

## Video Article

# T-maze Forced Alternation and Left-right Discrimination Tasks for Assessing Working and Reference Memory in Mice

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## Abstract

Forced alternation and left-right discrimination tasks using the T-maze have been widely used to assess working and reference memory, respectively, in rodents. In our laboratory, we evaluated the two types of memory in more than 30 strains of genetically engineered mice using the automated version of this apparatus. Here, we present the modified T-maze apparatus operated by a computer with a video-tracking system and our protocols in a movie format. The T-maze apparatus consists of runways partitioned off by sliding doors that can automatically open downward, each with a start box, a T-shaped alley, two boxes with automatic pellet dispensers at one side of the box, and two L-shaped alleys. Each L-shaped alley is connected to the start box so that mice can return to the start box, which excludes the effects of experimenter handling on mouse behavior. This apparatus also has an advantage that *in vivo* microdialysis, *in vivo* electrophysiology, and optogenetics techniques can be performed during T-maze performance because the doors are designed to go down into the floor. In this movie article, we describe T-maze tasks using the automated apparatus and the T-maze performance of  $\alpha$ -CaMKII<sup>+/−</sup> mice, which are reported to show working memory deficits in the eight-arm radial maze task. Our data indicated that  $\alpha$ -CaMKII<sup>+/−</sup> mice showed a working memory deficit, but no impairment of reference memory, and are consistent with previous findings using the eight-arm radial maze task, which supports the validity of our protocol. In addition, our data indicate that mutants tended to exhibit reversal learning deficits, suggesting that  $\alpha$ -CaMKII deficiency causes reduced behavioral flexibility. Thus, the T-maze test using the modified automatic apparatus is useful for assessing working and reference memory and behavioral flexibility in mice.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/3300/>

## Protocol

### 1. Apparatus setting

1. The automatic modified T-maze apparatus (O'HARA & Co., Tokyo, Japan) is constructed of white plastic runways with 25-cm high walls<sup>1</sup>. The maze is partitioned off into 6 areas (A1, A2, S1, S2, P1, P2) by sliding doors (s1, s2, s3, a1, a2, p1, p2) (Figure 1) that can be automatically opened downward. The stem of the T is composed of area S2 (13 x 24 cm) and the arms of the T comprise areas A1 and A2 (11.5 x 20.5 cm). Areas P1 and P2 comprise the connecting passages from the arm (area A1 or A2) to the start compartment (area S1).
2. The end of each arm is equipped with a pellet dispenser that automatically provides a sucrose pellet (20 mg, Formula 5 TUT, TestDiet, Richmond, IN, USA) as a reward. The pellet intake by the mouse is detected by the infrared sensor and is automatically recorded by a computer.
3. The Charge Coupled Device (CCD) camera is mounted above the apparatus to monitor the mouse's behavior, and images of the apparatus and mouse are captured by the computer.
4. Place the T-maze apparatus in a soundproof room (170 x 210 x 200 cm, O'HARA & Co., Tokyo, Japan) as possible. The apparatus is illuminated by fluorescent lights at 100 lux in our laboratory. The light intensity could be weaker than this lux level but should be held at a constant level during all the experiments.

### 2. Animal preparation

1. House about two to four mice per cage in a temperature-controlled room (23±2°C) with a 12-h light/dark cycle (lights on at 7:00 AM), according to guidance and protocols established by local Animal Care and Use Committee.
2. Transfer all the cages containing mice into the soundproof room from the housing room at least 30 min before the first trial begins.
3. All the experiments should be always performed during the same time period (e.g., 9:00 AM to 6:00 PM). During the testing period, the subjects from each genotype or experimental condition should be tested in counterbalanced order, since there could be a potential effect of the time in a day on the performance of the task.

### 3. Food restriction

1. Until the beginning of the experiment, give free access to standard pellet chow and water to mice.
2. From 1 week before the pre-training sessions, weigh mice daily and feed them with standard pellet chow to maintain 80% to 85% of their free-feeding body weight throughout the experiment.
3. Provide daily with eight sucrose pellets per mouse in addition to standard pellet chow in their home cage to habituate to the sucrose pellets until beginning the pre-training sessions.

