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Review Editor
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Dear Dr. Vineeta Bajaj,

Thank you for the thorough review of our manuscript (Ms. No. JoVE60751R1) entitled "**Assessment of memory function in pilocarpine-induced epileptic mice**" for publication in *Journal of Visualized Experiments*.

We have given careful consideration to your suggestions and have revised our manuscript accordingly including the revised manuscript with track changes. We hope we have satisfied the Reviewers' concerns and look forward to hearing your decision.

We would like to ask you a favor that the peer-review process can be completed by Nov. 15th or sooner if it's possible. We hope that you will now find our revised manuscript suitable for publication in *Journal of Visualized Experiments*. Thank you very much for your time and consideration.

Sincerely,

Kyung-Ok Cho
Associate Professor

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TITLE:

Assessment of Memory Function in Pilocarpine-induced Epileptic Mice

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KEYWORDS:

novel object location test, novel object recognition test, pattern separation test, spatial memory, cognition, behavioral test, pilocarpine, epilepsy, mouse

SUMMARY

This article presents experimental procedures for assessing memory impairments in pilocarpine-induced epileptic mice. This protocol can be used to study the pathophysiologic mechanisms of epilepsy-associated cognitive decline, which is one of the most common comorbidities in epilepsy.

ABSTRACT:

Cognitive impairment is one of the most common comorbidities in temporal lobe epilepsy. To recapitulate epilepsy-associated cognitive decline in an animal model of epilepsy, we generated pilocarpine-treated chronic epileptic mice. We present a protocol for three different behavioral tests using these epileptic mice: novel object location (NL), novel object recognition (NO), and pattern separation (PS) tests to evaluate learning and memory for places, objects, and contexts, respectively. We explain how to set the behavioral apparatus and provide experimental procedures for the NL, NO, and PS tests following an open field test that measures the animals' basal locomotor activities. We also describe the technical advantages of the NL, NO, and PS tests with respect to other behavioral tests for assessing memory function in epileptic mice. Finally, we discuss possible causes and solutions for epileptic mice failing to make 30 s of good contact with the objects during the familiarization sessions, which is a critical step for successful memory tests. Thus, this protocol provides detailed information about how to assess epilepsy-associated memory impairments using mice. The NL, NO, and PS tests are simple, efficient assays that are appropriate for the evaluation of different kinds of memory in epileptic mice.

INTRODUCTION

Epilepsy is a chronic disorder characterized by spontaneous recurrent seizures[1–3]. Because repetitive seizures can cause structural and functional abnormalities in the brain[1–3], abnormal seizure activity can contribute to cognitive dysfunction, which is one of the most common epilepsy-associated comorbidities[4–6]. Contrary to the chronic seizure events, which are transient and momentary, cognitive impairments can persist throughout epileptic patients' lives, deteriorating their quality of life. Therefore, it is important to understand the pathophysiologic mechanisms of epilepsy-associated cognitive decline.

Various experimental animal models of epilepsy have been used to demonstrate the learning and memory deficits associated with chronic epilepsy[7–12]. For instance, the Morris water maze, contextual fear conditioning, hole-board, novel object location (NL), and novel object recognition (NO) tests have frequently been used to assess memory dysfunction in temporal lobe epilepsy (TLE). Because the hippocampus is one of the primary regions in which TLE shows pathology, behavioral tests that can evaluate hippocampus-dependent memory function are often preferentially selected. However, given that seizures can induce aberrant hippocampal neurogenesis and contribute to epilepsy-associated cognitive decline[10], behavioral paradigms for testing dentate newborn neuronal function (i.e., spatial pattern separation, PS)[8,13] can also provide valuable information about the cellular mechanisms of memory impairments in epilepsy.

In this article, we demonstrate a battery of memory tests, NL, NO, and PS, for epileptic mice. The tests are simple and easily accessible and do not require a sophisticated system.

