tube containing the trapped female significantly more frequently as adults (4 months) than as juveniles (1 month) ($p = 0.00001$; mean 1 month 4.73 ± 0.95 times vs. 4 months 15.92 ± 1.64 times) (**Figure 5E**). Adult males also initiated more trapped female contact events. Male mice touched noses with the trapped female through the air holes significantly more frequently as adults (4 months) than as juveniles (1 month) ($p = 0.002$; mean 1 month 3.93 ± 1 times vs. 4 months $8.67 \pm .94$ times). (**Figure 5F**).

Ultrasonic recording equipment was used to listen for ultrasonic vocalizations above 20 kHz by female mice, and it was also used to listen for ultrasonic vocalizations by trapped male mice in a "bachelor-in-distress" reversal paradigm. No vocalizations were detected in either situation, which is expected, since previous research found no ultrasonic vocalizations mice during aversive stimuli exposure (such as physical restraint or electric shock)¹¹.

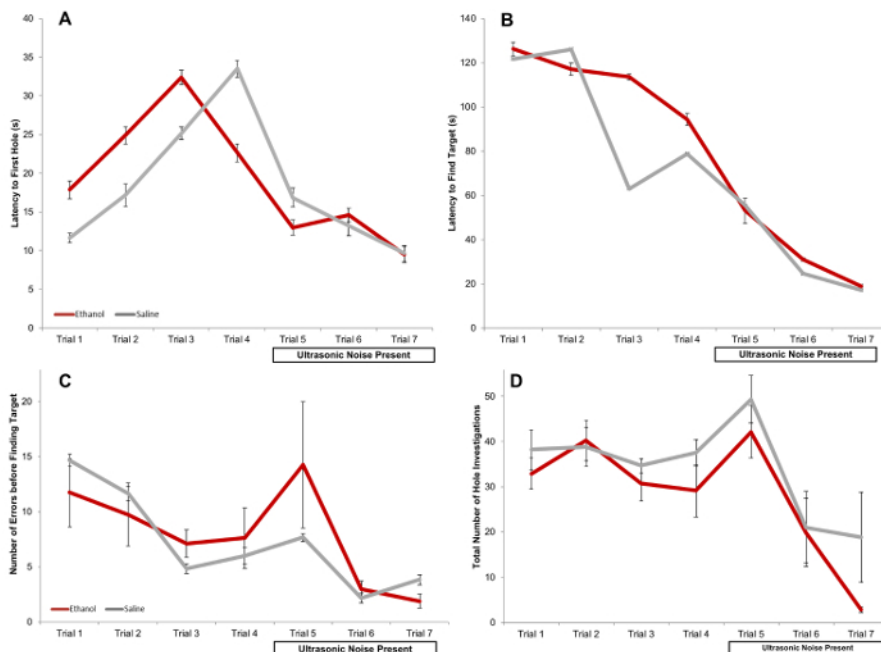


Figure 1: Barnes maze accuracy representative results by time and error rate. Mice were either injected with a 2.5 g/kg ethanol solution or with saline twice 2 hours apart at postnatal day 6. We trained the animals once daily for 7 days during adolescence, then performed a single long-term trial in adulthood¹¹. (A) The latency to reach the first hole (the time it takes for the mouse to investigate any first hole after starting the trial) reveals if the mouse is learning to find the escape hole. (B) The latency to find the target hole (scored by an initial lowering of nose across the plane of the target hole) is able to find the correct hole. (C) The number of wrong hole investigations (termed errors) the mouse makes before locating the correct hole indicates if the animal is able to go relatively straight to the target escape hole. (D) The total number of times a mouse explores an incorrect hole over the course of the trial gives a rudimentary measure of locomotor activity and exploratory behavior. Error bars represent SEM¹¹. [Please click here to view a larger version of this figure.](#)

