Video Article

# Morris Water Maze Test: Optimization for Mouse Strain and Testing Environment

Daniel S. Weitzner[1], Elizabeth B. Engler-Chiurazzi[2], Linda A. Kotilinek[3], Karen Hsiao Ashe[3,4,5], Miranda Nicole Reed[1,6]

[1]Department of Psychology, Behavioral Neuroscience, West Virginia University

[2]Department of Physiology and Pharmacology, West Virginia University

[3]Department of Neurology, N. Bud Grossman Center for Memory Research and Care, University of Minnesota

[4]Department of Neuroscience, N. Bud Grossman Center for Memory Research and Care, University of Minnesota

[5]GRECC, VA Medical Center

[6]Center for Neuroscience, Center for Basic and Translational Stroke Research, West Virginia University

Correspondence to: Miranda Nicole Reed at Miranda.Reed@mail.wvu.edu

URL: http://www.jove.com/video/52706
DOI: doi:10.3791/52706

Keywords: Behavior, Issue 100, Spatial learning, spatial reference memory, Morris water maze, Alzheimer's disease, behavior, tau, hippocampal-dependent learning, rTg4510, Tg2576, strain background, transgenic mouse models

Date Published: 6/22/2015

Citation: Weitzner, D.S., Engler-Chiurazzi, E.B., Kotilinek, L.A., Ashe, K.H., Reed, M.N. Morris Water Maze Test: Optimization for Mouse Strain and Testing Environment. *J. Vis. Exp.* (100), e52706, doi:10.3791/52706 (2015).

## Abstract

The Morris water maze (MWM) is a commonly used task to assess hippocampal-dependent spatial learning and memory in transgenic mouse models of disease, including neurocognitive disorders such as Alzheimer's disease. However, the background strain of the mouse model used can have a substantial effect on the observed behavioral phenotype, with some strains exhibiting superior learning ability relative to others. To ensure differences between transgene negative and transgene positive mice can be detected, identification of a training procedure sensitive to the background strain is essential. Failure to tailor the MWM protocol to the background strain of the mouse model may lead to under- or over- training, thereby masking group differences in probe trials. Here, a MWM protocol tailored for use with the F1 FVB/N x 129S6 background is described. This is a frequently used background strain to study the age-dependent effects of mutant P301L tau (rTg(TauP301L)4510 mice) on the memory deficits associated with Alzheimer's disease. Also described is a strategy to re-optimize, as dictated by the particular testing environment utilized.

## Video Link

The video component of this article can be found at http://www.jove.com/video/52706/

## Introduction

Transgenic mouse models have been instrumental in evaluating the pathophysiology of Alzheimer's disease (AD), as well as the potential of therapeutic interventions. Cognitive tasks, such as the Morris water maze (MWM), are commonly used with these models to identify the molecular correlates of memory deficits and to assess the efficacy of pre-clinical drugs. It is crucial, however, that the dynamic range of the cognitive task be wide enough to detect subtle treatment effects. With mouse models of AD, cognitive deficits are typically age-dependent, and mice display progressive declines in performance (*e.g.*, [1]). Use of a sensitive cognitive task can allow detection of subtle differences earlier in the animal's life, thereby reducing the costs associated with aging animals. For example, reducing the number of training trials in the hippocampal-dependent Barnes maze from 15 to 5 increased the difficulty of the task, resulting in the detection of deficits in the 3xTg model at an earlier age than previously reported [2]. Earlier detection of deficits not only offers considerable time and cost savings, it also increases the likelihood that the molecular changes underlying cognitive deficits can be identified.

One factor influencing the sensitivity of cognitive tasks is the genetic background strain of the mouse model. For example, BALB/c mice exhibit superior performance in learning and memory tasks compared to other strains, such as the C57BL/6 [3]. The F1 FVB/N x 129S6 background is used for two of the most widely employed models of AD, the Tg2576 and rTg(TauP301L)4510 models. This strain exhibits superior learning ability in the MWM relative to other strains, including B6/SJL mice [4]. Because of this superior learning ability, the use of a single probe after extensive training may mask group differences resulting from over-training. In addition, the sensitivity of probe trials may be age-dependent. We have previously shown that earlier probe trials, after limited hidden platform training, are more sensitive to differences in young Tg2576 compared to young transgene-negative littermate controls than are probe trials inserted after more extensive training [5]. In contrast, probe trials following extensive training are more sensitive in older (20-25 month) Tg2576 mice compared to older littermates than are earlier probe trials [5]. By interspersing probe trials throughout training, the likelihood that a sensitive trial will be identified is increased, particularly if longitudinal testing is performed and the sensitivity of a particular probe trial is age-dependent. **Figure 1** shows the superior performance of F1 FVB/N x 129S6 mice under the protocol optimized for this strain as compared to mice of the B6/SJL background trained under a protocol with more extensive training.

 

The MWM is generally thought to provide reliable measures that are reproducible across both time and laboratories [6]. For example, the primary protocol originally used by our Minnesota laboratory [1,7] was successfully implemented with minor modifications at West Virginia University [8]. Similarly, equivalent levels of impairment were observed in rTg(TauP301L)4510 mice relative to control littermates if housed under pathogen-free or conventional conditions [9]. However, the testing environment can influence the sensitivity of the MWM task. Factors such as room lighting, air vents, temperature gradients, and noises all contribute to environmental cues [4] that can ultimately influence performance. When our Minnesota laboratory and vivarium were moved to a new building, up to a 38% reduction in wild-type performance was observed, substantially reducing the dynamic range of the task and the ability to detect transgene-related deficits. This change in performance occurred despite designing the testing room to be of equivalent size and configuration, and using the same applied visual cues. A “re-optimizing” of the original protocol was required to increase the dynamic range of the MWM task in the new testing environment.

