3-2016

Motor and Hippocampal Dependent Spatial Learning and Reference Memory Assessment in a Transgenic Rat Model of Alzheimer's Disease with Stroke

Jennifer L. Au
Western University

Nina Weishaupt
Western University

Hayley J. Nell
Western University

Shawn N. Whitehead
Western University

David F. Cechetto
Western University, cechetto@uwo.ca

Video Article

Motor and Hippocampal Dependent Spatial Learning and Reference Memory Assessment in a Transgenic Rat Model of Alzheimer's Disease with Stroke

Jennifer L. Au[1], Nina Weishaupt[1], Hayley J. Nell[1], Shawn N. Whitehead[1], David F. Cechetto[1]

[1]Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, Western University

Correspondence to: David F. Cechetto at cechetto@uwo.ca

URL: https://www.jove.com/video/53089
DOI: doi:10.3791/53089

Keywords: Medicine, Issue 109, Morris water maze, cylinder task, beam walk, transgenic, rat, Alzheimer's disease, stroke

Date Published: 3/22/2016

Citation: Au, J.L., Weishaupt, N., Nell, H.J., Whitehead, S.N., Cechetto, D.F. Motor and Hippocampal Dependent Spatial Learning and Reference Memory Assessment in a Transgenic Rat Model of Alzheimer's Disease with Stroke. *J. Vis. Exp.* (109), e53089, doi:10.3791/53089 (2016).

Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disease that results in neurodegeneration and memory loss. While age is a major risk factor for AD, stroke has also been implicated as a risk factor and an exacerbating factor. The co-morbidity of stroke and AD results in worsened stroke-related motor control and AD-related cognitive deficits when compared to each condition alone. To model the combined condition of stroke and AD, a novel transgenic rat model of AD, with a mutated form of amyloid precursor protein (a key protein involved in the development of AD) incorporated into its DNA, is given a small unilateral striatal stroke.

For a model with the combination of both stroke and AD, behavioral tests that assess stroke-related motor control, locomotion and AD-related cognitive function must be implemented. The cylinder task involves a cost-efficient, multipurpose apparatus that assesses spontaneous forelimb motor use. In this task, a rat is placed in a cylindrical apparatus, where the rat will spontaneously rear and contact the wall of the cylinder with its forelimbs. These contacts are considered forelimb motor use and quantified during video analysis after testing. Another cost-efficient motor task implemented is the beam-walk task, which assesses forelimb control, hindlimb control and locomotion. This task involves a rat walking across a wooden beam allowing for the assessment of limb motor control through analysis of forelimb slips, hindlimb slips and falls. Assessment of learning and memory is completed with Morris water maze for this behavioral paradigm. The protocol starts with spatial learning, whereby the rat locates a stationary hidden platform. After spatial learning, the platform is removed and both short-term and long-term spatial reference memory is assessed. All three of these tasks are sensitive to behavioral differences and completed within 28 days for this model, making this paradigm time-efficient and cost-efficient.

Video Link

The video component of this article can be found at https://www.jove.com/video/53089/

Introduction

Alzheimer's Disease (AD) is the most prevalent form of dementia in the elderly population and a debilitating neurodegenerative disease. Histopathologically, AD presents itself as amyloid plaques, neurofibrillary tangles and neuronal loss. Amyloid plaques consist primarily of beta-amyloid peptide (Aβ) that has been produced through an altered proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase enzymes[1,2,3]. The cleavage product, Aβ, deposits in the brain creating pathological amyloid plaques and has toxic effects on the brain that can lead to the characteristic learning impairments and memory loss. All of these steps together are referred to as the "amyloid cascade hypothesis"[3,4]. While this hypothesis is important when investigating AD, other cellular changes have been found to precede these plaque formations that strays from the original amyloid cascade pathway. These other cellular changes are thought to contribute to early memory loss, learning impairments and other cognitive dysfunctions involved in AD prior to plaque formation[3,5,6].

With AD becoming increasingly prevalent, risk factors for developing AD are becoming an extremely important focus of research. Although age is the principal risk factor for sporadic forms of AD, other risk factors have been identified, including stroke[7,8]. Stroke is not only a risk factor, but it can also exacerbate already present dementias. For example, clinically, the progression of AD has been shown to be worse in patients who had previously experienced stroke[9]. Moreover, increased APP expression and Aβ accumulation has been found in experimental animal models of Aβ toxicity combined with induced stroke[10,11]. Since there is this important interaction between stroke and AD, it is essential that these two pathologies be further researched together in co-morbid models to better understand pathophysiology and behaviors implicated in both conditions.

To investigate co-morbid conditions, an appropriate model had to be developed in which a stroke could interact with Aβ to produce AD-like pathology. For the first time, an APP21 transgenic rat that has a mutated human APP gene incorporated into its DNA was used to achieve an appropriate model of AD. The mutations are the Swedish double missense and Indiana single missense mutations, which have both been implicated in familial forms of AD[4,8,12]. In the absence of an additional insult, this rat model ages without developing the characteristic Aβ plaques or neurofibrillary tangles[12]. Therefore, in an effort to induce AD-like behavioral pathology, a small stroke is introduced into the right striatum to mimic the small subcortical strokes often present in dementia patients[9]. The stroke in the APP21 transgenic rat embodies the co-morbid

condition and allows investigation of various types of behavioral changes implicated in both disease conditions. In particular, this induction of AD-like pathology and cognitive deficits in the adult rat allows us investigate the earliest molecular and cognitive changes preceding AD.

