Video Article

Utilizing the Modified T-Maze to Assess Functional Memory Outcomes After Cardiac Arrest

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Abstract

Background: Evaluating mild to moderate cognitive impairment in a global cerebral ischemia (i.e. cardiac arrest) model can be difficult due to poor locomotion after surgery. For example, rats who undergo surgical procedures and are subjected to the Morris water maze may not be able to swim, thus voiding the experiment.

New Method: We established a modified behavioral spontaneous alternation T-maze test. The major advantage of the modified T-maze protocol is its relatively simple design that is powerful enough to assess functional learning/memory after ischemia. Additionally, the data analysis is simple and straightforward. We used the T-maze to determine the rats' learning/memory deficits both in the presence or absence of mild to moderate (6 min) asphyxial cardiac arrest (ACA). Rats have a natural tendency for exploration and will explore the alternate arms in the T-maze, whereas hippocampal-lesioned rats tend to adopt a side-preference resulting in decreased spontaneous alternation ratios, revealing the hippocampal-related functional learning/memory in the presence or absence of ACA.

Results: ACA groups have higher side-preference ratios and lower alternations as compared to control.

Comparison with Existing Method(s): The Morris water and Barnes maze are more prominent for assessing learning/memory function. However, the Morris water maze is more stressful than other mazes. The Barnes maze is widely used to measure reference (long-term) memory, while ACA-induced neurocognitive deficits are more closely related to working (short-term) memory.

Conclusions: We have developed a simple, yet effective strategy to delineate working (short-term) memory via the T-maze in our global cerebral ischemia model (ACA).

Video Link

The video component of this article can be found at https://www.jove.com/video/56694/

Introduction

According to the American Heart Association (2017), cardiac arrest (CA)-induced mortality occurs every four minutes, and affects over 400,000 people per year in the United States[1]. It is well-documented that CA can cause neuronal brain injury as a result of insufficient blood perfusion[2,3,4]. CA-induced brain injury occurs in the ischemia-sensitive CA1 region of the hippocampus[5,6,7], affecting neurons that are critical to learning and memory[8,9,10,11,12]. Moreover, the loss of dendritic spine density, under ischemic conditions in the hippocampus (i.e. CA1 neurons), plays a critical role in spatial memory impairment[13,14,15]. Due to these pathological changes after CA, behavioral disorders such as: anxiety, depression, post-traumatic stress disorder, and memory loss are more prevalent. Although there have been advances in medical technology (i.e. efficient ambulatory service) that correlate with improved CA survival rates, most of the neuroprotective treatments (except for hypothermia) fail to improve functional outcomes after CA[16,17]. CA survivors typically have a poor quality of life, and are burdened with incremental medical spending[16].

Cognitive status assessments for cerebral ischemia via behavioral tests are important to determine both drug efficacy and ultimately develop a successful clinical trial. In the 1940s, Edward Tolman designed the first behavior trial to study hippocampus-based spatial memory[18]. Subsequently, different mazes (i.e. Morris water maze, radial maze, T- or Y-maze, and Barnes maze) were developed to evaluate hippocampal-

based spatial learning and memory in rats[19,20,21,22,23]. One of the more widely used behavioral test is the Morris water maze, which examines spatial learning and memory in rat models[24]. However, the Morris water maze requires the rat to swim and exert full motor function and control. For ischemia experiments such as the asphyxial cardiac arrest (ACA, a rat model of CA) model, cannulation of the femoral artery/vein are required to obtain vital blood pressure, blood gases and introduction of various drugs. Since femoral artery/vein cannulation can inhibit leg mobility rendering the rat's ability to swim properly, the Morris water maze may not be the most appropriate to test cognitive impairments under ACA.

The Barnes maze is the other widely used behavioral test to examine spatial learning and memory in rodent models. The Barnes maze does not require the exertion of full motor function and control, and thus less stressful than the Morris water maze. In the past, we performed experiments using the Barnes maze to determine if functional learning/memory differences occur between control or sham versus ACA-induced rats. The data obtained for the Barnes maze did not have the resolution to test cognitive impairments following mild to moderate ACA due to the fact that the Barnes maze is widely used to measure reference (long term) memory[25,26], while ACA-induced neurocognitive deficits more closely related to working (short-term) memory[27,28,29,30] suggesting that the Barnes maze is less viable for assessing memory function in our ACA model.