### 4. Habituation to the apparatus and pre-training

1. Place six sucrose pellets per mouse into the center of each one of the six compartments of the apparatus, and deposit one pellet into each tray of the food dispensers.
2. Place all mice in a cage into the apparatus and allow them to freely explore the apparatus with all doors open for 30 min.
3. From 1 day after the habituation, mice are daily subjected to pre-training. With all the doors closed and the pellet deposited in the food tray, place mouse into area A1. If the mouse consumes the pellet or 5 min elapse, transfer the mouse to area A2 and begin pre-training again. Such training is repeated five times a day, and continued until the mice consume more than 80% of the pellets.
4. After the pre-training sessions are complete, the mice are subjected to either a forced alternation task or left-right discrimination task.

### 5. Forced alternation task

1. In the forced alternation task, each trial consists of a forced choice run followed by a free choice run.
2. Run the application program (Image TM) for the onset of the task, and place a mouse in the start box (area S1).
3. Click the start button, and a forced-choice run starts. In this run, the doors of start box (door s2) and of either area A1 (door a1) or area A2 (door a2) are opened, and a sucrose pellet is automatically delivered to the food tray of the area with the door open. The mouse is allowed to enter the area and to consume the pellet. When the mouse has eaten the pellet, the door near the food tray of the arm that the mouse currently stays (either door p1 or p2) is opened. Then, the mouse approaches the door (either s1 or s3) neighboring the start box, and either the door p1 or p2 is closed and the door s1 (or s3) is opened so that the mouse can return to the start box. If the mouse fails to eat the pellet within 30 sec, the response is recorded as an "Omission Error". Then, the pellet is automatically removed from the food tray and the door of the arm that the mice stayed (either p1 or p2) is opened, and then the mouse can return to the start box.
4. Following the forced-choice run, the free-choice run automatically begins. The door s2 and both doors a1 and a2 are opened. The mouse is allowed to choose between the two arms. If the mouse enters the opposite arm that it was forced to choose in the forced-choice run, its response is considered to be "Correct" and the mouse receives a sucrose pellet. If the mouse fails to eat the pellet within 30 sec, the response is recorded as an "Omission Error", and the pellet is automatically removed from the food tray. If the mouse goes to the same arm as that visited in the forced-choice task, the mouse is confined within the area for 10 sec as a penalty ("Error" response). Then, the doors of p1 (or s1) and p2 (or s3) are opened, and the mouse can return to the start box.
5. A mouse is subjected to 10 consecutive trials in a session per day (cutoff time, 50 min). Control mice are trained daily to reach a group average of 80% correct response in a session. The group average of correct response is calculated by averaging the % correct responses of each mouse in a session in each group.
6. After control and/or experimental mice are trained to the criterion, you can further test the mice in the delayed alternation task by inserting 3-, 10-, 30-, or 60-s delays between the forced-choice and the free-choice runs.
7. After each session, return the mice to their home cage, and clean the apparatus with super hypochlorous water (pH 6-7) to prevent a bias based on olfactory cues.

### 6. Left-right discrimination task

1. In the left-right discrimination task, each mouse is given a free choice run of 10 or 20 trials. A sucrose pellet is always delivered to the food tray of one of the arms, namely, the goal arm. Mice have to learn to enter the goal arm. The location of the goal arm is invariable across trials and sessions, and is counterbalanced across control and experimental mice.
2. Run the application program (Image TM) for the onset of the task, and place a mouse into the start box (area S1).
3. Click the start button, and a free-choice run starts. In this run, the door s2 and both doors a1 and a2 are opened, and a pellet dispenser automatically delivers a pellet to the food tray of the goal arm. The mouse is allowed to freely choose between the left and right arms. When the mouse enters the goal arm, it is considered a correct response. If the mouse eats the pellet or 30 sec elapse, door p1 (or p2) is opened. When the mouse approaches the start box by passing through the area P1 (or P2), door s1 (or s3) is opened so that the mouse can return to the start box.
4. A mouse is usually subjected to 10 to 20 consecutive trials in a session per day (cutoff time, 50 min). Control mice are trained daily to reach a group average of 80% correct response in a session. The group average of correct response is calculated by averaging the % correct responses of each mouse in a session in each group.
5. After mice reach the criterion, you can give additional sessions to the mice either to assess retention memory and relearning by inserting a delay of several weeks between the sessions or to assess behavioral flexibility by placing the reward in the opposite, previously unbaited arm (i.e. reversal learning), as necessary.
6. After each session, return the mice to their home cage, and clean the apparatus with super hypochlorous water (pH 6-7) to prevent a bias based on olfactory cues.