Since the goal is to determine the first signs of behavioral changes and since both stroke and AD have very distinct behavioral pathologies, when studying the co-morbid model, behavior tasks need to assess a variety of behavioral phenotypes. There are a battery of relatively sensitive tests that can be done to analyze motor and cognitive behavior in rodent models that involve a variety of paradigms and equipment. To specifically analyze forelimb and hindlimb motor function, the cylinder task and beam-walk task have been implemented to detect motor deficits and monitor locomotion in this model. Other sensitive tasks designed to specifically assess fine forelimb motor skill (*i.e.* the staircase task and single pellet reaching task) require food deprivation[11,13,14]. To avoid any of the known effects of food deprivation on disease pathologies[15,16,17], these tests have been deemed unsuitable for this study. The cylinder task assesses the spontaneous use of the rat's forelimbs during rearing in a novel environment and can detect asymmetry between forelimbs in rats with unilateral stroke[10,18]. A major benefit to this task is that the apparatus can be utilized for other behavior tasks, such as the Porsolt forced swim task[19]. Contrary to the cylinder task, the beam-walk task also allows analysis of hindlimb and forelimb motor control, in addition to locmotion[10,14]. Beam walking includes a locomotor component, a balance component and skilled foot placement. Both of these tests are cost-efficient, straightforward, and time-efficient and elucidate the effects of stroke and AD on differences in limb functioning.

Aside from changes in motor function, AD involves memory deficits that can present in early stages of the disease progression. When addressing AD-like pathologies in a rodent model, it is crucial that hippocampal dependent learning and memory is assessed because the hippocampus is an important brain structure largely affected in AD[2]. The hippocampus is an essential brain region for spatial learning and memory and its function can be tested using various maze paradigms in rodents. One of the mostly widely used maze tasks for rodent models of different diseases is the Morris water maze[20]. The Morris water maze utilizes spatial cues to assist the rat in locating a stationary hidden platform and tests spatial reference memory when the platform is removed. A valuable advantage of the water maze setup is that it is highly adaptable depending on the proposed research question[20].

For the first time, the techniques described have been used to assess motor and cognitive function in a novel co-morbid rat model of stroke and AD. involving small strokes in an APP21 transgenic rat model. Co-morbidity was achieved by inducing vasoconstriction of the blood vessels in the striatum to produce a small stroke in APP21 transgenic rats. This stroke model has been well established as a co-morbid condition in an alternative rat model of AD[11]. Advancement into this novel APP21 transgenic rat model was intended to produce a more translationally valuable model. While the behavioural tasks are described using a co-morbid stroke and AD rat model, these tasks can be further applied to other models of stroke or models of other neurological diseases (*i.e.* Parkinson's disease). The general methodology described will be widely applicable to these other disease states, but behavior timelines and paradigms may require alteration based on the proposed research question and model. In addition to being adaptable, the tasks described are effective in demonstrating minor deficits, while also being cost and time-efficient.

Protocol

The appropriate institutional animal ethics committee should approve all of the behavioral procedures prior to starting experimentation. All animal work described here was approved by Western University Animal Use Subcommittee and follows the Canadian Council on Animal Care guidelines. These animal experiments were performed during the light phase.

1. Cylinder Task for Gross Forelimb Motor Assessment

1. Equipment Setup
    1. Acquire a Plexiglas cylinder with a perforated lid that will accommodate the size of the rat used for the experiment. The size of the cylinder should not allow the rat to reach the top when rearing and it should allow for 2 cm between the wall and the rat's nose and the base of the rat's tail. The standard size of a cylinder is 23 cm in diameter and 40 cm in height for a 6-month-old (400-600 g) rat.
    2. Place a mirror at a 45° angle below the cylinder apparatus with the cylinder sitting on a Plexiglas stand or some other form of support approximately 30 cm above table top.
    3. Set up a video camera on a tripod at an appropriate distance to visualize the entire diameter of the cylinder in the mirror on the video camera.
    4. Turn lighting to a dimmer setting and play white noise in the room (*i.e.* low volume, subtle music) to reduce the effect of sudden loud noises. This should help encourage movement and prevent freezing due to loud noises.

2. Experimental Procedure
    1. Move the rats into the experimental room 30 min before the start of first trial to acclimatize rats to the room with music playing and dimmer lighting.
    2. Write the corresponding animal and trial information (*i.e.* animal number, day in timeline and trial number) on a small white board. Place this board in front of the mirror.
    3. Press record on the video camera with the white board in front of the mirror, pick up the rat near the base of the tail, place the rat in the cylinder and secure the lid. Stand off to the side of the cylinder to avoid interfering with video recording.
    4. Remove the white board from in front of the mirror and let the video camera record the rat in the cylinder for 5 minutes (use a timer to record 5 min from when the white board is removed). This is one trial.
    5. Clean the cylinder with paper towel and water after a trial.
    6. Repeat steps 1.2.2-1.2.5 two more times to achieve a total of three trials. There should be a 20-30 min inter-trial time to decrease the chance of habituation to the cylinder. During this time, run cylinder trials for the other rats.

3. Video Analysis
    1. Import video camera files into video editing program (*i.e.* iMovie)
    2. Compile the videos into clips for each trial. Mute the volume of the video and decrease the speed of the video to 25% of the original.

3. Count the number of forelimb contacts with the wall of the cylinder for the left and right forelimb. For simultaneous left and right contacts, count these forelimb contacts as "both". Keep in mind that the video is recorded through a mirror, what appears to be the left forelimb in the video corresponds to the animal's right forelimb in reality.
4. Calculate the percent use of the affected forelimb (contralateral to the stroke) using the following equation: [{(affected contacts + ½ bilateral contacts)/total number contacts} x 100]. The performance of both wild type groups and the transgenic group without stroke are considered comparison groups to evaluate presentation of disease-induced problems.

2. Beam-walk Task for Gross Motor Assessment

1. Equipment Setup
    1. Acquire a smooth sealed wooden beam that is 2 cm wide and approximately 120 cm long (optimal width for a 200-600 g rat).
    2. Place two tables or shelving units 100 cm apart. The surfaces of each unit should be approximately 40 cm above the ground.
    3. Secure both ends of the beam to the table or shelving surface using tape. Approximately 1 m of unsupported beam length should now be elevated 40 cm above the ground.
    4. Set up a video camera on a stand, capturing the entire length of the beam. To enhance contrast in the video, consider introducing a black background behind the beam when using white rats.