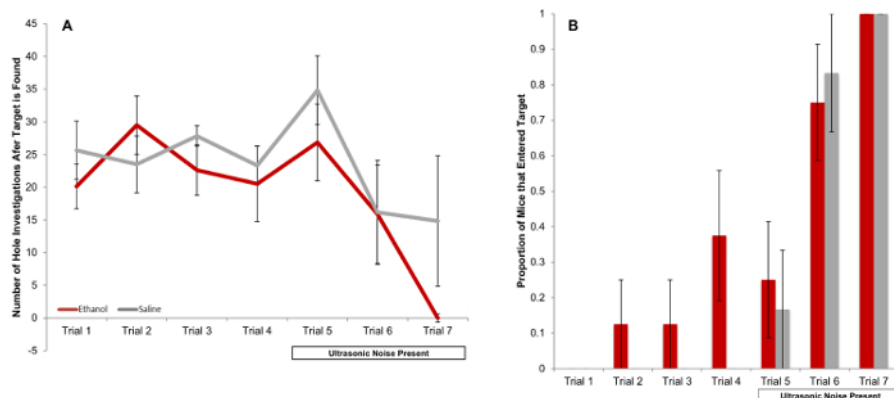


Figure 2: Successful Barnes maze completion. The latency to actually enter the target hole is commonly used as the criteria for successful maze completion, but animals may not enter the target hole after discovery. **(A)** A large difference between errors made before finding the target and the total number of hole explorations during a trial indicates poor motivation to enter the target escape chamber. In our experiment, an ultrasonic noise was added on Trial 5 to induce mice to enter the target. **(B)** Significantly more mice (saline and ethanol combined) entered the target on Trial 6 compared to Trial 4 (t -test, $p = 0.0014$). No ethanol mice kept exploring after finding the target by day 7. Error bars represent SEM. [Please click here to view a larger version of this figure.](#)