## 7. Image analysis

- Behaviors in the T-maze apparatus are recorded by a video camera attached to a computer and the image is stored in a TIFF format. The application used for acquiring and analyzing the behavioral data (Image TM) is based on the public domain Image J program (developed by Wayne Rasband at the National Institute of Mental Health and available at <http://rsb.info.nih.gov/ij/>), which was modified by Tsuyoshi Miyakawa (available through O'HARA & Co., Tokyo, Japan).
- The Image TM program automatically generates the text files for percentage of correct response, latency (sec) to complete a session, distance traveled during the session, and the number of omission errors in the session. Also, the trace images of a mouse, raw position data, and raw response data (correct, omission, or error) in each run are produced and saved.

## 8. Statistical analysis

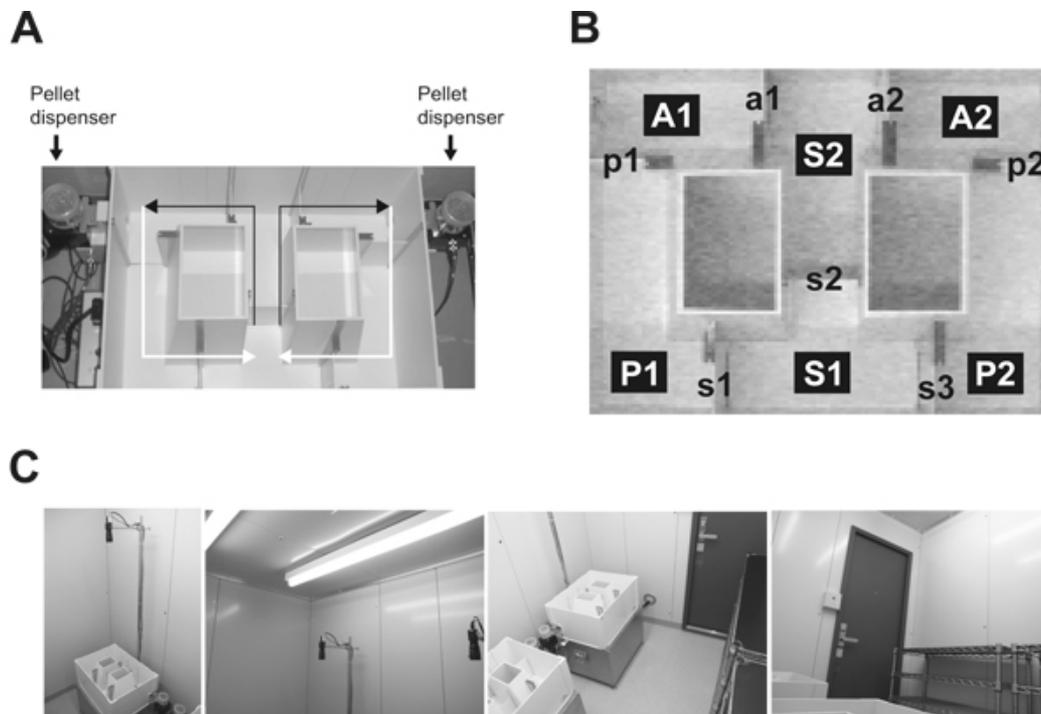
Analyze each behavioral data by two-way (EXPERIMENTAL CONDITION (e.g., GENOTYPE) x SESSION or EXPERIMENTAL CONDITION x DELAY) repeated measures analysis of variance.