We thus developed a modified T-maze using spontaneous alternation test to evaluate working (short-term) memory after ACA. The modified T-maze spontaneous alternation test's major advantage is its simplicity and minimal stress on the rats as compared to other behavioral tests due to the fact that the modified T-maze does not require prior animal training, as well as heavy computational analysis or sub-routines (i.e. video imaging of the rat) as required by the Morris water maze and Barnes maze. Here we show that the modified T-maze spontaneous alternation test is a simple and yet highly efficient behavioral trial paradigm that can offer enough resolution to accurately detect and evaluate hippocampal function in diseases that cause short-term memory loss (i.e. ACA).

Protocol

All experimental procedures were conducted in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (LSU Health Sciences Center-Shreveport) for the usage of male Sprague Dawley rats (300-350 g, 9-10 weeks old). Rats were fasted overnight before the ACA surgery.

1. T-maze apparatus design and setting

NOTE: Base the T-maze design on the Deacon and Rawlins' 2006 model[31].

1. Design 3D structure of the maze utilizing SketchUp[32]. To create a 3D structure of the T-maze, construct the start arm with an outside length of 200 mm, width of 165 mm, and height of 148 mm to fit within the printing dimensions of the 3D printer. Use a wall thickness of 5.5 mm and a floor thickness of 8 mm.
2. Print the maze using a 3D printer (see table of materials)[32]. If a 3D printer is not available in the laboratory, use other materials such as wood, medium-density fiberboard, or a plastic (i.e. polyvinyl chloride), which can be purchased from home improvement stores.
    1. Due to height restrictions in the print area, construct the walls of the maze in two separate 3D prints and join together upon maze assembly (i.e., a second wall height was added to the maze section to increase the height by 140 mm, for a total wall height of 280 mm). Each separate 3D print base contained a "T" shaped locking mechanism, where one section connected to the next.
    2. At the junction of the start arm with the goal arms, create a 165-mm wide section to join the width of the start arm with that of the goal arms. Construct the goal arms using a similar design method as the start arm; however, reduce the width of the arm to 100 mm per the design of Deacon and Rawlins.
    3. Please see **Figure 1** for detailed schematic/dimensions of the T-maze.
    4. Include a central partition into the design at the junction of the start arm and goal arms. Extend this partition from the back wall of the T-maze and 200 mm into the start arm to divide the goal arms. This partition also extended the height of the maze (**Figure 1**).

2. Asphyxial Cardiac Arrest (ACA)

1. Autoclave surgical tools (121 °C for 15 min) prior to initiation of surgery. Disinfect the surgical table by 70% ethanol for 15 min. Shave the animal hair at the site of surgery. Apply a betadine solution to skin surfaces for surgical operation.
2. **Anesthetization**
    1. Anesthetize rats with 4% isoflurane and 30:70 mixture of O_2 and N_2O (300 mL/min O_2 and 700 mL/min N_2O) via mask.
    2. Give rats endotracheal intubation for mechanical ventilation (After intubation, rats were connected to a ventilator).
    3. Maintain anesthesia by lowering isoflurane from 4% to 2% with a 30:70 mixture of O_2 and N_2O. Use the pinch-response method to determine depth of anesthesia.
    4. Apply ointment on eyes to prevent dryness while under anesthesia. Regulae the body temperature by a rodent heating pad with an anal probe as a temperature reference.