2. Experimental Procedure
    1. Move the rats into the experimental room 30 min before the start of first trial to acclimatize rats to the room with music playing and dimmer lighting.
    2. Place the rat's home cage or environmental enrichment tubes at one end of the beam and place the rat at the other end of the beam.
    3. Conduct a few non-recorded runs two days prior to experimental trials. Let the rat explore the area and guide the rat toward the beam by holding the base of the rat's tail.
    4. Once the rat has crossed the beam, move the cage or tube to the other end of the beam and repeat. When the rat crosses the beam freely in either direction, the training session is over. Keep the number of training runs consistent among all animals.
    5. Write the corresponding animal and trial information (*i.e.* animal number, day in timeline and trial number) on a small white board. Tape this white board to the wall behind the beam.
    6. Place the rat's home cage or environment enrichment tubing at one end of the beam.
    7. Press record on the video camera and pick the rat up by the base of the tail and place at the end of the beam opposite to the home cage or tubing.
    8. Record the entirety of the trial, which ends when the rat has successfully completed traversing the full length of the elevated beam. If the rat pauses midway across the beam, gently scruff the rat by the base of the tail or touch the rat's tail gently to promote movement across the beam. Do not push the rat forward in any manner.
    9. Re-do trials where the rat turns sideways while on the beam, repeatedly stops walking or walks inconsistently. If a rat falls and continues to hang on to the beam, gently scoop up the rat and place it back on the beam at the position of the fall and continue the trial.
    10. Move the home cage or tubing to the other end of the beam, change the trial # on the white board and record the subsequent trial.
    11. Repeat this procedure until a total of 6 trials have been completed, with 3 trials being recorded for each direction. All 6 trials for a rat can be recorded before starting the trials for the next rat.

3. Video Analysis
    1. Import video camera files into video editing program (*i.e.* iMovie).
    2. Compile the videos into clips for each trial and mute the volume of the video. Analyze each video clip frame by frame.
    3. Count the number of total steps the rat takes to walk the full length of the beam and the total number of left and right hindlimb and forelimb slips and total number of falls. The performance of both wild type groups and the transgenic group without stroke are considered in comparison to the co-morbid group to evaluate emergence of deficits unique to the co-morbid condition.

3. Morris Water Maze for Hippocampal-dependent Spatial Learning and Reference Memory

1. Equipment Setup
    1. Secure a video camera positioned above the center of a circular pool (148 cm diameter and 58 cm depth). Align the four designated quadrants properly with the outline of the pool in the tracking software program.
    2. Fill the circular pool with water approximately 36 cm deep. The water should be warmed to room temperature by filling the pool a few days prior to beginning the experimental protocol.
    3. Add black non-toxic acrylic paint until the water is opaque when using white rats. Use a light color, such as white, for darker colored rats.
    4. Surround the pool with blank wall surfaces, including room dividers if needed. When in the pool, the rat should not be able to see the experimenters.
    5. Cut 4 large different shaped spatial cues from different colors of poster board and attach one shape on the wall per designated north, east, southeast and southwest pool locations. These cues should be slightly higher than the edge of the pool.
    6. Turn on a radio at low volume in the northwest quadrant to prevent the rat from being distracted by unexpected loud noises during testing.
    7. Place a circular platform (11.5 cm diameter, surface 2-3 cm below water level) in the center of the target quadrant.
    8. Turn off main room lights and turn on a floor standing lamp on the opposite side of the room divider to the pool to illuminate the area.

2. Computer Configuration
    1. Use a tracking program that is designed for Morris water maze behavior tasks and set up protocol prior to starting experiments. An example of a possible program is provided in **Figure 4**.

2. Set 4 consecutive days with 4 trials of 90 sec each for the spatial learning experiment.
3. Set 2 separate probe experiments of 30 sec each at 24 hr after the last spatial learning trial and 1 week after the first probe experiment.
4. Set cued learning trials at 4 trials per day for 2 consecutive days starting 24 hr after the second probe experiment. Each trial should be 60 sec total.
5. For each stage, set the testing order so that there is a 20 min inter-trial interval between each trial for any given animal. Other animals can be run during this inter-trial interval, as long as every animal maintains a 20 min inter-trial interval.
6. Ensure that the platform position is to be defined by the experimenter and that all other zones are defined by the designated platform position.
7. Set the program to manually start and end each trial.

3. Spatial Learning Experiment
   1. Place the circular platform in the southwest quadrant. It should be aligned with the circular designated "platform" area in the quadrant on the computer program.
   2. Start positions around the pool should be randomized for each rat. Represent all start positions in each treatment group. No rat starts from the northeast position for any spatial learning trials in the paradigm presented to allow for a novel start position during probe testing.
   3. Hold the rat at the base of its tail and gently place it in the water along the wall of the pool at the designated start position and quickly move out of the rat's sight.
   4. Have the other experimenter start the tracking software as soon as the rat is in the water. A timer should start counting up from 0 on the tracking program.
   5. If the rat locates the platform, have the other experimenter stop the trial on the computer and leave the rat on the platform for at least 30 sec before retrieving it. If the rat jumps off the platform before the trial is stopped on the computer, the trial continues.
   6. If the rat does not find the platform in the allotted trial time, guide the rat to the platform using your hand (either making the rat follow your hand or guide the rat by the base of its tail). Hold the rat on the platform for 30 sec.
   7. Remove the rat from the pool by the base of its tail onto the experimenter's arm that is covered with a towel or let the rat climb onto a portable surface.
   8. Repeat steps 3.3.3-3.3.7 for a total of 4 trials per rat. There should be an inter-trial interval of 20 min for each rat.
   9. Return the rats to their home cage under a heat lamp for at least 10 min following the rat’s final spatial learning trial.
   10. Continue the exact same spatial learning protocol for day 2 through day 4 of spatial learning.
   11. On day 2-4, do not hold the rat on the platform any longer. Allow the rat to sit on the platform without assistance for 30 sec with the experimenters out of sight. This can be done during later trials on day 1 on occasion.