Here the original protocol tailored for use with the F1 FVB/N x 129S6 background [5] is described. Because some studies suggest stress is associated with poor MWM performance [10] and pre-handling can alleviate this stress-induced deficit in performance [11], a pre-handling protocol was designed to acclimate the mice to the introduction and removal of the pool prior to MWM testing. Following pre-handling, mice undergo visible platform training, in which a raised platform is marked with a flag. Visible platform training is used to identify mice with performance problems related to sensorimotor abnormalities. Using exclusion criteria described in the protocol section, performance-incompetent mice are removed from subsequent examinations of hidden platform training and probe trials. Impairments in hidden platform training and probe trials are interpreted as cognitive deficits because sensorimotor performance is factored out of the data. After completion of visible platform training, mice begin hidden platform training where the platform is submerged in water and remains in the same position relative to external cues. Trials in which the platform is removed (probe trials) are interspersed throughout hidden platform training to assess the influence of additional training. Because probe trials occur at the beginning of each day, before additional hidden platform training, probe trials measure the ability of the animal to remember the location of the platform following a 20 hr delay, considered a measure of reference memory [12]. Finally, ways in which this original protocol was re-optimized when changes in the testing environment disrupted control performance are described.

## Protocol

All experimental procedures were conducted in accordance with the standards of the Institutional Animal Care and Use Committee (IACUC) and approved by West Virginia University’s IACUC.

# 1. Pre-handling

1. Set Up the Pool
    1. Prop the pre-handling pool so it is raised to a comfortable height.
    2. Place 2 L of water (21 °C) in the pre-handling pool to a level of approximately 1 cm.
    Note: Do not add coloring.

2. Procedure
    1. Bring the mice from the vivarium to the testing room.
    2. Verify the subject identification (subject number, tail tattoo, ear clip, *etc*.).
    3. Mark the mouse’s tail with a permanent marker to distinguish mice within the cage that have undergone pre-handling testing from those that remain to be tested. Mark all mice in one cage with the same color.
    4. Place a mouse in the transfer beaker for approximately 5 sec. Then, gently pour the mouse out of the beaker into the pre-handling pool.
    5. Using a timer, allow the mouse to remain in the pool for 20 sec.
    6. After 20 sec, gently place the scoop (without angling it) in front of the mouse. Briefly allow the mouse to explore the scoop, and if needed, encourage the mouse to get on the scoop by gently sliding the scoop under the mouse while slowly lifting. Take care not to frighten the mice with the scoop.
    7. Once the mouse is in the scoop, transport it back to the holding cage.
    8. Repeat these procedures for each mouse in the cage once per day for 10 days. At regular intervals (*e.g.* after each animal), pick up fecal boli and bedding from the pool using a net.
    9. Rinse the transfer beaker with water at regular intervals (*e.g.* following the completion of a cage of mice).
    10. Once these steps are completed, rinse the transfer beaker with water several times, dump out water from the pool, and rinse the pool approximately two times with water.

# 2. Visible Platform Training

1. Setup the Mouse Performance Tracking Software *e.g.* Viewer (See **Table of Materials**).
    1. Calibrate the video to match the dimensions of the maze under the 'Configuration' –“Filters and Objects” tab.
    2. Set filters so that the software can differentiate a mouse from the background.
        1. Adjust the “sensitivity” beneath the “Background filter” area so that the tracking software identifies the animal.
        2. Click the “accept” option once the tracking software identifies the animal. Choose the “edit” option to blackout areas outside the maze area.
        3. Select the appropriate “animal filter” and “min. animal size”.

2. Creating the Run Sheets
    1. Create a run sheet listing each mouse, each potential location, and a space for notes containing information about aberrant behavior.

2. Predetermine locations for the visible platform test that are the same for each group of mice. Thus, for the first trial, pre-determine that the platform will be placed on the side opposite to where the mouse is placed into the maze, on the right side close to the wall. For the second trial, pre-determine that the platform will be placed in a different location along the back wall for each mouse and that this will continue for each of the 6 trials.

3. Set Up the Room
    1. Hang curtains around the room so that any spatial cues are obscured.

4. Set Up the Tub.
    1. Place the tub so it matches the configuration in the tracking software by ensuring the maze matches up with the configuration setup on the “Configuration” –“Zone Definition” tab. Note: It helps to ensure the tub is in the proper location prior to filling it with water as the tub is not easily relocated once filled.
    2. Fill the tub with tap water, and using a thermometer, ensure the water temperature is approximately 21 °C. Ensure that the water remains within one degree of this temperature throughout testing, as colder water can impact performance especially when using aged mice [8].
    Note: Do not add coloring.
    3. Using the predetermined locations, place the platform, which has a mounted flag that reaches a height of 13 cm and is 4.5 cm x 4.5 cm with a bold “S” shaped character embossed on it, in the first location of the tub so that it is approximately 1 inch above the surface of the water.

5. Procedure
    1. Take the first mouse from the cage, verify the subject identification, mark the tail using a permanent marker of the correct color, and place in the beaker.
    2. Gently place the mouse into the tub, start the timer, and ensure that the tracking software is tracking the animal. Allow the mouse up to 2 min to locate the platform. Once the mouse locates the platform, allow the mouse 20 sec on the platform.
    3. If the animal finds the platform and proceeds to jump or fall off, scoop the mouse and place the mouse on the platform. If the mouse continues to jump or fall off the platform, hold the mouse on the platform, so the mouse learns that remaining on the platform leads to escape from the tub.
    Note: The mouse should remain on the platform for a combined time of 20 sec; thus, if the mouse jumps off after 5 sec, the amount of time left on the platform is 15 sec and does not reset to 20 sec.
    4. If the animal fails to find the platform in the 2 min period, scoop the mouse and place it on the platform for 20 sec.
    5. Following 20 sec on the platform, remove the mouse from the platform as done in pre-handling training and place the mouse in the heated cage lined with paper towels and warmed to ~31 °C by a heating pad and heat lamp for 30 sec. Perform periodic assessment of body temperature with a rectal thermometer to ensure that certain strains of mice or mice at a certain age are not differentially susceptible to hypothermia induced by exposure to the water.
    6. After 2 min to the holding cage with the heating lamp, transfer the mouse to the holding cage with only a heating pad, but no heat lamp, to recover between trials. Eventually, place all mice from a single home cage into the same holding cage during testing.
    7. Clean the maze of debris with a net after each mouse has completed a trial, to disrupt olfactory cues.