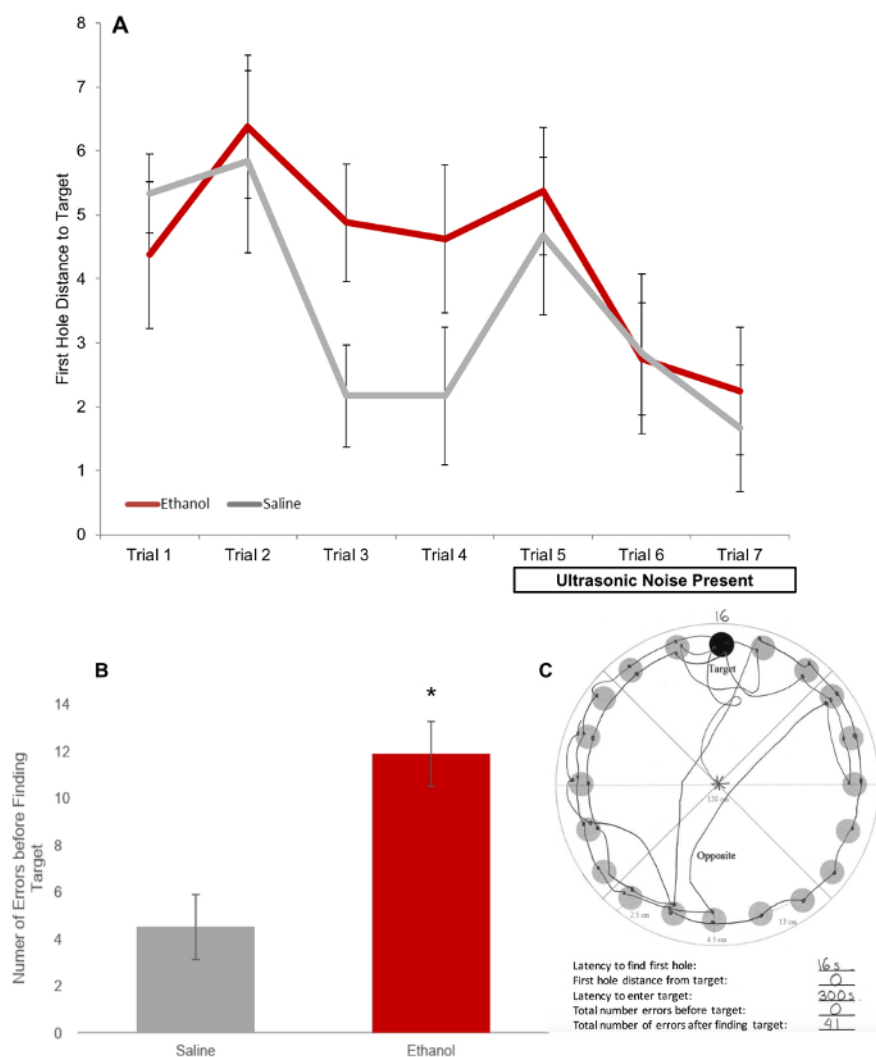


Figure 3: Barnes maze motivation. Mapping mouse paths can provide information about motivation. **(A)** The distance between the first explored hole and the target hole is a good indicator of memory for target location, as this measure tracks where the mouse goes first and how far they are away from the target. **(B)** Although no statistical difference in learning was found between the ethanol and control animals over the course of the training period, when these mice were re-run as adults using a single long-term trial (at 4 months), the ethanol-injected animals had a harder time remembering where the target hole was located. **(C)** A representative diagram of the Barnes maze surface allows researchers to map the path the mouse took, including the number of errors and a record of the first hole visited. This mouse went straight to the target within 16 seconds, but never entered it over the course of 5 minutes and explored 41 holes afterwards. This behavior indicates low motivation to enter the target. Error bars represent SEM. [Please click here to view a larger version of this figure.](#)

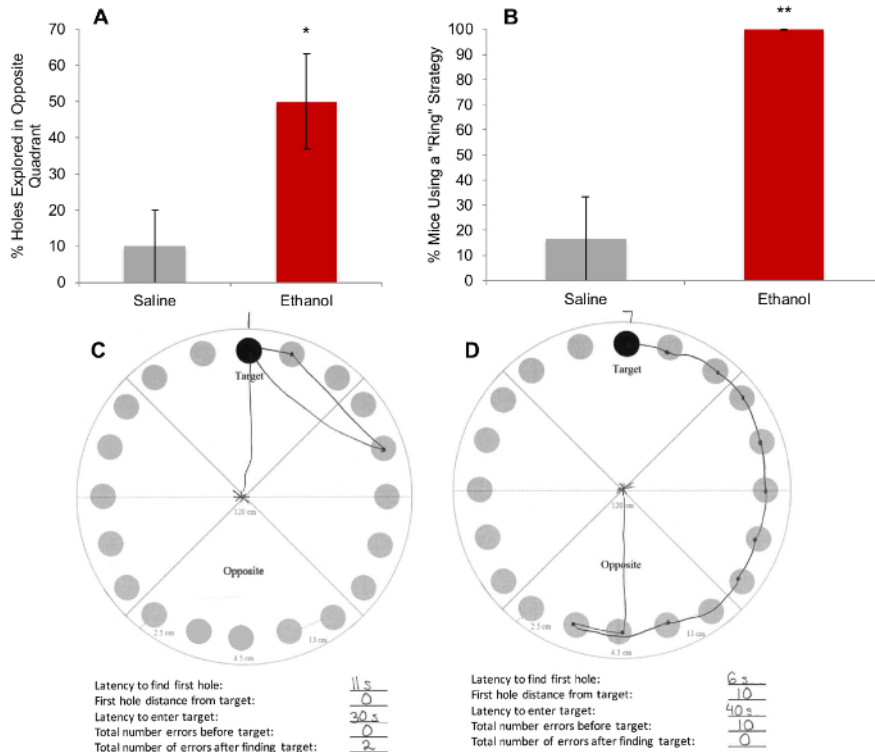


Figure 4: Barnes maze search strategy. Mapping mouse paths can uncover search strategies and provide information about learning strategy. (A) On the long-term adult trial, ethanol treated mice explored significantly more holes in the opposite quadrant from the target, indicating a failure to remember the correct general spatial location (* $p = 0.03$). (B) During the long-term trial, all of the ethanol-treated animals used a sequential hole-to-hole search in a ring-shaped fashion until the hole was found, whereas significantly less of the saline treated mice employed this technique (** $p = 0.004$) (B, D). Trial day 6 results are pictured for a (C) saline treated mouse and (D) ethanol treated mouse. Mice can target a general direction of the maze (C), which indicates that spatial learning is occurring and the animal is likely using external cues to head for the vicinity of the target hole. Typically, quadrant scores are much better for mice using this strategy. (D) The sequential hole-to-hole search is a ring strategy, where the mouse goes to any edge of the maze and looks in each hole to find the target. Quadrant scores are poor with great variation when mice use the ring strategy. Error bars represent SEM. [Please click here to view a larger version of this figure.](#)