PROTOCOL:

All experimental procedures were approved by the Ethics Committee of the Catholic University of Korea and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

1. Novel object location test (NL)

1.1. Prepare epileptic C57BL/6 or transgenic mice 4–6 weeks after pilocarpine injection.

NOTE: Acute seizures were induced by intraperitoneal (IP) pilocarpine injection, following the protocol detailed in our previous report[14].

1.2. Transfer the epileptic mice from the breeding room to the behavior room one day before the behavioral tests begin. Allow the mice to habituate for at least 12 h overnight.

1.3. In the behavior room, separate individual mice into new cages for single housing. Write the information for each animal on the cage card and keep the animals in the same cages throughout the behavioral testing. Multiple cages can be simultaneously transferred using a cart.

1.4. On the next day, begin 3 days of habituation sessions (H1–H3) in the early morning. Acclimate the animals to the low light in their home cages for at least 30 min.

1.5. Prepare an open field box with outside dimensions of 44 x 44 x 31 cm and inside dimensions of 43 x 43 x 30.5 cm. On day 1 of the habituation (H1), place an illuminometer in the center of the open field box and adjust the illuminance to 60 lux.

1.6. Spray the floor and walls of the open field box with 70% ethanol and wipe down with a clean paper towel to remove possible olfactory cues. Then wait for at least 1 min until the residual alcohol has dried completely.

1.7. Evaluate the locomotor activity of each mouse by performing an open field test.

1.7.1. To record and track the behavior of each experimental mouse, use animal behavior video tracking software (see **Table of Materials**).

1.7.2. Once the video tracking software is opened, calibrate the size of the open field box. Then, set the zone for tracking. Set 3 s of latency and 15 min of acquisition time to avoid tracking an experimenter's hands. Insert the information about each experimental mouse (group, gender, age, etc.).

1.7.3. Then, gently place an experimental mouse in the open field box facing the wall. Do this by placing it on the cage lid to minimize handling-associated stress and anxiety. Then, release the mouse near the wall of the open field box that is the farthest from where the objects will be during the familiarization session (step 1.9.3.).

NOTE: Once the mouse is in the open field box, the mouse tracking software will automatically detect it and start recording. For optimal tracking of the exploration, the camera can be placed directly above the open field box.

1.7.4. After 15 min of recording, return the animal to its home cage by placing it on the cage lid. Clean the open field box with 70% ethanol spray and wipe down with a clean paper towel between trials. Restore the bright light and measure the animal's total distance moved using the video tracking software according to the manufacturer's instructions.

1.7.4.1. Open the video tracking software and video clips. Then, click **Analysis** to calculate the total distance moved based on the calibration of the open field box size.

1.8. On day 2 and day 3, perform the habituation sessions (H2, H3) by repeating steps 1.3 to 1.7.

1.9. On day 4, perform the familiarization session (F1).

1.9.1. In the dim light, place each mouse in the empty open field for 3 min. After that brief rehabituation, return the animal to its home cage.

1.9.2. During the habituation, thoroughly clean the objects with 70% ethanol and wipe them down with a paper towel. Wait for at least 1 min for the residual alcohol to dry completely.

1.9.3. Place two identical objects (rubber dolls, object A) in the open field arena 5 cm away from the adjoining walls. Fix the objects with double-sided tape. Introduce the experimental mouse into the open field box, facing the wall farthest from the objects.

1.9.4. Allow free exploration for 20 min and manually measure the time spent exploring both objects using two stopwatches. Once the mouse reaches the minimum exploration time (30 s) for both objects, stop the F1 session and transfer the animal to its home cage. If the mouse fails to explore the objects for 30 s within 20 min, remove the mouse from the open field box and exclude it from further sessions.

1.9.5. After the animal is removed from the open field box, thoroughly clean the floor and walls of the box with 70% ethanol spray and wipe them down with a paper towel.

NOTE: Measure the time when the mouse touches the objects with its whiskers, snout, or front paws. Do not quantify as exploratory time any behaviors in which the animal's snout does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object.