6. Repeat these steps for each mouse in the group. After each mouse has completed the first trial, move the platform to the second predetermined location.
Note: The platform will remain in the same location for each mouse during a single trial and will be moved to the other predetermined locations for the ensuing trials. This is done to ensure that mice 1) learn there is a platform to escape from, 2) swim directly to the visible platform, thus demonstrating intact visual competence, and 3) have no motor deficits.
7. Perform these procedures for 6 trials for the first 3 days of testing. Thus, perform visible training on testing days 1, 2, and 3, with approximately 10 min between each trial (**Figure 2**).
8. During visible platform training, use the animal tracking software to measure latency to reach the platform, track the pathlength of the animal, and swim speed. Obtain these measurements from the “Water maze” tab under “Experimental list” and export directly to an excel spreadsheet.
Note: The use of a hand timer can also be beneficial to ensure the accuracy of the tracking software.

## 3. Morris Water Maze Hidden Platform Training

1. Set Up the Room
    1. Hang up curtains in the room to obscure spatial cues in the laboratory.
    2. Place cues strategically on the curtains. Ensure that the cues are large and contain contrasting colors (ex.: black and white) for better visibility. Hang cues at a distance and height where they are visible to mice from inside the tub. Note: The cues are static and do not move during the course of testing.

2. Setup the Mouse Performance Tracking Software
    1. Calibrate the video to match the dimensions of the maze under the 'Configuration' –“Filters and Objects” tab.
    2. Set the filters so that the software can differentiate the mice from the background.
        1. Use the same procedures as described in the visible platform testing (steps 2.1.2.1-2.1.2.3) to set up the filters for Morris water maze hidden platform training.

    3. Create four equal quadrants inside the maze
        1. Click the “Ellipse” option under the “Zone Definition” tab first, and create a circle that matches the maze on the screen. Note: On this tab, the actual maze set up is displayed, so it allows the option to match the computer setup with the actual maze setup.

2. Click the “Rectangle” option and create four equal squares. Place these squares on the screen to create four equal quadrants within the newly created circle.

4. Create the platforms in the maze.
    1. Select the “Ellipse” option, and create a circle the size of the platform that will be used. Note: Placing the platform in the maze beforehand can be beneficial to create an ellipse the exact size of the platform.
    2. Place the newly created platform location in the center of the target quadrant. (it is recommended to name this object “target” to distinguish the correct platforms from the rest). Critical step: In the box just below “Grid”, select the “stop” option under “trigger” for the target platform. This will cause the program to stop once the mouse reaches the platform.
    3. Create three other identical platform areas, and place them in the exact locations in the other quadrants. Do not select “stop” in the “trigger” option.

3. Creating the Run Sheets
    1. Create a run sheet listing each mouse, each trial, a space to write down the time to reach the platform, and a space to note aberrant behavior (**Table 1**).
    2. Pre-determine the release point of each mouse in a pseudorandom manner. Use pseudo-random selection such that the distance to the platform is equal each day, each of the 4 locations is used equally, and the angle from the start to platform (*i.e.*, from left or right) is balanced within and between days.
    Note: the location of the platform on the run sheet to ensure the location of the platform does not move from the original position.
    Ensure that the platform is in the center of one of the four zones that were created on the tracking software.
    Note: If longitudinal testing is being conducted at various ages, move the platform to a new location at each age.

4. Set Up the Maze
    1. Adjust either the water maze or the camera such that there is a match between the maze and the configuration setup on the “Configuration” –“Zone Definition” tab.
    2. Label the four unseen quadrants of the maze (N, S, E, W). Check that these match with the configuration in the tracking software to ensure mice are being released from the correct starting points.
    Critical Step: Place these labels outside of the maze and out of view of a swimming animal given that cues within the maze constitute a non-spatial, non-hippocampal dependent task.
    3. Fill the maze with tap water (approximately 21 °C) so that the platform is approximately 5 mm below the surface of the water.
    4. Place the platform in the predetermined location.
    Note: The location of the platform will remain the same for all mice being tested across all days and trials.
    5. Use non-toxic white tempera paint to make the water opaque. Do this to ensure that the top of the platform is invisible from the animals’ eye level while swimming.

5. Procedure
    1. Take the mouse from the cage, mark the tail using a permanent marker of the correct color, and place in the beaker.
    2. Gently pour the mouse into the maze, so that it enters facing the wall. Note: Each mouse will be placed in the same starting location for a unique trial.
    3. At the initial release of the mouse, begin the timer and stand in an area where the tester is not easily visible by the mice. Ensure that animal tracking software is properly tracking the animal.
    4. Once the animal reaches the platform, allow it to remain on the platform for 15 sec.
    Note: This allows the animal to orient to its spatial location within the room.
    5. After 15 sec on the platform, remove the animal from the maze and return the mouse to the heated cage.
    6. If the mouse finds the platform and proceeds to jump or fall off prior to 15 sec, scoop the mouse and place it back on the platform for the remainder of the 15 sec so the mouse learns to associate the platform with escape.
    7. If the mouse does not locate the platform within 60 sec, gently scoop the mouse and place it on the platform. Allow it to remain on the platform for 15 sec, then remove the animal with the scoop and bring it back to the heated cage.
    8. Clean the maze of debris with a net to disrupt olfactory cues.
    9. Repeat these procedures for the remaining mice in the group. Thus, for testing days 4 through 9, conduct four trials of hidden platform training per day with approximately 20 min between each trial.
    10. Change the starting location pseduorandomly for each trial. Thus, during trial 1, release all mice from the same starting location, then repeat the procedure from a different starting position for the ensuing trials. Each day, vary the release points to ensure animals do not develop a non-spatial, non-hippocampal dependent motoric strategy. For example, if mice are released from N, S, E, and W points on day one, do not release them in that same order the following day.
    11. During hidden platform training, use the animal tracking software to measure latency to reach the platform, the pathlength of the animal, and the percentage of time or distance the animal spends in each maze quadrant. Obtain these measurements from the “Water maze” tab under “Experiment list” and can be exported directly to an excel spreadsheet.
    Note: The use of a hand timer ensures the accuracy of the tracking software.

## 4. Probe Trials

1. Setup the Mouse Performance Tracking Software
    1. Use the same procedures as in the Morris water maze hidden platform procedure (steps 3.2.1-3.2.4.3) with one exception. Critical step: For probe trials, ensure the “stop” option for “trigger” is turned off during probe trials. This will prevent the program from stopping when a mouse crosses the platform area.

2. Creating the Run Sheets

1. Create a run sheet listing each mouse, each trial, and a space to note aberrant behavior. Pre-determine the release point during the probe trials such that they alternate between the two sides opposite the platform. Thus, pre-determine the release points such that mice are not released from the two sides that are adjacent to the platform.