4. Probe Experiment
   1. Remove the platform from the pool. Ensure the previous platform position remains defined on the computer (circle in the southwest quadrant).
   2. Place the rat in the water along the wall of the pool at the northeast position and quickly move out of the rat's sight. Use of the novel northeast start position ensures that the rat recalls the platform position independent from previously trained start positions.
   3. Have the other experimenter start the tracking software as soon as the rat is in the water. A timer should start counting up from 0 on the tracking software.
   4. Retrieve the rat from the southwest quadrant of the pool by the base of its tail and hold on a towel-covered arm or let the rat climb onto a portable surface.
   5. Return the rats to their home cage under a heat lamp for at least 10 min following each rat’s probe trial.
   6. Repeat this experiment (steps 3.4.1-3.4.5) 1 week later.

5. Cued Learning Experiment
   1. Use tape to secure a 4 cm diameter white spherical cue on a stand that is 8.5 cm tall on the platform (total height of cue is 12.5 cm). The top of the spherical cue will be at least 8.5 cm above water level.
   2. Remove the spatial cues from walls surrounding the pool.
   3. Randomize platform positions and start positions for each rat in each group. All platform and start positions should be represented for each treatment group.
   4. Place platform in the designated platform area and define in the tracking software.
   5. Place the rat in the water along the wall of the pool at the designated start position and quickly move out of the rat's sight.
   6. Have the other experimenter start the tracking software as soon as the rat is in the water. A timer should start counting up from 0 on the tracking software.
   7. Once the rat reaches the platform, have the other experimenter stop the trial on the computer. If the rat jumps off the platform, the trial continues. Before retrieving the rat, allow the rat to sit on the platform for 15 sec.
   8. Retrieve the rat from the pool by the base of its tail and hold on a towel-covered arm or let the rat climb onto a portable surface.
   9. Check the platform position for the next rat and move the platform into the corresponding area defined in the tracking software.
   10. Repeat steps 3.5.4-3.5.9 for the following 3 trials. There should be an inter-trial interval of 15 min.
   11. Return the rats to their home cage under a heat lamp for at least 10 min following each rat’s final cued learning trial.
   12. Continue the same protocol for day 2 of cued learning.

6. Data Analysis
   Note: Time spent in zones, distance traveled in zones, average speed, number of entries into zones and the time to first entry into zones are often used.
   1. Analyze the time and distance to reach the platform zone and the average speed per day of spatial and cued learning separately. For probe experiments, analyze the latency to first entry into the target zone as a raw time or a percent change from probe 1 to probe 2.

Representative Results

The behavioral tasks described were used to demonstrate the effects of stroke in an APP21 transgenic rat model of Alzheimer's disease. The combination of stroke and the APP21 transgene is expected to result in greater motor deficit in the affected limbs, as well as increased memory deficits.

The cylinder task assessed gross forelimb motor function and is represented as the use of the affected forelimb. Furthermore, the beam-walk task was used to specifically assess hindlimb motor function and locomotion. Since the stroke was induced in the right striatum, the left forelimb is expected to show a motor deficit if one is present. The data presented in both **Figure 2** and **Figure 3a** and **3b** does not statistically demonstrate that co-morbid rats have a forelimb or hindlimb deficit, respectively. While these animals do not appear to have forelimb or hindlimb motor deficits, they do appear to have minor differences in motor function pertaining to locomotion. In the beam-walk task total steps were significantly increased in the co-morbid transgenic rats with stroke (p <0.05, **Figure 3c**), suggesting that the beam-walk task is sensitive enough to pick up minor changes in gait and locomotion. The small striatal stroke model used produces small strokes that are likely too small to produce any major motor deficits, but deficits have been demonstrated before in other stroke models with these two tasks[10,14]. Here, these tasks can simply monitor motor function and locomotion when investigating the parameters presented.

Hippocampal dependent spatial learning and reference memory can be effectively assessed using Morris water maze. There were no apparent differences in learning between groups (**Figure 5a**, **Figure 5b**), therefore differences in learning cannot account for any differences in memory performance. APP21 transgenic rats with stroke demonstrated a robust long-term reference memory deficit compared to transgenic rats without stroke and wild type rats with stroke (p <0.05, **Figure 5c**).

Cued learning is completed to ensure that rats have equal ability to use visual spatial cues to locate the platform in the Morris water maze. As demonstrated in **Figure 6a** and **Figure 6b**, no differences were observed in the latency or path length to reach the platform during cued learning between groups. Furthermore, the average swim speed was consistent between groups (**Figure 6c**) and demonstrates that the motivation to escape and swim abilities was equal between groups.

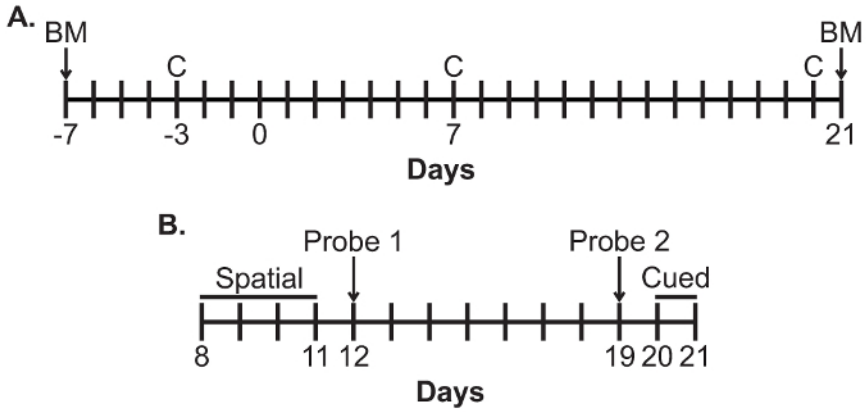


Figure 1: Timeline for motor and cognitive behavior assessments. Stroke-inducing surgery day is assigned day 0 and all testing days are in reference to this day. (**A**) Pre-surgery and post-surgery testing for cylinder task (**C**) and beam-walk task (BM). (**B**) Morris water maze spatial learning, probe testing and cued learning. Please click here to view a larger version of this figure.