## 9. Representative Results

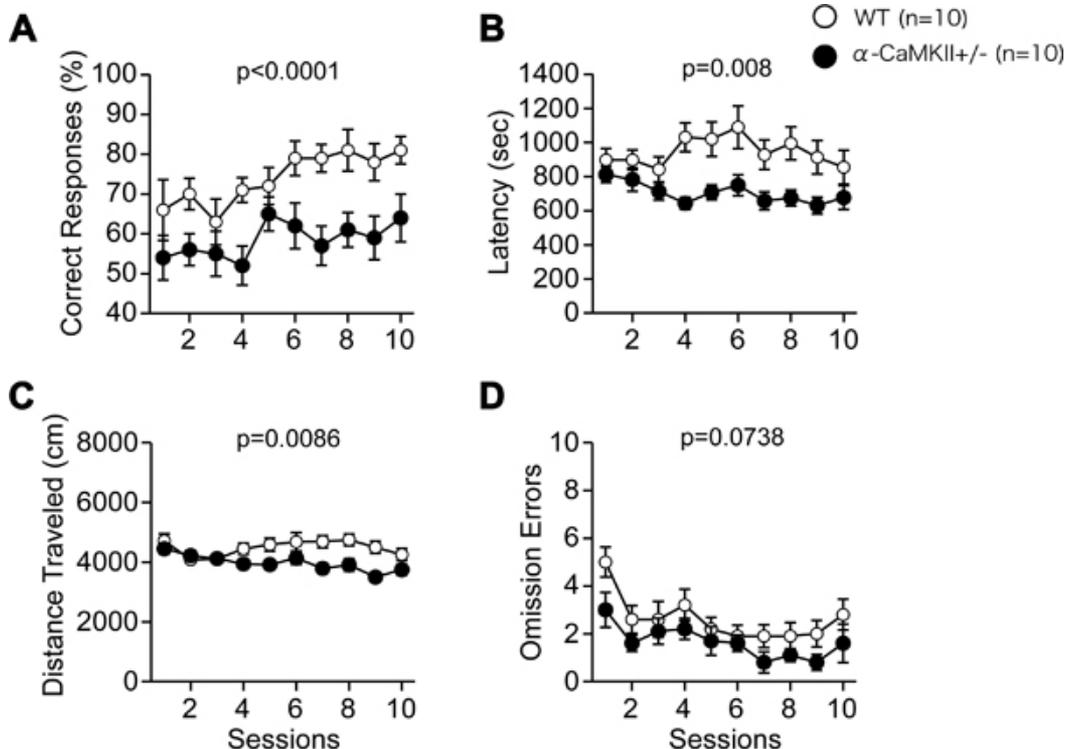
An example of T-maze performance by  $\alpha$ -CaMKII<sup>+/-</sup> male mice and their wild-type control littermates (C57BL/6J background) (11-18 weeks old, n=10 per group for forced alternation or left-right discrimination task) is shown in Figures 2-4. Because  $\alpha$ -CaMKII<sup>+/-</sup> mice exhibit high levels of aggression toward cage mates<sup>2,3</sup>, both the mutants and control mice were singly housed in a plastic cage (22.7 x 32.3 x 12.7 cm) after weaning. The experiments were approved by the Institutional Animal Care and Use Committee of Fujita Health University.

In forced alternation task, control mice will increasingly learn to make correct choices, and can usually reach the criterion of a mean 80% correct response in about 1 to 2 weeks (Figure 2A). Compared to the control mice,  $\alpha$ -CaMKII<sup>+/-</sup> mice showed a significantly lower percentage of correct responses (GENOTYPE:  $F(1,18)=29.04$ ,  $p<0.0001$ ) and shorter latency (GENOTYPE:  $F(1,18)=8.88$ ,  $p=0.008$ ; GENOTYPE x SESSION:  $F(9,162)=2.24$ ,  $p=0.0218$ ) and traveled a shorter distance (GENOTYPE:  $F(1,18)=8.67$ ,  $p=0.0086$ ; GENOTYPE x SESSION:  $F(9,162)=3.19$ ,  $p=0.0014$ ) than control mice (Figure 2A, B, and C). No significant effect of genotype was found in omission errors (Figure 2D). Also, in the delayed alternation task, the correct choice percentages of  $\alpha$ -CaMKII<sup>+/-</sup> mice were significantly lower than those of wild-type mice at any delay time (GENOTYPE:  $F(1,18)=38.781$ ,  $p<0.0001$ ; DELAY:  $F(3,54)=8.074$ ,  $p=0.0002$ ; GENOTYPE x DELAY:  $F(3,54)=0.223$ ,  $p=0.88$ ; Figure 3). These results indicate that the mutants displayed impaired performance compared to control mice although the mutant mice could perform the task faster than controls, suggesting that  $\alpha$ -CaMKII deficiency induces a working memory deficit.