3. **Endotracheal intubation**
    1. Place the rat in the induction chamber. Anesthetize the rats with 4% isoflurane and 30:70 mixture of O_2 and N_2O.
    2. Remove the rat from the induction chamber. Place anesthetized animal in the supine position with the rat's face towards the anesthesia mask.
    3. Gently move the tongue towards either the left or right of the animal with the left thumb and index finger.
    4. Glide a 14-gauge flexible intravenous catheter (49 mm-long) over a 17-gauge blunt tip pipetting needle (93 mm-long with 10-degree angle at the needle's tip). Insert the 17-gauge blunt tip pipetting needle into the trachea.
    5. Gently pull out the 17-gauge pipetting needle from the trachea. Connect the 14-guage catheter hub to the ventilator. Adjust ventilator stroke volume to 0.67 mL/100 g and respiratory rate of 60 breaths/min.

6. Maintain head and body temperature at 37 °C during the entire procedure by a rodent heating pad with anal probe as a temperature reference.

4. **Femoral arterial and venous catheterization**
   1. Shave hair near the inguinal area (either side) and apply betadine to skin surfaces for surgical operation.
   2. Placed the rat in the supine position. Make an incision (10 mm) in the inguinal area with surgical scissors.
   3. Separate the connective tissue by blunt tip forceps until the inguinal ligament is exposed. Use a hemostat to grasp inguinal ligament. The femoral artery and vein are underneath the inguinal ligament.
   4. Use blunt tip forceps to separate the connective tissue until the femoral artery and vein are exposed.
   5. Gently separate the femoral nerve which runs along the femoral artery via fine tip forceps. Carefully separate the femoral artery and vein as a unit via fine tip forceps.
   6. Use fine tip forceps to separate the femoral artery from the vein.
   7. Place 2 pieces of 5-0 silk suture (one towards the leg and the other towards the body) under the vein.
   8. Tie a loose knot on the side near the body. Use a hemostat to hold and pull the suture as far as possible towards the opposite sides of the body.
   9. Tie a loose knot on the side near the leg. Hold and pull the suture towards the leg via a hemostat to allow the vein to fill with blood.
   10. Make a small incision in the vein (approximately 0.1 mm) by micro-dissecting scissors (at a 45° angle). Soak up any blood with sterilized gauze.
   11. Attach a blunt tip needle syringe (filled with saline with 20 U/mL heparin) to a PE-50 catheter. Fill the PE-50 catheter with saline with 20 U/mL heparin. Cut the PE-50 catheter with dissection scissors at a 45° angle to create a point or sharp end. Use blunt tip forceps to hold the end of the PE-50 catheter. Gently insert the PE-50 catheter into the femoral vein.
   12. After the catheter is fully inserted, slowly administer 0.1 mL of heparin/saline to ensure that there is no leak. Tie firm suture knots (single-knot) to stabilize the PE-50 catheter. Keep the PE-50 catheter for continuous intravenous (IV) injection of various drugs.
   13. Use a 1 mL syringe connected with a 23 gauge Luer stub adapter to administer vecuronium bromide (0.67 mg/kg, administered every 10 min) via the femoral vein to immobilize the rat throughout the procedure.
   14. Place 2 pieces of 5-0 silk suture (one towards the leg and the other towards the body) under the artery.
   15. Tie a loose knot on the side near the leg. Use a hemostat to hold and pull the suture as far as possible towards the leg.
   16. Tie a loose knot on the side near the body. Hold and pull the suture towards the body via a hemostat to allow the artery to fill with blood.
   17. Make a small incision in the artery (approximately 0.1 mm) by micro-dissecting scissors (at a 45° angle).
   18. Attach a blunt tip needle syringe (filled with saline with 20 U/mL heparin) to a PE-50 catheter. Fill the PE-50 catheter with saline with 20 U/mL heparin. Cut the PE-50 catheter with dissection scissors at a 45-degree angle to create a point or sharp end. Use blunt tip forceps to hold the end of the PE-50 catheter. Use blunt tip forceps to hold the end of the PE-50 catheter. Gently insert the PE-50 catheter into the femoral artery.
   19. After the catheter is fully inserted, slowly draw back the syringe to ensure that catheter is functional. Tie firm suture knots (single-knot) to stabilize the PE-50 catheter. Keep the PE-50 catheter for continuous recording of arterial pressure and blood gases.