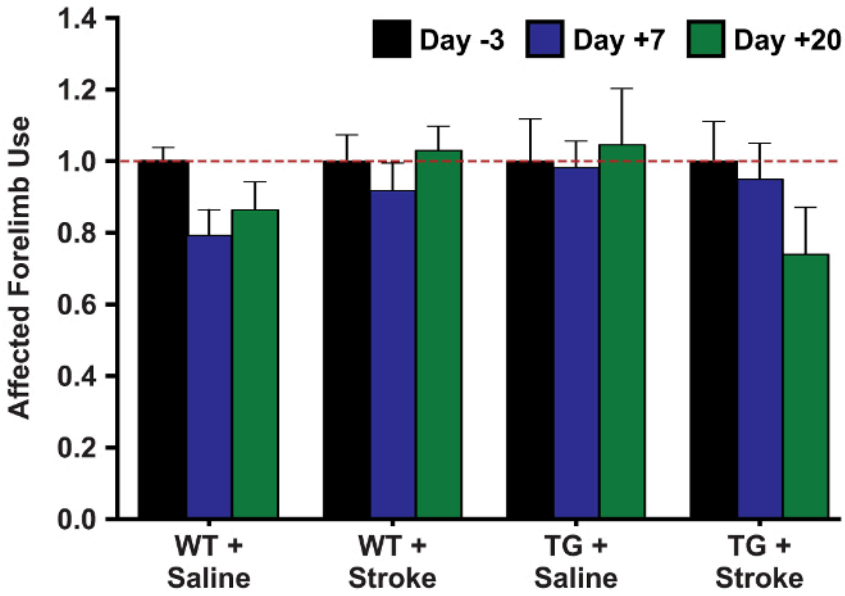


Figure 2: Cylinder Task. Percent use of affected forelimb was calculated using the equation in step 1.3.4 and standardized to day -3 baseline values. The red dotted line annotates the 1.0 value that represents equal use of each forelimb in the cylinder task. Wild type is abbreviated as WT and transgenic is abbreviated as TG. Animal numbers are as follows: WT + saline (n = 7), WT + stroke (n = 8), TG + saline (n = 8), TG + stroke (n = 6). All values are presented as mean ± SEM. (Two-way ANOVA, Tukey's post hoc). Please click here to view a larger version of this figure.

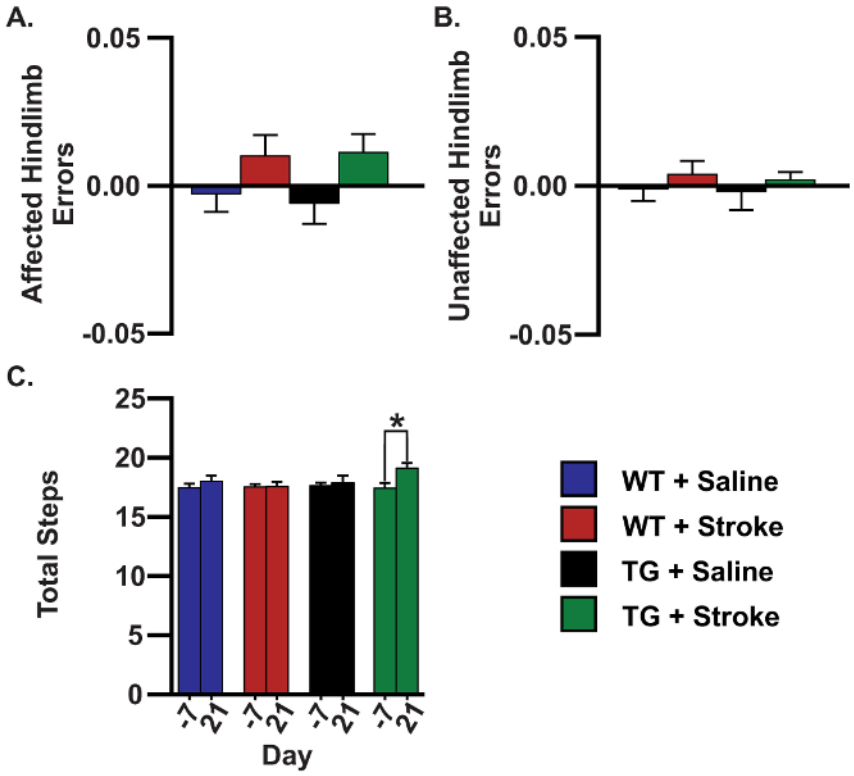
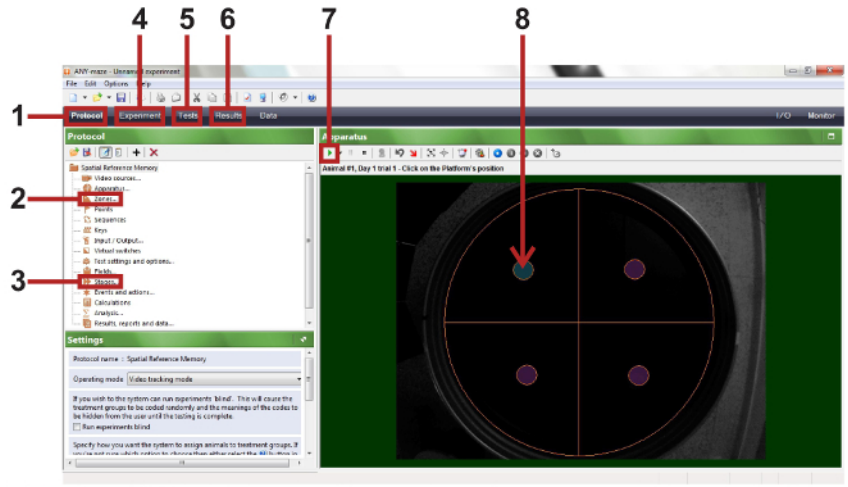


Figure 3: Beam-walk task. The sum of all slips made with the (**A**) affected and (**B**) unaffected hindlimb across 5 trials is presented as a ratio of the total number of steps taken to cross the beam. Values were standardized to baseline values as follows: post-surgery ratio - pre-surgery ratio. (**C**) The total steps to cross the beam on day -7 and day 21. Wild type is abbreviated as WT and transgenic is abbreviated as TG. Animal numbers are as follows: WT + saline (n = 6), WT + stroke (n = 6), TG + saline (n = 6), TG + stroke (n = 5). All values are presented as mean ± SEM and asterisks indicate statistical significance. (One-way ANOVA, Tukey's post hoc, p <0.05). Please click here to view a larger version of this figure.