3. Procedure
    1. Conduct probe trials similar to hidden platform training, with the exception that no platform is in the maze.
    2. Release a mouse from a predetermined release point. During the probe trial, remove the platform and the mouse has 60 sec to swim in the maze.
    3. During testing, conduct 4 probe trials. Alter the release point of the mice between the probe trials. Thus, change the release point of the current probe trial from that of the previous probe trial.
    4. During the probe trials, use the animal tracking software to track the pathlength of the animal, the percentage of time the animal spends in each quadrant of the maze, and the number of times an animal swims over the previously platformed area. Obtain these measurements from the “Data analysis” tab and the “Water maze” tab under “Experimental list”, and export directly to an excel spreadsheet.
    Note: The use of a hand timer can also be beneficial to ensure the accuracy of the tracking software.
    5. Conduct a probe trial prior *to* hidden platform training on days 6, 7, 8 and 10. (**Figure 2**). After these probe trials, carry out 4 trials of hidden platform training each day. Thus, perform a probe first, immediately followed by hidden platform training for all mice using the same procedures as described in section 3. In contrast, do not perform additional hidden platform training after the probe trial on day 10.

## 5. Analyses

1. Visible Platform Training
    1. Conduct repeated measures ANOVAs separately for (a) pathlength, (b) latency to find the platform, and (c) swim speed, with transgene (or another variable, such as treatment) as the between-subject variable and days or training blocks as the within-subject variable.
    Note: For more complex designs, a consultation with a statistician can be useful. See [8,13] for information on group sizes sufficient to properly power this task with commonly used models of Alzheimer’s disease.
    2. Performance Incompetence
        1. Identify performance-incompetent mice, including mice that demonstrate visual or motor incompetence, or animals that do not acquire the procedural components of the test, and remove these mice from subsequent statistical analyses of hidden platform and probe performance.
            1. Calculate the mean latency and swim speed for the last day of visible platform training for each mouse. Remove mice with means 2 standard deviations above the group mean, as this may be indicative of motor or visual impairments.
            2. Identify and remove mice failing to orient to or follow the escape scoop, or mice exhibiting aberrant behavior, such as cork-screw swimming or floating.

2. Morris Water Maze Hidden Platform Training
    1. Conduct repeated measures ANOVAs on (a) pathlength, (b) latency to find the hidden platform, (c) swim speed, and (d) percent time or percent distance in quadrant with transgene as the between-subject variable and trials as the within-subject variable.

3. Probe Trials
    1. Swim speed
        1. Conduct repeated measures ANOVAs on swim speed across probe trials with transgene as the between-subject variable and trials as the within-subject variable.

    2. Platform crossing index
        1. Using the probe trial configuration for each mouse, platform crossing index (PCI) is computed using the following formula: PCI = number of times the mouse crosses the target location – average crossings of the equivalent location in the other 3 quadrants.
        Note: This is conducted to determine whether the mouse is using a spatial search strategy, as indicated by more crossings over the trained platform location, or a non-spatial strategy of thigmotaxic swimming, as indicated by approximately equal crossings over all four locations.
        2. Conduct repeated measures ANOVAs on PCI with transgene as the between-subject variable and trials as the within-subject variable.

    3. Percent time or percent distance in quadrant
        1. Compute the percent time or distance each mouse spends in the four quadrants of the maze. Conduct repeated measures ANOVAS on the percent of time, or distance, with transgene as the between-subject variable and trials as the within-subject variable. Use the percent time to determine whether a spatial search strategy is being used; findings of approximately 25% time in the target quadrant indicate mice are performing at chance level and not using a spatial search strategy.

    4. O/N forgetting
        1. Compare the latency of platform area crossing on the probe trial to the latency to locate the platform on the final trial of the previous day. Note: If the latency of the platform crossing on the probe trial is significantly longer than the latency to find the platform during the last trial of hidden platform on the previous day, then O/N forgetting of the platform location is occurring.

4. *Post Hoc* Analysis

1. Follow significant RMANOVAs with *post hoc* comparisons of transgene at each trial.

## 6. Example of Re-optimization for a New Testing Environment

1. Pre-handling. Conduct pre-handling in the housing room. Place the cages without lids on a transport cart for the entire session.
   Note: Objectives for modification of the pre-handling procedure are to more gradually introduce handler manipulations and exposure to the transportation devices (beaker and scoop) and to increase the time mice are in the home cage without microisolator lids and outside of their cage in an open field and brighter light conditions.
   1. Days 1 and 2
      1. Allow mice to acclimate to hands being in the cage, followed by gentle touching, followed by being lifted for tattoo inspection and marking of the tail. Starting on Day 2 of pre-handling, place supplemental nesting material in the cage to provide additional material for nest building and shelter while lids have been removed [9].
   2. Days 3 and 4
      1. Place the transport beaker, scoop, and flag of the visible platform in the pre-handling box and allow mice to explore for 2 trials at 2 min per trial.
      2. Do not use water in this protocol during pre-handling. Rather, ensure the box contains enough clean bedding to cover the floor and is scented with a small amount of female and male soiled litter if both genders are being tested. Change the bedding each day.
   3. Day 5
      1. Place mice in the pre-handling box containing lightly soiled litter and the visible platform flag for 3 trials, for 20 sec each. Use the beaker and the scoop to transport the mice to and from the box.

2. Visible Platform Training
   1. Set water temperature at 27 °C (routinely used for the Minnesota testing environment).
   2. Return the mice to the home cage upon acquiring the cued platform.
   3. Allow mice up to 60 sec to acquire the visible platform.
   4. Conduct training sessions consisting of 3 trials per day for 5 days.

3. Hidden Platform Training
   1. Modify visual cues. Except for one large curtain to obscure the handler, either eliminate or make narrower other curtains surrounding the pool so as to have a more open room. Place additional objects about the room (*e.g.*, exposed shelving, black fabric poster with a white symbol, a black and white beach ball, large black funnels, notebooks, and a black stuffed animal), resulting in a balanced but more varied visual cue-set than previously used.
   2. Begin hidden platform training 72 hr following completion of visible platform training, with an interval of approximately 30 min between trials.
   3. Conduct training sessions consisting of 2 trials for 8 days.