1.10. On day 5, perform the NL testing session.

1.10.1. Transfer the mouse from its home cage to the open field area for rehabituation for 3 min. Then return the animal to its home cage.

1.10.2. During the habituation, thoroughly clean the objects with 70% ethanol and wipe them down with a paper towel. Wait for at least 1 min for the residual alcohol to dry completely.

1.10.3. Move one object (rubber doll, object A) to the diagonal position, 5 cm away from the adjoining walls. Fix the object with double-sided tape. Transfer the experimental mouse on its cage lid to the open field area and place it facing the wall of the open field box.

NOTE: Counterbalance the location of the object moved to reduce any potential innate preference for a certain direction. For example, change the location of the preferred object from the familiarization session for half of the experimental animals, and for the rest of the animals, move the less preferred object from the familiarization session.

1.10.4. Allow 10 min of free exploration and record with a video tracking system. Measure the time spent exploring each object using two stopwatches and calculate the discrimination ratio as

$$\frac{(\text{time spent exploring the moved object} - \text{time spent exploring the stationary object})}{\text{total time spent exploring both objects}}$$

NOTE: Measure the time when the mouse touches the objects with its whiskers, snout, or front paws. Do not quantify as exploratory time any behaviors in which the animal's snout does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object.

1.10.5. Grab the tail of the experimental mouse and place it on its cage lid for transfer to its home cage. For 3 days (days 6–8), let the mouse rest with free access to food and water.

1.10.6. Once the animal is removed from the open field box, thoroughly clean the floor and walls of the box with 70% ethanol spray and wipe down with a paper towel.

2. Novel object recognition test (NO)

2.1. On day 9, perform a 15 min habituation session by repeating steps 1.2–1.7.

2.2. On day 10, perform the familiarization session (F1).

2.2.1. In dim light, place the mouse in the empty open field for 3 min. After rehabituating the mouse to the open field area, temporarily return it to its home cage.

2.2.2. During the habituation, thoroughly clean the objects with 70% ethanol and wipe down with a paper towel. Wait at least 1 min for the residual alcohol to dry completely.

2.2.3. Place two identical objects (50 mL plastic tubes filled with 40 mL of water, object B) in the open field 5 cm away from the adjoining walls. Fix the objects with double-sided tape. Introduce the experimental mouse into the open field box facing the wall farthest from the objects.

2.2.4. As the animal is exposed to the two different objects (50 mL plastic tube filled with 40 mL of water, object B; glass Coplin jar, object C) in the NO test, counterbalance the object during the F1 session. For example, present two identical objects (glass Coplin jars, object C) for half of the animals in the group.

2.2.5. Allow free exploration for 20 min and manually measure the time spent exploring both objects using two stopwatches. Once the mouse reaches the minimum exploration time (30 s) for both objects, stop the F1 session and transfer the animal to its home cage. If the mouse fails to explore the objects for 30 s within 20 min, remove it from the open field box and exclude it from further sessions.

2.2.6. After the animal is removed from the open field box, thoroughly clean the floor and walls of the box with 70% ethanol spray and wipe them down with a paper towel.

NOTE: Measure the time when the mouse touches the objects with its whiskers, snout, or front paws. Do not quantify as exploratory time any behaviors in which the animal's snout

does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object.

2.3. On the next day (day 11), perform the NO testing session.

2.3.1. Transfer the mouse from its home cage to the open field for rehabituation for 3 min, and then return the animal to its home cage.

2.3.2. During the habituation, thoroughly clean the objects with 70% ethanol and wipe down with a paper towel. Wait for at least 1 min for the residual alcohol to dry completely.

2.3.3. Replace one object (50 mL plastic tube filled with 40 mL of water, object B) with another object (glass Coplin jar, object C) 5 cm away from the adjoining walls. Fix the objects with double-sided tape. Transfer the experimental mouse on the cage lid to the open field, and place it facing the wall. Counterbalance the objects presented together during the NO test. For example, replace one glass Coplin jar (object C) with a 50 mL plastic tube filled with 40 mL of water (object B) for the mice exposed to the two glass Coplin jars (object C) during the familiarization session.