1. Protocol Tab: Setup for Apparatus, Camera and Experiment
2. Platform and Zone Position Setup
3. Experimental Protocol Setup
4. Experiment Tab: Animal Number Setup
5. Tests Tab: Define Animal Groups and Run Experiment
6. Results Tab: Obtain Raw Data Results
7. Play Button to Start Experimental Trial
8. Designated Platform Location in Target Zone

Figure 4: Program view demonstrating sections required for setup and running an experiment. A few highlighted features of the tracking program used for Morris water maze. The apparatus view with the video of the pool featured above will only appear as presented when in the Tests tab. Please click here to view a larger version of this figure.

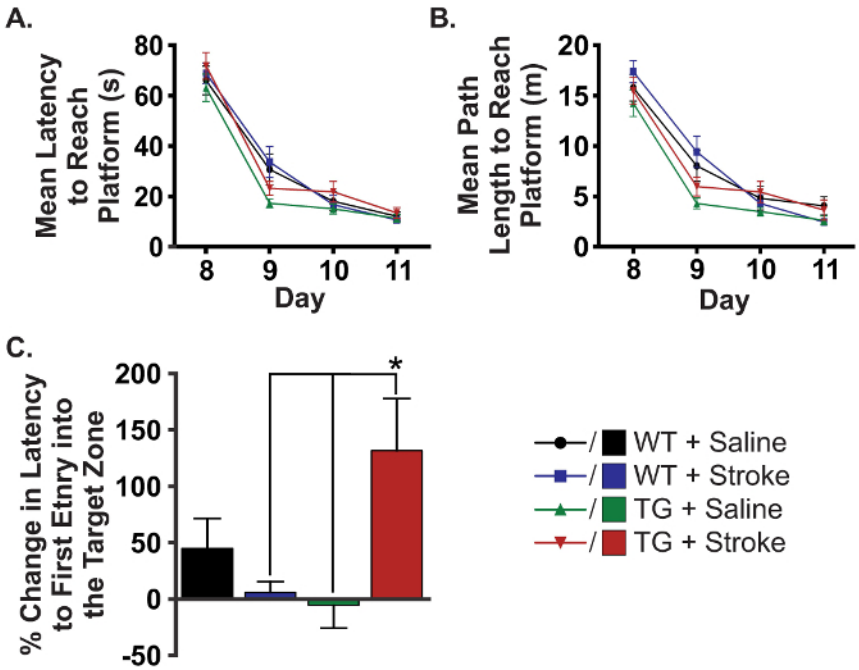


Figure 5: Spatial learning and reference memory in Morris water maze. Spatial learning was measured by (**A**) latency and (**B**) path length to reach the platform. (**C**) Reference memory was measured as percent change of latency to first entry into the target zone on the day 19 probe test compared to the day 12 probe test. Wild type is abbreviated as WT and transgenic is abbreviated as TG. Animal numbers are as follows: WT + saline (n = 8), WT + stroke (n = 7), TG + saline (n = 7), TG + stroke (n = 8). All values are presented as mean ± SEM and asterisks indicate statistical significance. (Two-way ANOVA for spatial learning and one-way ANOVA for probe testing, Tukey's post hoc, p <0.05). Please click here to view a larger version of this figure.

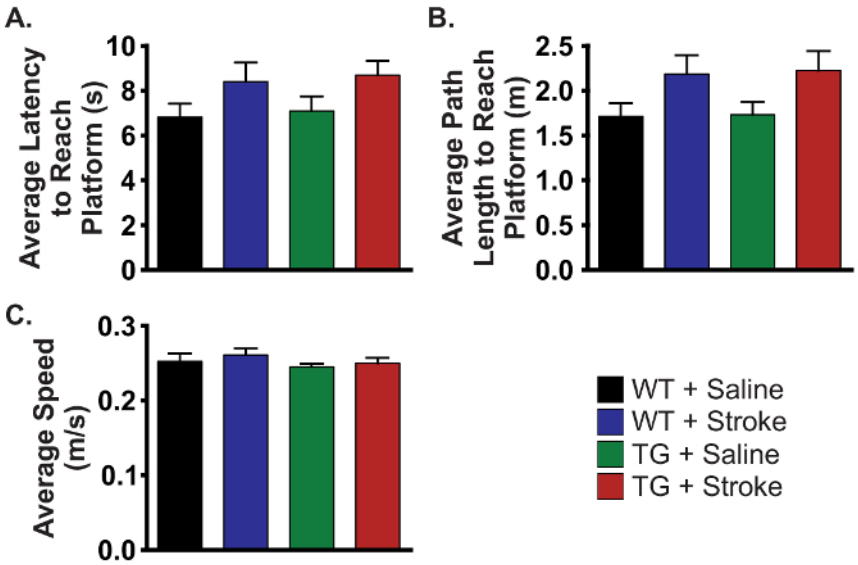


Figure 6: Cued learning in Morris water maze. (**A**) Latency and (**B**) path length to reach platform are stated as an average of all eight trials of learning. (**C**) Swim speed is presented as an average of all eight trials of learning. Wild type is abbreviated as WT and transgenic is abbreviated as TG. Animal numbers are as follows: WT + saline (n = 8), WT + stroke (n = 7), TG + saline (n = 7), TG + stroke (n = 8). Values are presented as the mean ± SEM. (One-way ANOVA, Tukey's post hoc). Please click here to view a larger version of this figure.