4. Probe Trials
   1. Perform probe trials 72 hr following hidden training trials 8, 12, and 16.Include a 3-day interval between trials 8-9, and 12-13 of training.
   2. Set the probe duration time for 30 sec.

## Representative Results

We have used the Morris water maze to study the effects of beta-amyloid (Tg2576 mice) and mutant P301L tau (rTg(TauP301L)4510 mice) on spatial reference memory (*e.g.*, [1,5,7,8]). **Figure 3** is the representative result reported in our study examining the effect of adult-onset P301L tau expression on learning and memory [8], utilizing testing Environment A. To assess motor and visual capabilities, mice were compared across visible platform training blocks, where each training block consisted of 3 trials. Pathlength in visible platform training did not differ between controls and TauP301L mice (**Figure 3A**), suggesting transgene positive and negative mice exhibit comparable swimming and both groups can see the visual cue (flag) marking the platform. No mice were identified as performance-incompetent based on the exclusion criteria. Next, performance in hidden platform training block was compared, where each block consists of 1 day (4 trials) of training. As the mice learned the location of the platform, the pathlength and time to find the platform decreased. However, pathlength was significantly longer in TauP301L mice compared to controls at each training block (**Figure 3B**), suggesting spatial learning was impaired in TauP301L mice. Four probe trials, in which the platform was removed, were interspersed throughout hidden platform training and took place at the beginning of the day, prior to the start of hidden platform training. Thus, these probe trials measured spatial reference memory. Comparing across these four probe trials, Controls significantly improved with additional training (**Figure 3C**), as indicated by increased time in the target quadrant. In contrast, TauP301L mice did not improve with additional training. Thus, the greatest differences between the two groups at this age occurred at probe trial 4. These data indicate that P301L tau expression is associated with both spatial learning and spatial reference memory deficits. While the water maze task may be relatively stable to some procedural differences, the F1 FVB/N x 129S6 background strain may be particularly sensitive to certain environmental changes. The first protocol outlined was also successfully utilized in Environment B (*e.g.*, [1,7]). However, wild-type probe performance was significantly lower when the first protocol was used in a third location, Environment C. The re-optimized protocol significantly improved wild-type probe performance (**Figure 4**).

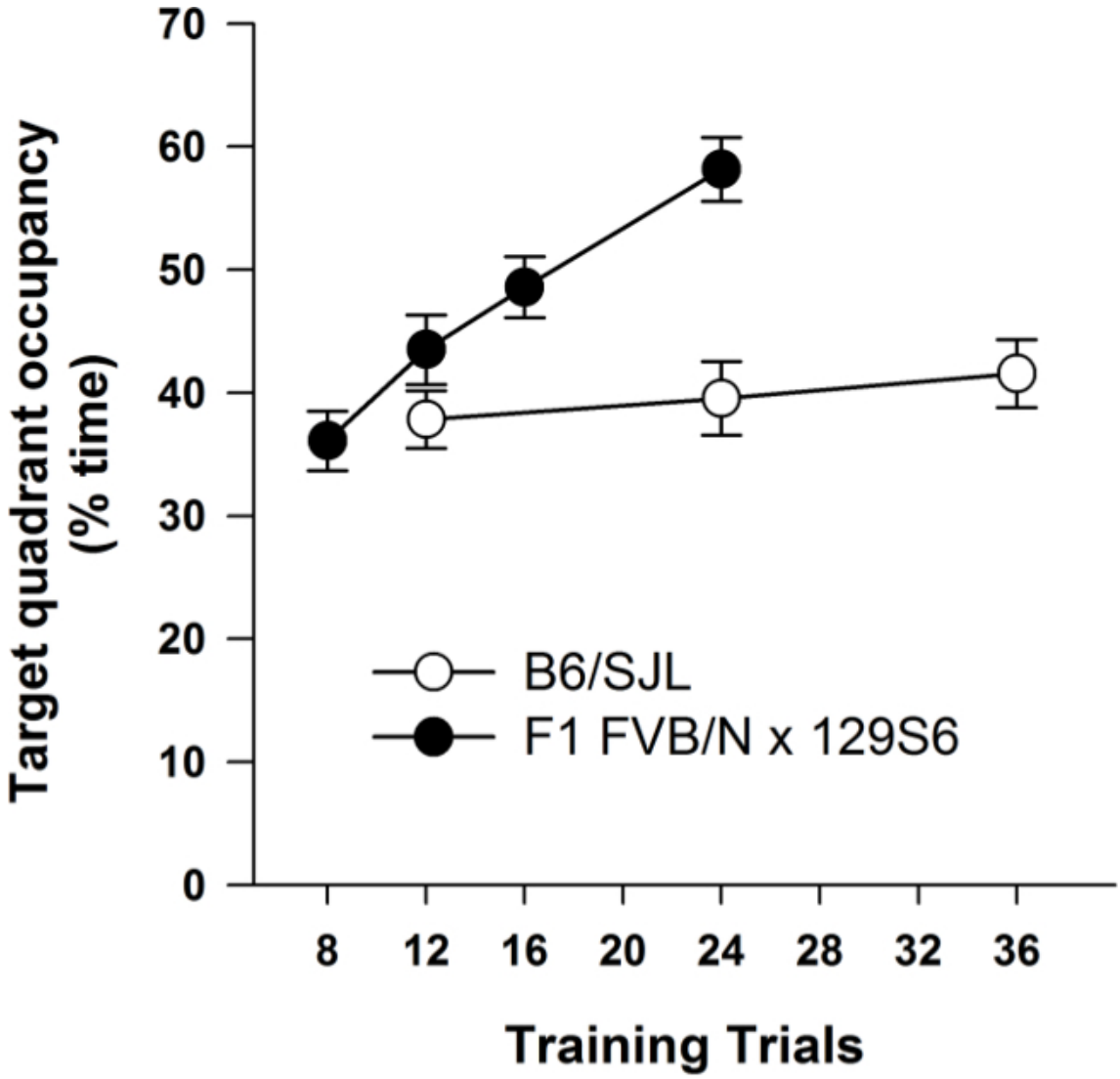


**Figure 1: Optimization of Protocol for Background Strain.** Wild-type 7-8 month old mice were trained using the same cues and testing environment. F1 FVB/N x 129S6 (N = 24) and B6/SJL (N = 16) mice first received 18 and 24 visible platform training trials respectively, delivered at 6 and 8 trials per day respectively. Both strains received 4 hidden platform training trials per day. For F1 FVB/N x 129S6 mice, probe trials were performed 20 hr following 8, 12, 16, and 24 training trials. For B6/SJL mice, probe trials were performed 20 hr following 12, 24, and 36 training trials.