5. **Asphyxial Cardiac Arrest (ACA) procedure**
   1. Adjust physiological parameters (i.e. pO_2, pCO_2, blood pressure, and pH value) as needed by modulating stroke volume, O_2 or N_2O levels. Use normal physiological ranges of these parameters: pO_2: 100 mmHg, pCO_2: 35-40 mmHg, blood pressure: 100 mmHg, and pH: 7.4.
   2. Use a 1 mL syringe connected with a 23 gauge Luer stub adapter to administer vecuronium bromide (0.67 mg/kg, I.V.) via femoral vein and wait for 2 min. Make sure the blood pressure is at or around 100 mmHg before performing ACA.
   3. Induce apnea (6 min) by disconnecting the endotracheal tube (14-guage catheter hub) from the ventilator. Further block the endotracheal tube by a 1 mL syringe to ensure complete apnea.
      NOTE: The 6-min asphyxia time is defined as the period between ventilator disconnection and the beginning of resuscitation. Complete cardiac arrest is defined as a mean arterial pressure lower than 10 mmHg.
   4. During the last min of apnea, adjust respiratory rate of the ventilator to 80 breaths/min, and increase O_2 to 2 L/min with 0% N_2O. This action will blow out any remaining isoflurane or N_2O remaining in the ventilator.
   5. min following apnea, remove 1 mL syringe from the endotracheal tube. Re-connect the endotracheal tube to the ventilator.
   6. Use a 1 mL syringe connected with a 23 gauge Luer stub adapter to administer epinephrine (0.005 mg/kg, I.V.) via femoral vein and administer manual chest compressions by the thumb, index, and middle fingers on the animal's chest in a light circular motion on the x and z-axis (200/min) until return of spontaneous circulation (mean arterial pressure ≥ 50 mmHg)[33,34,35].
   7. Use another 1 mL syringe connected with a 23 gauge Luer stub adapter to administer sodium bicarbonate (1 meq/kg, I.V.) via femoral vein immediately after return to spontaneous circulation (50 mmHg or higher)[33,34,35] to alleviate respiratory acidosis.
   8. Measure blood gases again 10 min after resuscitation to determine the acid-base status (pH after ACA should be around 7.35 to 7.40)
   9. Use a hemostat to clamp the femoral artery and vein. Slowly and gently remove arterial and venous catheters using blunt tip forceps. Ligate femoral artery/vein with a 5-0 silk suture to prevent bleeding. Close the skin overlying the surgical site using a 3-0 silk suture. Use the interrupted suturing technique to minimize the chances of the wound reopening.
   10. Wait until the rat breathes itself (usually 30 min to 60 min after resuscitation), disconnect the rat from ventilator and gently remove the endotracheal tube.
   11. Place the rat in the baby incubator (27 °C, 50% humidity) overnight. Place softened food (made by soaking them in the water) and water into the baby incubator overnight.
   12. Transfer the rat to the individual cage and return the rat to animal facility with regular chow and water. T-maze tests start 3 days after ACA.

3. T-maze

1. **Animal preparation**
    1. The day before surgery (sham or ACA), handle each rat for 5 min. Never elevate rats from their cage (480 mm x 250 mm x 200 mm, plastic transparent cage) when handling (**Figure 2**).
    2. After handling the rat, gently pick the rat up by its tail with one hand with the other hand supporting its' legs. Let them jump from the hand to the cage (100 mm height) 5 times. Separate each rat into individual cages, so they will not dominate for food and/or fight.
    3. Three days after sham or ACA surgery (**Figure 2**), transfer the rats with the cage into a quiet and dark room before the start of the first run. Only turn on a low power desk lamp and place it at the corner of the testing room to maintain minimum illumination. Allow the rat to adapt to darkness for 10 min.
    4. Perform all experiments in the afternoon to avoid any effects of diurnal variation on rats' performance. Do not advise the operator on which rat received sham or ACA surgery.