Discussion

The combination of stroke and Alzheimer's disease results in very distinct behavioral pathologies that can affect both motor and cognitive function depending on the severity of each condition. Thus, it is necessary to make use of a variety of behavioral tasks to determine the individual contributions of these conditions, as well as give some insight into the combined and potentially interactive effects in the co-morbid condition. The data presented demonstrate three cost-efficient, time-efficient and sensitive behavior tasks to assess motor function and hippocampal dependent spatial learning and reference memory in a novel co-morbid APP21 transgenic rat model with stroke. In addition to the data presented, these tasks have been verified in more severe stroke models[10,14,18], as well as in models of AD[8,16] and should be widely applicable to various models of both diseases.

That being said, no task is without limitations. For the motor tasks specifically, some troubleshooting may be required if rats become habituated to the cylinder and the beam. In the cylinder task, a motivation may be required to ensure the rat achieves the appropriate amount of forelimb contacts with the wall of the cylinder. To achieve this result, the application of a non-toxic scent on the perforated lid or wall of the cylinder can motivate the rat to rear and contact the wall of the cylinder with its forelimbs. For example, a little bit of peanut butter or vanilla extract can be smeared on the inside wall close to the top of the cylinder to further encourage sedentary rats to explore the cylinder walls. A ring of coloured tape can also be applied on the inside of the cylinder ¾ from the bottom of the cylinder. Another way of promoting rearing involves removing a sedentary rat and re-introducing it to the cylinder after some time has passed, which has been achieved with an inter-trial interval in the protocol. Furthermore, depending on the height of the cylinder compared to the length of the rat, removing the perforated lid halfway through a trial can prompt rearing and forelimb contact with the cylinder wall. This should not be done if there is a possibility the rat will be able to escape from the cylinder during testing. These motivations may help increase the number of rears, but should not influence the use of the left and right forepaws during wall contact. If there is still a major concern about an inadequate number of forelimb contacts after implementing these suggestions, a trial can be ended after the rat has made a total of 10 rears regardless of the length of time. This would be different from the 5 minute trials completed in the protocol above, as each rat would reach a total of 10 rears at different times. With a model of stroke and AD co-morbidity, it is important to be aware that changes in cognitive performance, anxiety and activity levels may develop in these rats. While such changes do not directly affect the primary outcome of the cylinder task (spontaneous forelimb use during rearing), recording other observations may constitute valuable evidence for non-motor-related behavioral changes that may develop. In the cylinder task, such behaviors can potentially be observed by analyzing increased or decreased rearing behavior, as rearing is a form of motivated exploratory behavior. Furthermore, other measures such as time spent grooming, turning and landing can provide insight on anxiety and other physical deficits, respectively.

In regards to the beam-walk task, using the home cage enrichment tubing is usually enough motivation for the rat to walk across the beam. If a rat continues to stop midway along the beam, a fragrant food reward or treat, such as peanut butter or precision sugar pellets, could be introduced in addition to the enrichment tubes at the opposite end. If treats are given, ensure that all rats receive about the same amount of treats on testing day, independent of their performance. Additionally, rather than running all six trials for a rat before moving onto the next rat, trials could be staggered. For example, the first two trials could be completed for all rats before starting the next two. This may prevent the increased number of stops midway across the beam that can occur on later trials when some rats become too comfortable with the beam environment. Another possible solution if frequent stopping occurs is to decrease the number of trials. This could be implemented by placing a mirror behind the beam to analyze both left and right limbs in each trial, thus allowing the number of trials to be decreased. Furthermore, habituation to the beam may occur after multiple testing sessions and can result in rats refusing to cross the beam and remain seated on the beam, despite all efforts to motivate the animal. Due to this problem, beam assessments are not ideal for repeated testing in long-term

experiments. In protocols that utilize repeated testing, compensation can also become a concern in addition to motivation. To overcome issues of compensation while crossing the beam, a tapered beam can be used rather than a regular wooden beam[21].

Again, cognitive changes, including increased anxiety and changes in overall activity levels and motivation, may occur in this animal model. Therefore, it is important to take note of any irregularities among the experimental groups regarding the animals' motivation to cross the beam as well as their speed and non-locomotor behavior (stopping, sitting, trembling, orientation) while traversing the beam.

Behavioral Alzheimer's disease research requires short-term and long-term memory testing, which has been achieved here using the Morris water maze. Many protocols consider 24 hr after spatial learning to be long-term memory[21], but with this protocol a timeline of 24 hr after spatial learning is considered short-term memory and one week is considered long-term memory. During this one-week time period in-between probe trials, the rats should not be exposed to other behavioral tasks or unnecessary stressors to avoid interference with Morris water maze spatial reference memory.

In regards to spatial learning, the experimenters should be persistent in requiring the rat to sit on the platform without jumping for a certain amount of time. If the rat successfully sits on the platform but jumps off as the experimenters come into sight, the rat should be placed or guided back to the platform and required to sit on the platform until the experimenter retrieves the animal. To enforce the learning of the platform as the only way to escape the water, rats should not be picked up while swimming, unless in a probe trial setting. If rats manage to jump from the platform onto the rim of the pool, consider moving the platform further towards the center of the pool to discourage learning of an alternate route of escape.

To ensure there are no spatial biases during spatial and cued learning, the start positions and cued platform positions for each rat in a treatment group should be randomized. Each treatment group should have a representative number of rats starting from each starting position for spatial learning or following the same start and platform position paradigm for cued learning. For assigning start positions, Vorhees and Williams present a very detailed set of randomized start positions for spatial learning and randomized start and platform positions for cued learning[20]. This can be used directly or as a guideline to assign positions to each rat prior to beginning Morris water maze testing.