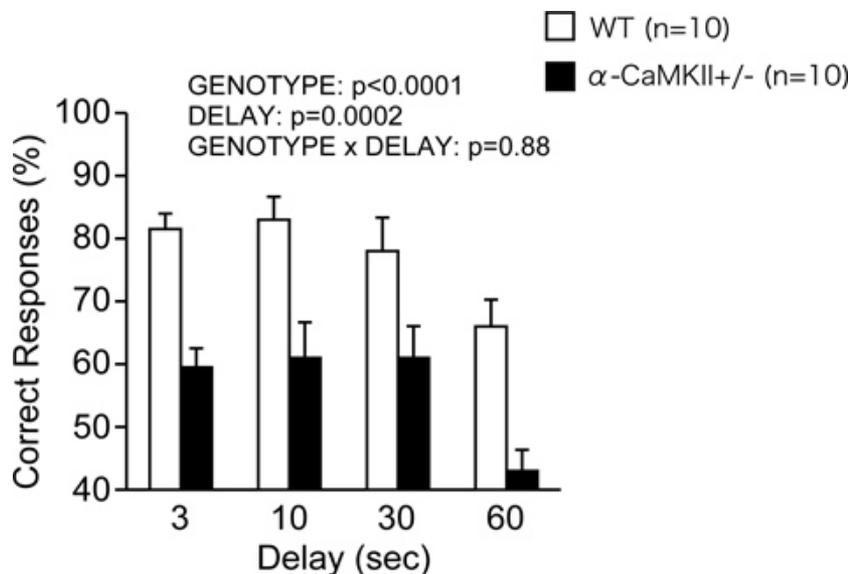
In the left-right discrimination task, the correct choice percentages of the  $\alpha$ -CaMKII<sup>+/-</sup> mutants gradually increased across sessions, similar to control mice (Figure 4A). Also, when a 1-month delay was inserted between sessions, there were no significant differences in the percent correct between the mutant and control mice. As in the forced alternation task,  $\alpha$ -CaMKII<sup>+/-</sup> mutants showed a significantly shorter latency to complete a session (GENOTYPE:  $F(1,18)=12.12$ ,  $p=0.0027$ ) and shorter distance traveled in the apparatus during a session (GENOTYPE:  $F(1,18)=25.08$ ,  $p<0.0001$ ; GENOTYPE x SESSION:  $F(15,270)=2.83$ ,  $p=0.0004$ ) than control mice across the training sessions (Figure 4B and C). These data indicate that  $\alpha$ -CaMKII deficiency does not affect reference memory as assessed by this task. In the reversal-learning sessions, however,  $\alpha$ -CaMKII<sup>+/-</sup> mutants showed a significantly lower percentage of correct responses (GENOTYPE:  $F(1,18)=10.92$ ,  $p=0.0039$ ; GENOTYPE x SESSION:  $F(5,90)=5.54$ ,  $p=0.0002$ ; Figure 4A) and had more omission errors (GENOTYPE:  $F(1,18)=17.12$ ,  $p=0.0006$ ; Figure 4D) than control mice. These findings suggest that  $\alpha$ -CaMKII<sup>+/-</sup> mutant mice have reduced behavioral flexibility.