2. **Spontaneous alternation**
    1. Spread a thin layer of bedding (~10 mm thick) to cover the entire floor of the maze. Then place the rat at the start arm (bottom of the "T"), which is the starting point of each run, and allow each rat 3 min to explore the right or left goal arm.
    2. Once the rat commits to a particular goal arm (all four paws of the rat have entered the goal arm), block the "T" junction between the start arm and the opposing goal arm (**Figure 1**) to prevent the rat from entering the opposing goal arm. Leave the rat in the maze for 30 seconds, then pick up the rat and place it back in its cage for a minimal time (~30 seconds). Then remove the "T" junction block (125 mm X 230 mm X 65 mm, made by a 3D printer) from the T-maze.
    3. Place the rat at the start arm and repeat 3.2.2. Alternation is defined as: when the rat enters the opposite arm as compared to the previous run[36]. Have rats perform 4 runs per day as follows:
    1^{st} run
    2^{nd} run
    10-min break
    3^{rd} run
    4^{th} run
    4. Change the bedding during the 10-min break and between animals to eliminate scent bias. Clean the T-maze with 75% ethanol followed by distilled water at the end of each experimental day.
    5. Repeat steps 3.2.1. - 3.2.4. for two more days (12 runs in total) as in **Figure 2**.

3. **Alternation rate and side preference rate calculations**
    1. Calculate the % Alternation and the % Side Preference, where
    L: the rats choose the left arm
    R: the rats choose the right arm
    Correct choice: the 2^{nd} run is different from the 1^{st} in a given set (each set contains two runs)
    Incorrect choice: the rats choose the same arm similar to previous run

$$\frac{\text{The number of correct choices}}{\text{Total sets performed}} \text{ X } 100\% = \% \text{ Alternation}$$

$$\frac{\text{The number of preferred side that the rat has chosen}}{\text{Total runs performed}} \text{ X } 100\% = \% \text{ Side preference}$$

Example:
Day 1: L L / L L
Day 2: L L / L R
Day 3: R L / L L
Alternation: 2 (correct choices) /6 (total sets performed) * 100 = 33.33%
Side preference: 10 (L, preferred side) /12 (total runs performed) * 100 = 83.33%

4. **Post-operative care:**
    1. Give rats buprenorphine (0.01 mg/kg IP) every 12 h for 2 days after surgery. Observe rats for up to 1 h after cardiac arrest.
    2. Attach rats to the ventilator and heating pad until it has regained sufficient consciousness to maintain sternal recumbency. To maintain animals' body temperature after surgery, place the rat in a baby incubator (set at 27 °C, 50% humidity).
    3. Provide softened chow (made by soaking in water) to animals for the first 24 h after surgery. If the rats were not drinking water, administer bacteriostatic saline (100 mL/kg/day, I.P.) until the animal recovers and is drinking water freely.
    4. Give the rats topical antibiotic with pain relief (bacitracin and lidocaine ointment) at all wounds. Move rats back to the animal facility after they fully recover.

5. **Euthanasia method**
    1. Use 5% isoflurane and 100% N_2O to euthanize the animals at the end of experiment.

Representative Results

ACA (global cerebral ischemia) mainly causes working (short-term) memory deficits[28,29]. To assess the function of learning and memory after ACA, we used the modified spontaneous alternation test to evaluate working (short-term) memory[30]. Results from spontaneous alternation test suggest that the alternation rate from three consecutive days in the ACA group (26.19 ± 4.96%) was significantly lower as compared to the control group (62.96 ± 6.07%) (*p0.05)[35] due to the fact that rats submitted to ACA developed a side bias as compared to control (82.14 ± 4.57% v. 62.89 ± 2.86%, *p0.05) (**Figure 3a and 3b**)[35], thus presented with lower spontaneous alternation rate. Results were expressed as means ± S.E.M. Data analyzed using one-way ANOVA followed by Turkey's post-hoc test[37]. $p < 0.05$ (95% confidence level) was considered statistically significant.