NOTE: Counterbalancing the location of the object replaced can be also performed to reduce the potential innate preference for a certain direction. For example, for each cohort of the animals exposed to the set of two objects (object B or object C), change the preferred object in the familiarization session for half of the experimental animals, and for the rest of the animals, replace the object less preferred in the familiarization session.

2.3.4. Allow 10 min of free exploration and record it using a video tracking system. Measure the time spent exploring each object using two stopwatches and calculate the discrimination ratio.

NOTE: Measure the time when the mouse touches the objects with its whiskers, snout, or front paws. Do not quantify as exploratory time any behaviors in which the animal's snout does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object.

2.3.5. Grab the tail of the experimental mouse and place it on the cage lid for transfer to its home cage. For 3 days (days 12–14), let the mouse rest with free access to food and water.

2.3.6. Once the animal is removed from the open field box, thoroughly clean the floor and walls of the open field box with 70% ethanol spray and wipe down with a paper towel.

3. Pattern separation test (PS)

3.1. On day 15, perform the first familiarization session (F1) for the PS test.

3.1.1. Transfer the mouse from its home cage to the open field area for rehabituation for 3

min, and then return it to its home cage.

3.1.2. During the habituation, thoroughly clean the objects and the gridded floor plate with 70% ethanol and wipe down with a paper towel. Wait for at least 1 min for the residual alcohol to dry completely.

3.1.3. Place the floor plate (42.5 x 42.5 x 0.5 cm) with the wide grid (5.5 x 5.5 cm) in the open field box and place two identical objects (plastic T-flasks filled with 50 mL of water, object D) in the open field 5 cm away from the adjoining walls. Fix the objects with double-sided tape. Introduce the experimental mouse into the open field box facing the wall farthest from the objects.

3.1.4. As the animal is exposed to two different objects (plastic T-flask filled with 50 mL of water, object D; glass bottle, object E) in the PS test, counterbalance the object during the F1 and F2 sessions. For example, present two identical objects (glass bottles, object E) on the wide grid floor for half of the animals in the group.

3.1.5. Allow free exploration for 20 min, and manually measure the time spent exploring both objects using two stopwatches. Once the mouse reaches the minimum exploration time total (30 s) for both objects, stop the F1 session and transfer the animal to its home cage. If the mouse fails to explore the objects for 30 s within 20 min, remove it from the open field box and exclude it from further sessions.

NOTE: Measure the time when the mouse touches the objects with its whiskers, snout, or front paws. Do not quantify as exploratory time any behaviors in which the animal's snout does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object.

3.1.6. After completion of the first familiarization session (F1), thoroughly clean the objects and the floor plate with 70% ethanol spray and remove them from the open field box.

3.2. On the next day (day 16), perform the second familiarization session (F2) for the PS test.

3.2.1. Transfer the mouse from its home cage to the open field area for rehabituation for 3 min, and then return the animal to its home cage.

3.2.2. During the habituation, thoroughly clean the objects and gridded floor plate with 70% ethanol and wipe down with a paper towel. Wait for at least 1 min for the residual alcohol to dry completely.

3.2.3. Place the floor plate (42.5 x 42.5 x 0.5 cm) with the narrow grid (2.75 x 2.75 cm) in the open field box and place two identical objects (glass bottles, object E) in the open field 5 cm away from the adjoining walls. Fix the objects with double-sided tape. Introduce the experimental mouse into the open field box facing the wall farthest from the objects.

3.2.4. For counterbalancing, present two identical objects (plastic T-flasks filled with 50 mL of water, object D) on the narrow grid floor.