For the analysis of Morris water maze data, the suggestions in the protocol above represent how the data presented here was acquired. The data collected in step 3.6 of the above Morris water maze protocol can be used to calculate various outcome measures that may be useful to describe a cognitive deficit. For example, beyond the numerical data set, tracking software also offers the experimenter the opportunity to analyze track plots, which can give additional insights into the animals' search strategies. Additionally, the percentage of time spent or distance traveled within the target quadrant in relation to all quadrants can be used as a measure of reference memory in probe trials. It is important to keep in mind that these co-morbid animal models may exhibit some motor deficits. Comparing the swim speed among the experimental groups can give an indication whether a potential motor deficit is impacting the animals' ability to perform in the Morris water maze. Furthermore, to exclude motor performance from becoming a confounding factor in water maze outcome, it is recommended to look at path length to reach platform in addition to latency and swim speed as demonstrated in **Figure 5** and **6**. If swimming ability is compromised in any way, path length is the most accurate measure to assess hippocampal function.

While there are various different timelines that can be applied to all of these behavioral tasks described, the methodology of running each experiment should remain the same. The data presented here was achieved with a 21 day post-stroke recovery time point, which captures the early events after stroke and their potential interaction with Aβ metabolism in the brain. While the data presented here was in a co-morbid model of stroke and Alzheimer's disease, these tasks can be applied to or adapted to suit various research questions and models. While the cylinder test is a less-modifiable standard procedure, the beam walk task can be somewhat adjusted to the severity of the expected motor deficit by choosing appropriate beam widths. The water maze is the most versatile test of all paradigms mentioned herein. For example, choosing various intervals between the end of the acquisition phase and the spatial reference memory probe trial can easily test short-term and long-term memory. Working memory and strategy shifting, two components of executive function, can also be tested using the water maze setup. For assessing working memory, the inter-trial interval can be reduced to under 1 min during acquisition learning of a new platform location. Furthermore, having animals learn a second, new location of the platform after successful acquisition of an initial platform location can test mental flexibility or strategy shift. Considering all of these potential modifications, there is a lot of flexibility with these behavior tasks, which is another major benefit in addition to all the aforementioned benefits.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This work was made possible with funding from the Canadian Institutes of Health Research (CIHR) and the authors would like to thank CIHR for their funding support.

References

1. Auld, D., Kornecook, T., Bastianetto, S., Quirion, R. Alzheimer's disease and the basal forebrain cholinergic system: relations to β-amyloid peptides, cognition, and treatment strategies. *Prog Neurobiol.* **68** (3), 209245 (2002).
2. Huang, Y., Mucke, L. Alzheimer Mechanisms and Therapeutic Strategies. *Cell.* **148** (6) (2012).
3. Querfurth, H., LaFerla, F. Alzheimer's disease. *New Engl J Med.* **362** (4), 329-44 (2010).
4. Karran, E., Mercken, M., Strooper, B. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov.* **10** (9), 698-712 (2011).
5. Akiyama, H., *et al.* Inflammation and Alzheimer's disease. *Neurobiol Aging.* **21** (3) (2000).

6. Butterfield, D., Drake, J., Pocernich, C., Castegna, A. Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid β-peptide. *Trends Mol Med.* **7** (12), 548554 (2001).
7. Kalaria, R. The role of cerebral ischemia in Alzheimer's disease. *Neurobiol Aging.* **21** (2), 321-330 (2000).
8. Selkoe, D. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev.* **81** (2), 741-766 (2001).
9. Snowdon, D.A., *et al.* Brain infarction and the clinical expression of alzheimer disease: The nun study. *JAMA.* **277** (10), 813 (1997).
10. Clarke, J., *et al.* Overexpression of APP provides neuroprotection in the absence of functional benefit following middle cerebral artery occlusion in rats. *Eur J Neurosci.* **26** (7), 1845-1852 (2007).
11. Whitehead, S., Hachinski, V., Cechetto, D. Interaction Between a Rat Model of Cerebral Ischemia and β-Amyloid Toxicity Inflammatory Responses. *Stroke.* **36** (1), 107-112 (2005).
12. Agca, C., *et al.* Development of transgenic rats producing human β-amyloid precursor protein as a model for Alzheimer's disease: Transgene and endogenous APP genes are regulated tissue-specifically. *BMC Neuroscience.* **9** (1), 28 (2008).
13. Bayona, N.A., Gelb, A.W., Jiang, Z., Wilson, J.X., Urquhart, B.L., & Cechetto, D. F. Propofol neuroprotection in cerebral ischemia and its effects on low-molecular-weight antioxidants and skilled motor tasks. *Anesthesiology.* **100** (5), 1151-9 (2004).
14. Langdon, K., Clarke, J., & Corbett, D. Long-term exposure to high fat diet is bad for your brain: exacerbation of focal ischemic brain injury. *Neuroscience.* **182**, 82-7 (2011).
15. Brownlow, M., *et al.*Partial rescue of memory deficits induced by calorie restriction in a mouse model of tau deposition. *Behav Brain Res.* **271**, 7988 (2014).
16. Halagappa, V., *et al.* Intermittent fasting and caloric restriction ameliorate age-related behavioral deficits in the triple-transgenic mouse model of Alzheimer's disease. *Neurobiol Dis.* **26** (1) (2007).
17. Maalouf, M., Rho, J., & Mattson, M. The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. *Behav Brain Res.* **59** (2) (2008).
18. Tillerson, J. L., *et al.* Forced limb-use effects on the behavioral and neurochemical effects of 6-hydroxydopamine. *Neuroscience.* **21** (12), 4427-35 (2001).
19. Sierksma, A., *et al.* Improvement of spatial memory function in APPswe/PS1dE9 mice after chronic inhibition of phosphodiesterase type 4D. *Neuropharmacology.* **77** (2013).
20. D'Hooge, R., De Deyn, P. P. Applications of the Morris water maze in the study of learning and memory. *Behav Brain Res.* **36** (1), 60-90 (2001).
21. Vorhees, C., Williams, M. Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc.* **1** (2), 848-858 (2006).