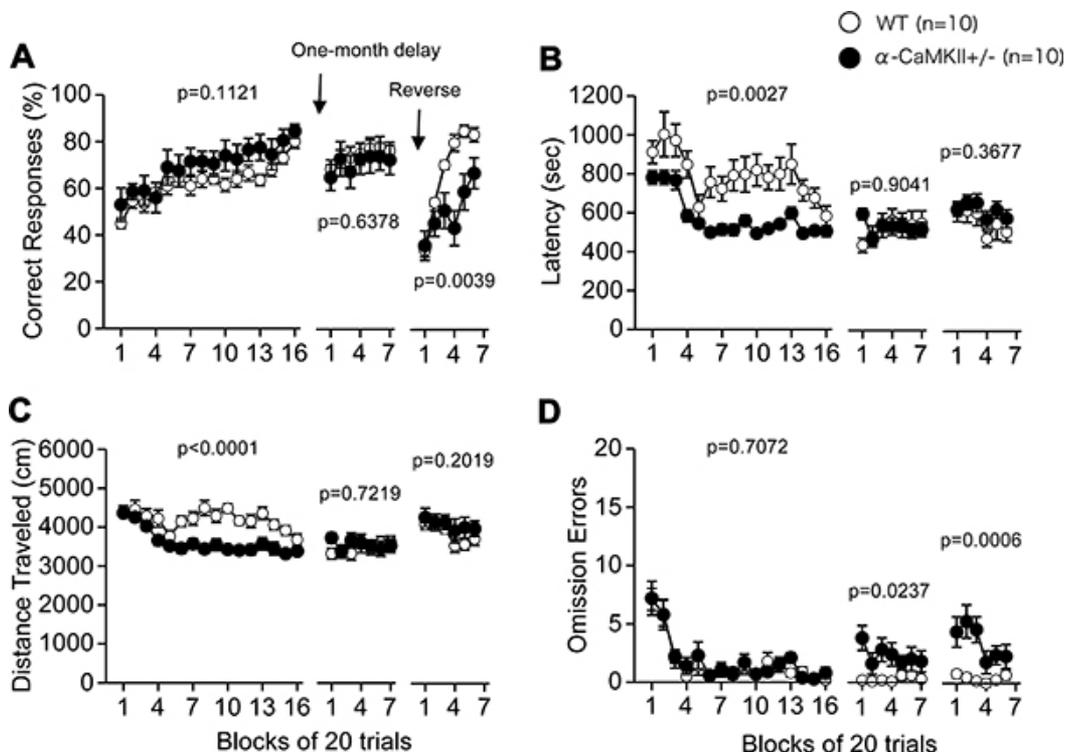
**Figure 1.** (A) T-maze apparatus for forced alternation and left-right discrimination tasks. The figure is cited from Takao *et al.* (2008). (B) The image was captured by a CCD camera mounted above the apparatus. The T-maze is partitioned off into 6 areas (A1, A2, S1, S2, P1, P2) by sliding doors (s1, s2, s3, a1, a2, p1, p2). (C) Configuration and orientation of the apparatus and extra-maze cues in a soundproof room. Two apparatuses are placed facing in the same direction toward a wall in a soundproof room, and objects, such as a door of the room, fluorescent lights on the ceiling, walls of the room, CCD cameras of the apparatuses, and racks to accommodate mouse cages are set.



**Figure 2.** T-maze forced alternation task. Mice received 10 daily trials per session. Data of (A) percentage of correct responses, (B) latency (sec), (C) distance traveled (cm), and (D) number of omission errors represented as means with standard errors for each block of two sessions, and were analyzed by a two-way repeated measures ANOVA.  $\alpha$ -CaMKII<sup>+/-</sup> mice showed a lower percentage of correct responses ( $p < 0.0001$ ) and a shorter latency ( $p = 0.008$ ), and traveled a shorter distance ( $p = 0.0086$ ) than control mice across sessions.



**Figure 3.** T-maze forced alternation task with delays of 3, 10, 30, and 60 sec. Approximately 24 h after the final training session, mice were subjected to five delay sessions. The percentage of correct responses for each delay is represented as means with standard errors, and were analyzed by a two-way repeated measures ANOVA.  $\alpha$ -CaMKII<sup>+/-</sup> mice showed a lower percentage of correct responses than control mice at any delay time ( $p < 0.0001$ ).