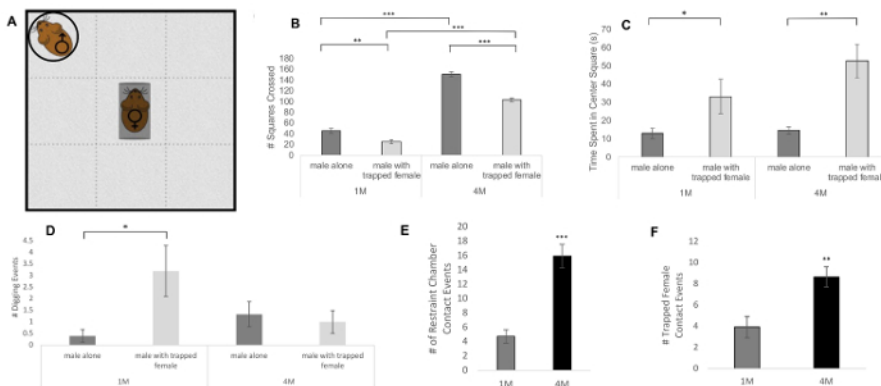


Figure 5: Damsel-in-distress. (A) The Damsel-in-Distress arena is divided into 9 squares. The male mouse is placed in a corner and male behavior is observed. The female mouse, trapped in a cylinder container, is placed in the central square of the arena. (B) Exploratory behavior within the arena increases between adolescence (1 month = 1M) and adulthood (4 months = 4M). Overall, male mice explored less in the presence of a trapped female. In addition, juvenile mice explored less than adult mice in the presence of the trapped female. (C) Social responsiveness can also be measured by the time a mouse spends in the center square where the trapped mouse is held. Both juvenile and adult males spent more time in the center square in the presence of the trapped female. (D) Juvenile males spent more time digging during their time in the arena in the presence of a trapped female mouse than when alone. (E) Male mice touched the tube containing the trapped female significantly more frequently as adults than as juveniles. (F) Adult males also initiated more trapped female contact events than juveniles. Error bars represent SEM (*** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$). [Please click here to view a larger version of this figure.](#)

Discussion

The Barnes maze and the Damsel-in-distress experiments are inexpensive, quick, and relatively easy ways to evaluate spatial learning, locomotor activity, and prosocial behavior in mice. Other advantages include the absence of overt stressors, pain, or food restriction for the

animal. Like most learning/memory paradigms, a disadvantage of the Barnes maze is the number of trials required for the animals to learn where the target hole is located and enter.

Data collection:

Data should be consistently gathered when observing the mouse's activities and behaviors in both paradigms. For the Barnes maze, this includes recording the time the first hole is reached, the number of errors/wrong holes it explores, the time at which it reaches the target hole, and the time at which it enters into the target hole. In addition, researchers should map the path of each mouse on the maze. Data can be tracked using a paper map of the Barnes maze at the same time that the mouse is running the maze, and the timing and hole numbers can be confirmed using video footage. When tracking the animal, it may be helpful to change pen colors once every minute to keep track of potentially overlapping hole explorations. Computer-assisted tracking systems are available to ensure accuracy and reliability, such as idTracker, Ctrax, or tracktor, and a few are free or open-source.

For the Damsel-in-Distress assay, recorded data should include how many squares the male enters, the time it takes for the male to reach the female, how much time the male spends in the center square when the trapped female is present compared to only the empty cylinder, how many contact events there are with the cylinder, the number of times the animals touched noses through the chamber, and how many times digging events occurred overall and in the center square.

Constants in behavioral testing:

As with all behavioral tests, it is critical that conditions are kept constant between and within animal trials to ensure that environmental variables do not confound the results of the study. Visual, auditory, or olfactory stimuli in the experimental environment can cause behavioral changes in the mice, whether intended or not¹³. As such, care should be taken to shield the mice from distracting external stimuli¹⁴. Both the arena used for the Damsel-in-Distress scenario and the Barnes maze should be cleaned after each trial with water and sanitized with 70% ethanol to ensure that no lingering indicators of previous mice influence a later trial. In addition, bedding should be changed after each Damsel-in-Distress assay. Data collection should always be recorded on video to allow the experimenters to review the data and ensure accurate analysis. It can be a challenge for the researcher to remain hidden behind a curtain or otherwise out of sight while collecting the data, but this is important to maintain a constant, distraction-free setting for the mice.