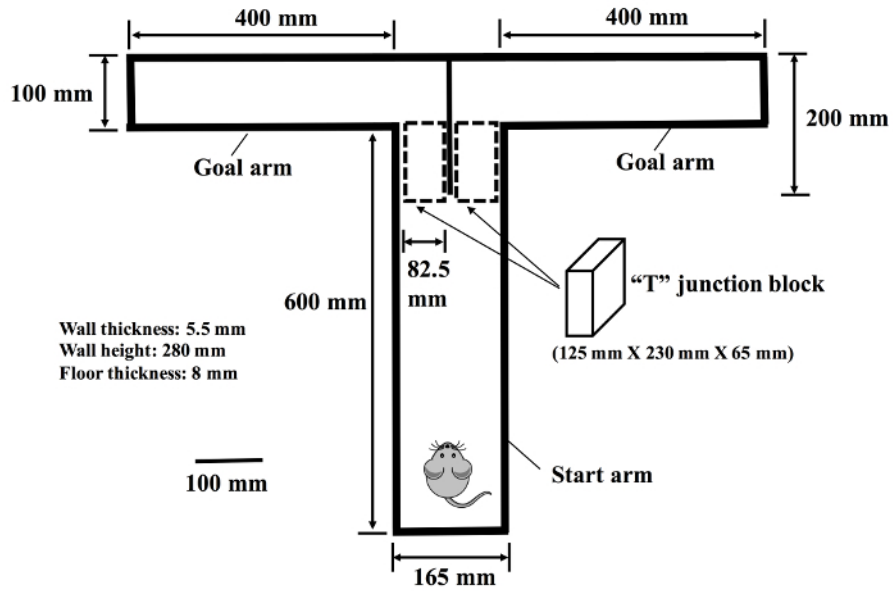

Figure 1. The T-maze design.
The T-shaped platform of the maze (for rats) was built with a 600 mm x 165 mm start arm and 400 mm x 100 mm goal arms at the upper apex of the "T". The thickness of the walls was 5.5 mm x 8 mm (floor thickness). A central partition was at the junction of the start arm to the goal arm, where this partition extended from the back wall of the T-maze and 100 mm into the start arm dividing the goal arms. The dotted squares represent the location of the "T" junction block that blocked either arm. Please click here to view a larger version of this figure.

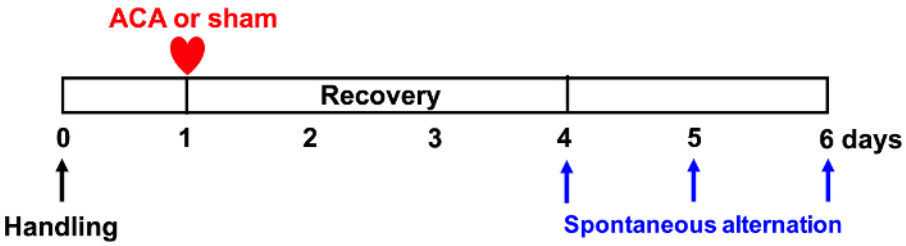

Figure 2. Experimental timeline for ACA/sham surgery and T-maze.
Before the day of the ACA surgery, rats were handled 4 times (5 min/each time, at day 0) to acclimatize the rat to human touch. After ACA surgery, the rats were permitted to heal and stabilize over the following 3 days. After recovery, the rats performed the spontaneous alternation tests for 3 consecutive days (12 runs, 4 runs/day). Each rat performs 4 runs per day. The experimental timeline is the same for sham surgery. Please click here to view a larger version of this figure.