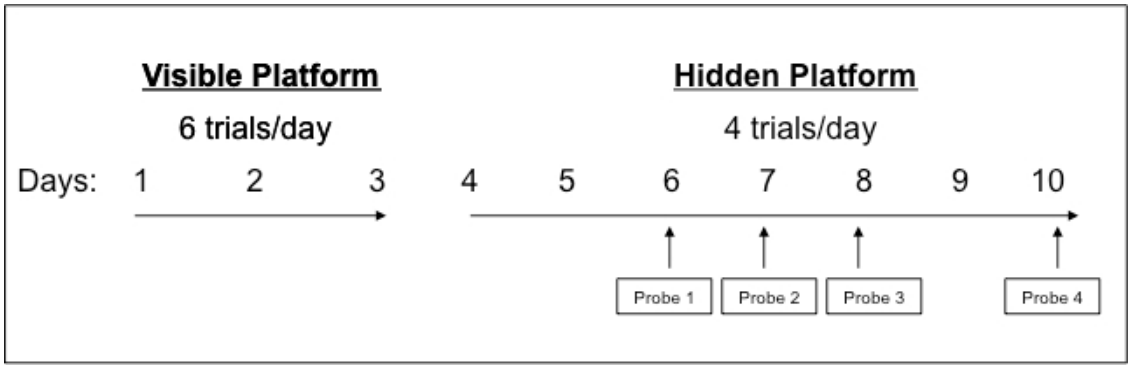


**Figure 2: Timeline.** Mice received visible platform training for 3 days, 6 trials per day, followed by hidden platform training for 6 days, 4 trials per day. Four probe trials were performed 20 hr after 8, 12, 16, and 24 hidden training trials.

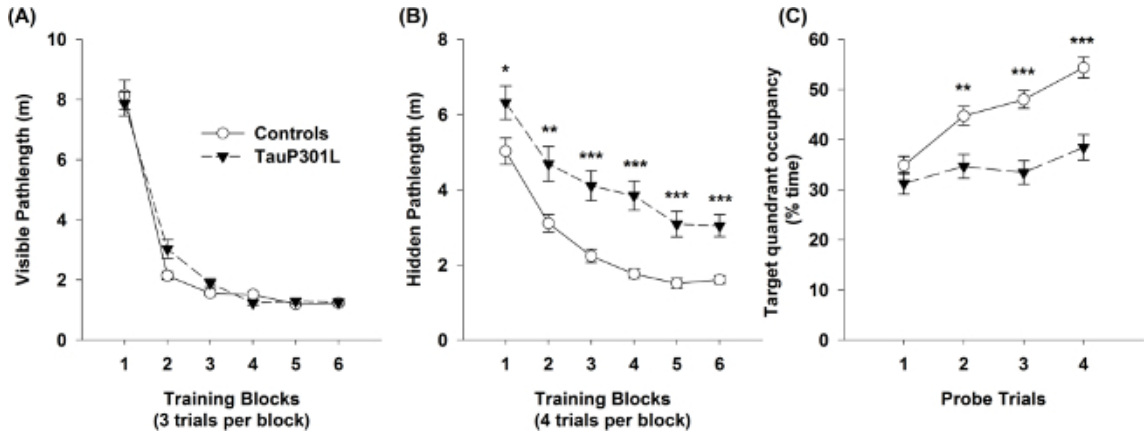


**Figure 3: Representative Results for the Morris Water Maze.** TauP301L mice carrying the human P301L tau gene were examined at approximately 6.5 months of age after three months of P301L tau expression (*n* = 41 tauP301L and *n* = 46 controls with an approximately equal number of males and females in each group). (**A**) Pathlength in visible platform training did not differ between controls and TauP301L mice (*p*s > 0.05). (**B**) During hidden platform training, TauP301L mice demonstrated significantly longer pathlengths across all training blocks (Transgene: $F(1, 83) = 41.96$, $p < 0.0001$; Transgene × Block: $F(5, 415) = 0.6141$, $p = 0.69$). (**C**) Controls improved across the four probe trials, whereas TauP301L mice did not (Transgene: $F(1, 83) = 29.1$, $p < 0.0001$; Transgene × Trial: $F(3, 270) = 4.91$, $p = 0.008$). Each training block consisted of 3 trials for visible platform training or 4 trials for hidden platform training. Tukey post-hoc analyses: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Portions of **Figure 3** reprinted from Hunsberger *et al*., Effect size of memory deficits in mice with adult-onset P301L tau expression, Behav Brain Res, Vol. 272, pp. 181-95. Copyright 2014, with permission from Elsevier.