Barnes maze paradigm design and modifications:

There are multiple published paradigms in use for the Barnes maze. Barnes maze training protocols typically range from 7-15 days, but the protocol can be shortened. Sometimes truncating the number of training sessions to make learning the task harder can isolate differences that longer protocols miss¹⁵. It can be used with only an overhead light (as in the original Barnes study), or other elements can be added (such as a brighter light, a fan, or a noise) in order to make the mice more motivated to enter the escape chamber^{3,16}. If you observe poor motivation to enter the target box during a pilot study, the addition of these elements should be considered during the actual training.

Entry into the correct target hole will likely occur less rapidly without these elements, but researchers should be aware that these additions may be stress-inducing stimuli. The presence or absence of aversive stimuli can be manipulated to assess motivation to enter the target hole. Our study showed that prenatal ethanol had an effect on the likelihood that a mouse would enter the escape hole during the training period when only the bright light was used, and this phenomenon was distinct from actually learning where the target hole was located.

As seen in days 1-4 of our protocol, the mice were not very motivated to enter the hole, though they seemed to find it easily enough. However, when the ultrasonic noise was placed above the maze on day 5, scores improved on most Barnes measures, including the likelihood that mice would enter the target chamber after finding it. The noise appeared to dramatically reduce the number of extraneous hole investigations on trial day 6 and 7, and by day 7 no ethanol mice kept exploring after finding the target (**Figure 2A**). Though it is possible this is a natural consequence of learning the maze, the animals did appear more agitated after the introduction of the ultrasonic noise and less likely to explore the maze. In order for the chamber to remain a rewarding situation for the mice, the researchers must also be careful to turn off the aversive stimuli (cover the hole and turn off the noise) and allow the animal to remain undisturbed in the chamber for the allotted amount of recovery time before removing the animal.

The design of the Barnes maze can have an impact on the learning and memory assessment. The current protocol uses extra-maze cues. In addition to the asymmetry of the testing room, posters of four simple black-on-white-background shapes (square, triangle, circle, cross) were mounted on the walls of the room as visual cues. The Barnes maze can be solved with or without the distal cues on the walls of the testing room or can be solved using a serial search strategy. Because of this, using only a "first hole distance from target" measurement may miss relevant differences in the way that the mouse solves the maze and may not differentiate spatial from non-spatial abilities¹⁷. Multiple analysis measures should be used, such as also examining the number of holes explored in the opposite square or the number of errors before the target. The pattern of search strategy can also be analyzed.

Different inbred strains of mice can have variable performance on the Barnes maze, possibly due to differences in visual acuity. If a strain of mice with poor visual acuity is chosen for use in the Barnes maze, the addition of a small wall around the edge of the maze may increase thigmotaxis (wall-hugging behavior) and increase the use of a non-visual search strategy. Males had better learning performance than females in DBA/2J and C3H/HeJ strains, and although only males were used in the current protocol, C57/Bl6 mice have not been found to display gender differences in memory on the Barnes maze¹⁸.

Damsel-in-Distress paradigm design and modifications:

For the Damsel-in-Distress experiment, a major limitation is that there is no way for the exploratory mouse to free the female mouse, or "damsel," from the tube in the center of the arena. Redesigning the chamber to contain a release mechanism would be an interesting future modification. A disadvantage of the Damsel-in-Distress is the absence of comparative studies using the paradigm, as it is a new assessment for measuring prosocial behavior.

The Damsel-in-Distress could be further modified to assess sociability (defined as the inclination to spend time with another mouse), preference for social novelty (the inclination to investigate a novel mouse compared to a familiar mouse), mating or aggressive behaviors (trapping a female in estrus or a novel male in the chamber), and evolutionary biology issues, such as the impact of shared genetic background on altruistic behavior tendencies (the response to a trapped littermate compared to a trapped non-littermate mouse)¹⁹.

Disclosures

The authors have nothing to disclose.

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