**Figure 4.** T-maze left-right discrimination task. Mice received daily 10 or 20 trials in a session. Data of (A) percentage of correct responses, (B) latency (sec), (C) distance traveled (cm), and (D) number of omission errors are represented as means with standard errors for each block of 20 trials, which were analyzed by a two-way repeated measures ANOVA. During the initial training sessions and the relearning sessions 1 month after the last training session, the percentage of correct responses did not significantly differ between  $\alpha$ -CaMKII<sup>+/-</sup> mutant and control mice. Mutant mice, however, showed a significantly lower percentage of correct responses than control mice during the reversal learning sessions ( $p = 0.0039$ ).

## Discussion

Forced alternation and left-right discrimination tasks using the T-maze are used extensively to assess working and reference memory, respectively, in rodents<sup>4,5</sup>. In T-maze tasks, it is known that rodents can use different strategies to perform the tasks, based on spatial and non-spatial cues, such as extra-maze cues, configuration of the room cues, orientation of the maze, and so on<sup>6,7,8</sup>. Orientation of the maze in a room

and its stability, absence or presence of polarizing cues in the room, and ability of rodents to see cues in the room may affect strategies. Thus, researchers need to consider configuration and orientation of apparatus and cues in a room in conducting an experiment and an interpretation of behavioral data. In our laboratory, we place two apparatus facing in the same direction toward a wall in a soundproof room and set objects, such as a door of the room, fluorescent lights on the ceiling, walls of the room, CCD cameras of the apparatuses, and racks to accommodate mouse cages, that may serve as extra-maze spatial cues for mice (see Figure 1C).

In many cases, the T-maze tests have been manually conducted by a human experimenter as follows: In each trial, the experimenter places a sucrose pellet on food tray, and opens the guillotine doors of the apparatus to start the test. Then, when a mouse enters either of the arms, the experimenter closes the doors, records the mouse behavior, and transfers the mouse from the arm to the start box by hand. The possible confounding variables of handling interacting with mouse genotype or experimental condition may affect T-maze performance. During the past decade, the modified T-maze test for a continuous alternation task that does not involve manual transfer of the subject from the goal arm back to the start box has been used.<sup>9-11</sup> Even when the apparatus, test protocols, and many environmental variables are vigorously equated, standardized behavioral tests do not always produce similar results in the different laboratories<sup>12,13</sup>. Specific experimenters performing the testing may be unique to each laboratory and can also influence the behavior of mice. In addition, a human experimenter is generally apt to make errors, such as misplacing a sucrose pellet, opening or closing other doors, as well as mistakes in tracking the trial number and timekeeping. To reduce the influence of confounding variables and the occurrence of human errors, we have developed and used the automated T-maze apparatus controlled by a video-tracking system with the Image TM program. The improved T-maze apparatus also has advantages that allow us to use microdialysis, electrophysiology, and optogenetics techniques during T-maze performance because the doors are designed to go down into the floor. Thus, the automated apparatus is a useful tool to facilitate studies of the neurobiology of working and reference memory in rodents.

To enable automatic and successive execution of a series of trials in a session, our protocols have some potential drawbacks. For example, in forced alternation task, the time for the mice to go back to S1 from A1 or A2 could potentially affect their performance. It may not be a serious problem, though, since staying P1 or P2 area itself can be a spatial cue and a long or short stay in either area in a forced-choice run may not change a memory load. Another potential problem is that odor trail made by the mice, instead of spatial working memory, could be used. However, after a few trials, odor trails could be overwritten multiple times and would become difficult to be utilized as cues. Also, in left-light discrimination task, odor trails may serve as olfactory cues for the mice to find the location of a reward across successive trials. The cues could influence learning and memory process across trials in a session, which may potentially be a problem. However, mice cannot use the odor trail strategy in the very first trial in a session and so the performances of the first trials would serve as an index that is devoid of a potential use of the odor trail strategy.