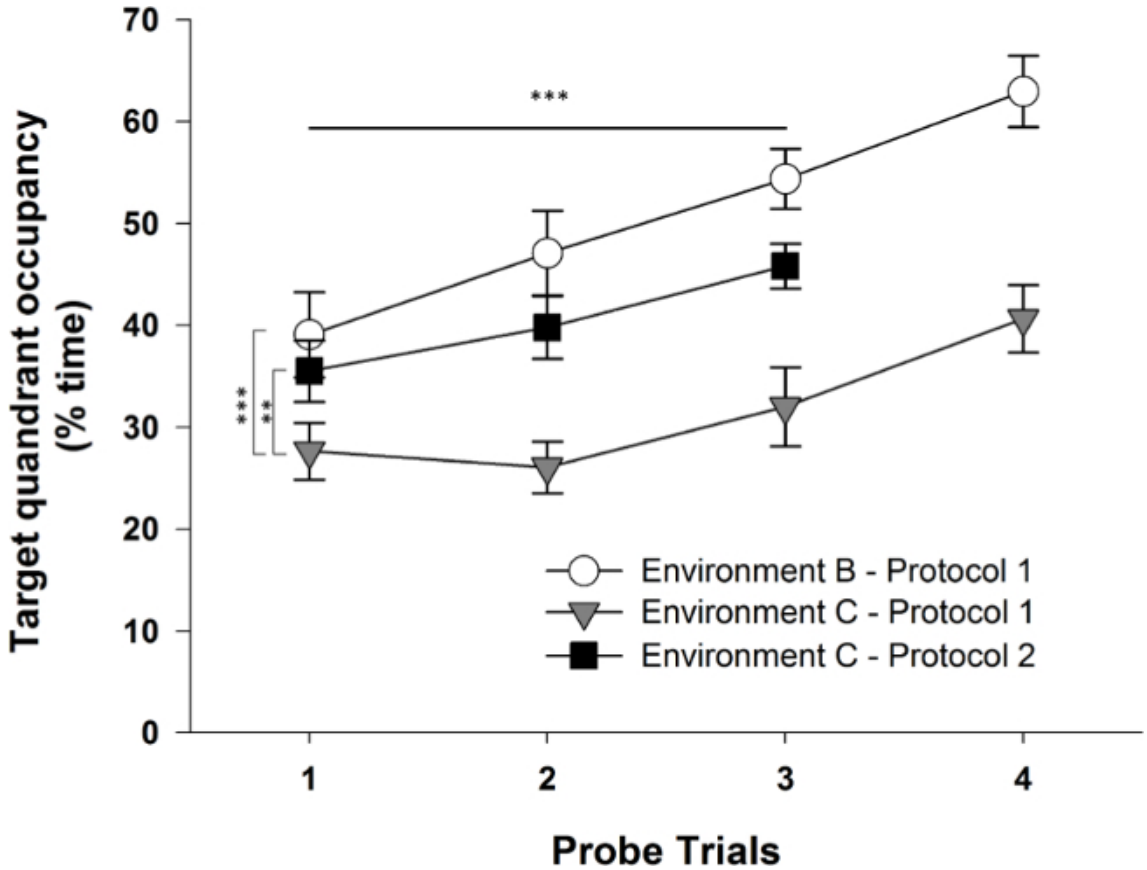


**Figure 4: Probe Scores Are Significantly Affected by Testing Environment and Training Protocol.** Three groups of tau negative F1 FVB/N x 129S6 mice trained using various combinations of training protocol and testing environment generated significantly different probe scores following 8, 12, and 16 training trials (Group: $F(2, 42) = 14.89$, $p < 0.0001$; Group x Trial: $F(4, 84) = 1.10$, $p = 0.36$). Mice trained utilizing Protocol 1 and the same set of applied cues displayed significantly lower probe scores under Environment C compared to Environment B (Environment: $F(1, 25) = 28.58$, $p < 0.0001$; Environment x Trial: $F(2, 50) = 1.93$, $p = 0.16$). Mice trained under Environment C displayed significantly higher probe scores when modified cues and the re-optimized Protocol 2 were utilized compared to mice trained utilizing the original cues and Protocol 1 (Protocol: $F(1, 30) = 15.32$, $p < 0.001$; Protocol x Trial: $F(2, 60) = 0.91$, $p = 0.41$). $^{**}p < 0.001$; $^{***}p < 0.0001$.

| Day 1 | | | Morris Water Maze - Cohort: | | | | | Date: _____ |
|---|---|---|---|---|---|---|---|---|
| Platform in SE quadrant | | | Tester: Start time: Finish time: Water temp: | | | | | 1m max trial time/15s on platform/ *placed on platform |
| Group 1 | | | | | | | | |
| Trial 1 | | | | | | | | |
| Animal ID | Marking | Cage # | End time | Release point | Platform location | Find platform? | Time | Notes (i.e. odd behavior) |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| | | | | W | SE | | | |
| 20 minute ITI | | | | | | | | |
| Trial 2 | | | | | | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| | | | | E | SE | | | |
| 20 minute ITI | | | | | | | | |
| Trial 3 | | | | | | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| | | | | N | SE | | | |
| 20 minute ITI | | | | | | | | |
| Trial 4 | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |
| | | | | S | SE | | | |

**Table 1: Hidden Platform Run Sheet.** An example run sheet in which the platform is located in the southeast (SE) quadrant is provided. The experimenter should write the mouse's ID number, any identifying marks on the tail or ears, and the cage grouping. The release point is indicated on the sheet and should be pseudo-randomly determined as described in Step 3.3.2. Record whether the mouse finds the platform and the time to find the platform. Odd behavior, including floating or thigmotaxis, should noted.

## Discussion

The MWM task is widely used to assess spatial learning and memory. However, the robustness of this task can be influenced by many factors and requires optimization for both background strain and testing environment. As shown in **Figure 4**, the same training protocol and applied visual cues used in two different testing rooms (equivalent size and layout) yielded significantly different probe performance. Since many features of the testing room might contribute to spatial cues [4], it was speculated that the two rooms were significantly different in some unknown way which made the test more difficult. In modifying the extra-maze cues, we opted to effectively increase the number of visual cues. In addition, by minimizing the use of curtains, any auditory or olfactory cues that might exist may have been altered. The light level of the two testing rooms were equivalent, however the home cage light level in the new vivarium was lower, resulting in a higher light level differential when going from home cage to testing room. It has been suggested that BALB/c mice are able to perform the MWM task providing the light levels are sufficiently low [14]. However, attempts to improve performance by lowering testing room light levels were unsuccessful (unpublished observations). To date, it is not known what factor(s) contributed to the decline in performance in the new testing environment, but the modified pre-handling, cues, and training protocol have resulted in a significant increase in probe scores.

If possible, it is advised to test a group of wild-type or control mice of the same background strain and age as the planned experiment to assess rate of learning and optimal probe placement for the particular testing environment. Ideally, the first probe is placed when approximately half of the mice are displaying a probable positive search bias (%-time in target≥35) and the last probe when most control mice show a positive search bias and have reached performance plateau. This strategy has been used to determine probe placement for superior performers (F1 FVB/N x 129S6) and a background which learned more slowly (mixed C57BL/6 x FVB/N x 129S6), where approximately half of the mice had target quadrant occupancies of ≥35 after 8 trials or 18 trials respectively with a group mean of approximately 35%-time for the first probe. Subsequent probes should be higher than the first if set in this fashion, ideally with at least a 15-point difference compared to baseline of 25%-time. If comparing probe scores which are moderately low (~35%-time), ensure that this represents a significant bias for the target and hence a valid difference, by comparing the target to non-target quadrants [4,5]. In addition, the minimum group mean should not be substantially lower than 25%-time in the target quadrant, which would be considered baseline or chance performance. Comparing target to non-target occupancy may help to identify if there are room biases, *e.g.*, mice spending more time in the opposite quadrant than either of the adjacent quadrants would be an unexpected search pattern.