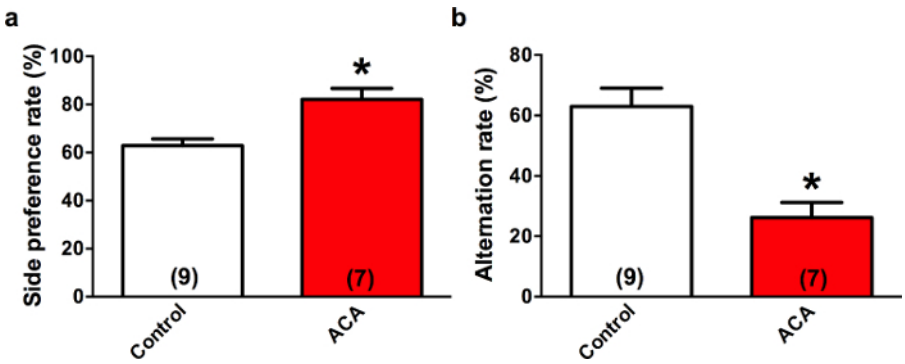

Figure 3. Short-term memory deficits after ACA.
An increase in side preference rate can be observed in rats subjected to ACA (a). Spontaneous alternation rate was decreased in rats with ACA as compared with control (b). Numbers in parentheses indicate the animals used per group. Results were expressed as means ± S.E.M. *P < 0.05 indicates significantly different from control. Statistical analysis was evaluated by one-way ANOVA with Tukey's post hoc test. This figure has been modified from Lee et al., 2017[35]. Please click here to view a larger version of this figure.

Discussion

Modifications were made in the present study as compared to Deacon and Rawlins' protocol[31]. The 3D printer was used to build the T-maze. The 3D-printing provides affordable and cost-effective alternatives to commercialized T-maze. To reduce rats' anxiety during the test, the T-maze was performed in the dark room with minimum illumination. Once the rat entered one of the goal arms, we gently blocked the opposing arm. This avoids possible stress from the test, as well as possible damage to rats' tail while lowering the guillotine door. Any unnecessary stress will inhibit the rat's performance on subsequent runs. Thus, we utilized a "T" junction block as the gate in the present study to eliminate any grinding noise from sliding plastic guillotine doors during test. This step can significantly reduce rats' anxiety during the T-maze experiment.

Habituation is a critical step for successful T-maze studies since anxious rats are not motivated to run in the maze. Thus, it is imperative to ensure that the rats acclimatize to human touch, height, and moving from the cage to the maze before the experiments. Anxious rats make squeaking or hissing sound when operators try to touch them. Additionally, anxious rats either present with long freezing responses or refuse to run in the maze during the entire T-maze experiments. They usually spend most of the time in the start arm without exploring the maze. If the rats do not run in the maze, gently touch their tail and they will run. The other possible reason is that the rats received insufficient habituation. Gently put the rat back in the cage and wait for 10 min to decrease their anxiety. If the rat fails to complete the run again, that rat is excluded from the study. If the rats cannot complete a single run in 3 min, put it back to its cage and wait for 10 min. If the rat still fails to complete the run, the rat must be excluded from the study. The rats should not perform the test for an extended duration or frequency or stay in the testing room for more than an hour, otherwise they will stop running due to lack of novelty.

Previous studies have shown that 70% of rats exhibit left or right bias[38,39]. This lateral bias worsens T-maze performance in the rat[39]. Thus, we specified the inclusion and exclusion criteria for rats in the present study. We have tested a total of 15 rats received ACA surgery in our T-maze study. The average alternation rate in ACA-treated rats is 29 ± 4 %. Since control or sham rats do not have any hippocampal injury or learning/memory deficits, we hypothesized that control or sham rats should have better T-maze performance (alternation rate ≥ 29 ± 4 %) as compared to ACA-treated animals. Therefore, control or sham rats with alternation rate < 29 % (the total number of alternation ≤ 1), which suggest the rats either have side preference or failed to learn in the maze should be excluded from the study. We have applied this exclusion criteria to 25 control rats. 6 of them (24%) performed with high side preference rate (85 ± 3%) and low spontaneous alternation rate (lower than 29%) were excluded from the studies.

The T-maze spontaneous alternation experiment is highly dependent on the rats' natural tendency to explore novelty. Thus, the major limitation of the T-maze spontaneous alternation protocol is that the rat will eventually cease to run in the maze. Based on our past experiences, the rat is only willing to run in the maze for three consecutive days. Since asphyxial cardiac arrest mainly results in short-term memory deficits, we can extrapolate our current findings to other brain injury/diseases (i.e. Alzheimer's disease, Parkinson's disease, and transient ischemic attack) that are also related to short-memory deficits.

Disclosures

The authors have nothing to disclose.

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