As shown in the representative results, the percent correct responses of the control C57BL/6J mice gradually increased across sessions in both tasks. The findings confirm that C57BL/6J mice can learn to make correct choices in the modified automatic T-maze. In this study, the mice stayed at around 80% correct choices and not more even after extensive training (see Figure 2A). Considering that they keep showing some omission errors throughout the trainings, their motivation may not be so high for the mice as to reach higher level of performance. In the forced alternation task,  $\alpha$ -CaMKII<sup>-/-</sup> mice showed a lower percentage of correct responses than control mice. Thus, the mutant mice displayed impaired performance compared to the control mice in this task. This result is consistent with the previous findings obtained in the eight-arm radial maze test<sup>2,14</sup>, providing further evidence that  $\alpha$ -CaMKII deficiency induces the deficits in working memory and that the forced alternation task in the automated T-maze apparatus accurately detects working memory deficits of the mutant mice. In the left-right discrimination task, the results indicate that  $\alpha$ -CaMKII deficiency does not affect reference memory. As shown in the results of the reversal-learning sessions, however,  $\alpha$ -CaMKII deficiency may reduce behavioral flexibility. The mutant mice also displayed more omission errors than control mice during the reversal learning sessions. The increase in the number of omission errors could reduce the opportunity to learn which arm is associated with the reward. Therefore, the delayed learning acquisition could be due to the increase in the number of omission errors during the initial sessions, but not to impaired reversal learning. Another possibility is that the mutants could be confused by the change in rules, which might induce errors of omission and interfere with executive function. Thus, to draw a reasonable conclusion, omission errors should be examined as well as correct choice percentage.

The Image TM program generates the additional results for the latency and distance traveled to complete a session as well as the percentage of correct response and the number of omission error. The differences in the latency and distance traveled to complete a session may be interpreted as a difference in locomotor activity level, impulsive tendency to choose the arms, motivation to perform the task, habituation level to the task, different learning strategy and etc. Regarding the representative results,  $\alpha$ -CaMKII<sup>-/-</sup> mice showed shorter latency and shorter distance traveled than those of the controls. In fact,  $\alpha$  CaMKII<sup>-/-</sup> mice showed a hyperlocomotor activity compared to the control mice<sup>3</sup> and this phenotype could underlie the differences in the indices.

In our laboratory, we have assessed more than 36 strains of genetically engineered mice and wild-type control mice in a T-maze test using the automated apparatus to elucidate the relation between genes, brain, and behavior<sup>15,16</sup>. We have obtained a large set of raw data of more than 1200 mice, and have reported the data for the T-maze performance in several strains of mutant mice<sup>3,16-22</sup>. The data of strains already published in the research article are included in the "Mouse Behavioral Phenotype Database" as a public database (URL: <http://www.mouse-phenotype.org/>). Some of the studies demonstrated that mice with mutant *Dtnbp1*<sup>1</sup>, *Nrd1*<sup>20</sup>, or *Plp1*<sup>21</sup> genes show working memory deficits. Thus, our standardized protocol for the T-maze tasks with the automated apparatus is suitable for detecting genetic effects on memory function between mutant and wild-type control mice. The behavioral test protocols need to be standardized, replicated, and the results compared across laboratories. The improved T-maze apparatus leads to the automation of test procedures, which can contribute to the standardization of protocols used across laboratories.

As shown in this video article, the current version of the apparatus and program can allow us to test black or agouti mice, but not albino mice. Now, we are producing a modified version of the system to enable albino mice to be tested. The system has an advantage that *in vivo* microdialysis, *in vivo* electrophysiology, and optogenetics experiments can be performed during T-maze testing, since the doors are designed to go down under the floor. For instance, some researchers may try to investigate the electrophysiological properties of neurons in hippocampus during the choice of arms although some improvements of the apparatus might be needed to minimize electrical noise from the doors and pellet removal mechanism actuators.

Taken together, T-maze forced alternation and left-right discrimination tasks using the modified automatic apparatus are useful for assessing working and reference memory and behavioral flexibility in mice.

## Disclosures

No conflicts of interest declared.

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