Some recommendations while conducting testing include limiting the number of people testing each cohort of animals to 1 individual to reduce variability in handling and testing styles between experimenters, and carrying out testing at around the same time each day. Also, it has been suggested that mice receiving 2 trials per day learn nearly as quickly as those receiving 4 trials per day [15]. It is essential that animals from different strains/backgrounds/treatment groups be represented in each testing group if more than one cohort needs to be run during a day. Finally, care must be taken to ensure the mice do not become hypothermic, as hypothermia can affect performance and is sex and background strain dependent [16]. Though the inter-trial interval used here (20 min) should be sufficient to prevent hypothermia, other methods include adjusting the water temperature, placing the mice in a heated holding cage between trials, or some combination of the two as needed. However, it should be noted that water temperature can influence performance in both directions. For example, proestrous rats perform better under warm conditions (33 °C), whereas estrous rats perform better under cold conditions (19 °C) [17]. Thus, care must be taken to control for water temperatures across experiments. Should hypothermia be a concern for a particular animal, the experimenter could hand dry that animal with a paper towel in the event that they fail to groom post-swim or give any indication of hypothermic behavior. Periodic assessment of body temperature can ensure the conditions are sufficient to prevent hypothermia.

Another procedural consideration pertains to visible platform training. Younger mice or another strain may need fewer visible platform training trials. A suggested guideline is to train until the control group has reached floor of performance during the final 3-6 consecutive training trials, providing the experimental group displays equivalent performance. If the experimental design includes both young and old mice, set the visible platform training duration for the group, which requires the highest number of training trials to reach floor of performance [5]. In addition, the visible platform can be effective towards acclimatizing mice to the handling and test procedure and may obviate the need for pre-handling if the mice are not very young when first tested or human handling is not excessively aversive to the strain being tested.

Broad benefits of the Morris water maze include its relative insensitivity to motivational factors as compared to food-based tasks, its validity as a measure of hippocampal-dependent spatial navigation and reference memory, and its cross-species efficacy [15]. A potential limitation of the

technique is that, because this protocol is tailored for a specific background strain, it may not be effective with other animals or other background strains of mice. Additionally, as part of the protocol, attempts are made to create and strategically place identifiable cues throughout the mazing room. However, it is unclear what exact height is optimal when placing cues around the room. Thus, cues that are large and are distinguishable from one another are necessary for effective training.

In summary, optimizing the MWM task for use with a particular background strain and testing environment can significantly increase the dynamic range of the task, resulting in considerable time and cost savings.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

This work was supported by the National Institute of General Medical Sciences (Reed/Engler-Chiurazzi - U54GM104942), the National Institute for Neurological Disorders and Stroke (Ashe – R01NS33249, R01NS63249 and R01NS79374), CoBRE (Engler-Chiurazzi - P20GM109098), the Alzheimer's Association (Reed - NIRG-12-242187), a WVU Faculty Research Senate Grant (Reed), a WVU PSCOR grant (Reed), and internal funds from the WVU College of Medicine Dean's Office (Engler-Chiurazzi). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or Alzheimer's Association.

## References

1. Ramsden, M., *et al.* Age-dependent neurofibrillary tangle formation, neuron loss, and memory impairment in a mouse model of human tauopathy (P301L). *The Journal of Neuroscience.* **25**, 10637-10647 (2005).
2. Attar, A., *et al.* A shortened barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. *PLoS One.* **8**, e80355 (2013).
3. Johnson, J. M., Bailey, J. M., Johnson, J. E., Newland, M. C. Performance of BALB/c and C57BL/6 mice under an incremental repeated acquisition of behavioral chains procedure. *Behavioural Processes.* **84**, 705-714 (2010).
4. Crawley, J. N. *What's wrong with my Mouse Behavioral phenotyping of transgenic and knockout mice.* 94-95 Wiley-Liss (2000).
5. Westerman, M. A., *et al.* The relationship between abeta and memory in the Tg2576 mouse model of Alzheimer's disease. *The Journal of Neuroscience.* **22**, 1858-1867 (2002).
6. D'Hooge, R., De Deyn, P. P. Applications of the morris water maze in the study of learning and memory. *Brain Research Reviews.* **36**, 60-90 (2001).
7. Santa Cruz, K., *et al.* Tau suppression in a neurodegenerative mouse model improves memory function. *Science.* **309**, 476-481 (2005).
8. Hunsberger, H. C., *et al.* Effect size of memory deficits in mice with adult-onset P301L tau expression. *Behavioural Brain Research.* **272**, 181-195 (2014).
9. Yue, M., Hanna, A., Wilson, J., Roder, H., Janus, C. Sex difference in pathology and memory decline in rTg4510 mouse model of tauopathy. *Neurobiology of Aging.* **32**, 590-603 (2011).
10. Sandi, C. The role and mechanisms of action of glucocorticoid involvement in memory storage. *Neural plasticity.* **6**, 41-52 (1998).
11. Hölscher, C. C. impairs performance in spatial water maze learning tasks. *Behavioural Brain Research.* **100**, 225-235 (1999).
12. Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. *Journal of Neuroscience Methods.* **11**, 47-60 (1984).
13. Reed, M. N., Liu, P., Kotilinek, L. A., Ashe, K. H. Effect size of reference memory deficits in the morris water maze in Tg2576 mice. *Behavioural Brain Research.* **212**, 115-120 (2010).
14. Huang, Y., Zhou, W., Zhang, Y. Bright lighting conditions during testing increase thigmotaxis and impair water maze performance in BALB/c mice. *Behavioral Brain Research.* **226**, 26-31 (2012).
15. Baker, R. *Water maze.* Available from: http://www.watermaze.org (2013).
16. Ivonen, H., Nurminen, L., Harri, M., Tanila, H., Puolivali, J. Hypothermia in mice tested in Morris water maze. *Behavioural Brain Research.* **141**, 207-213 (2003).
17. Rubinow, M. J., Arseneau, L. M., Beverly, J. L., Juraska, J. M. Effect of estrous cycle on water maze acquisition depends on the temperature of the water. *Behavioral Neuroscience.* **118**, (4), 863-868 (2004).

 