3.2.5. Allow free exploration for 20 min and manually measure the time spent exploring both objects using two stopwatches. Once the mouse reaches the minimum exploration time total (30 s) for both objects, stop the F2 session and transfer the animal to its home cage. If the mouse fails to explore the objects for 30 s within 20 min, remove it from the open field box and exclude it from further sessions.

NOTE: Measure the time when the mouse touches the objects with its whiskers, snout, or front paws. Do not quantify as exploratory time any behaviors in which the animal's snout does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object.

3.2.6. After completion of the second familiarization session (F2), thoroughly clean the objects and floor plate with 70% ethanol spray and remove them from the open field box.

3.3. On the next day (day 17), perform the PS testing session.

3.3.1. Transfer the mouse from its home cage to the open field area for rehabituation for 3 min, and then return it to its home cage.

3.3.2. During the habituation, thoroughly clean the objects and gridded floor plate with 70% ethanol and wipe down with a paper towel. Wait for at least 1 min for the residual alcohol to dry completely.

3.3.3. Place the floor plate with the narrow grid (2.75 x 2.75 cm) in the open field box and place two different objects (plastic T-flask filled with 50 mL of water, object D; glass bottle, object E) on the floor plate 5 cm away from the adjoining walls. Fix the objects with double-sided tape. Transfer the experimental mouse on the cage lid to the open field area and place it facing the wall.

3.3.4. Counterbalance the objects presented together during the PS test. For example, place each object (object D, object E) on the narrow grid floor to make the object E a novel object in this context. Counterbalancing the location of object D or object E (a novel object on the narrow floor pattern) can be also performed to reduce the potential for an innate preference for a certain direction. For example, replace the preferred object from the second familiarization session for half of the experimental animals, and for the rest of the animals, replace the less preferred object from the second familiarization session.

3.3.5. Allow 10 min of free exploration and record using a video tracking system. Measure the time spent exploring each object using two stopwatches and calculate the discrimination ratio.

NOTE: Measure the time when the mouse touches the objects with its whiskers, snout, or front paws. Do not quantify as exploratory time any behaviors in which the animal's snout

does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object.

3.3.6. Grab the tail of the experimental mouse and place it on the cage lid for transfer to its home cage.

4. Cresyl violet staining

4.1. After completing all the behavioral tests, anesthetize the animal by injecting a cocktail (4:0.5) of ketamine (50 mg/mL) and xylazine (23.3 mg/mL) dissolved in saline at a dose of 110 mL/kg body weight (IP; 1 mL syringe; 26 G needle). Check for the depth of anesthesia by the lack of a response to a toe pinch.

4.2. Once the animal is deeply anesthetized, perform transcardial perfusion with 4% paraformaldehyde to fix the brain[15].

4.3. After the transcardial perfusion is finished, decapitate the animal with a pair of scissors[15]. Then, remove the skull using a pair of iris scissors to expose the brain. After the brain is isolated, postfix it in 4% paraformaldehyde overnight, followed by cryoprotection in 30% sucrose in 0.01 M phosphate-buffered saline.

4.4 Make coronal sections (30 µm) from the snap-frozen brain using a cryostat.

4.5. Mount the brain tissues on slides and perform a series of hydration steps from 100% ethanol to tap water by washing for 3 min sequentially in 100%, 95%, 90%, 80%, 70% ethanol.

4.6. Incubate the tissue slides in 0.1% cresyl violet solution for 15 min.

4.7. Remove excessive stain by immersing the tissue slides in 95% ethanol/0.1% glacial acetic acid, and then dehydrate the tissues with solutions of 100% ethanol, 50% ethanol/50% xylene, and 100% xylene.

4.8. Coverslip the tissue slides using a commercially available xylene mounting medium.

REPRESENTATIVE RESULTS:

A general experimental schedule and setup for evaluating cognitive function are shown in **Figure 1**. Six weeks after the introduction of pilocarpine-induced acute seizures, mice were subjected to the NL, NO, and PS tests in that order separated by 3 day rest periods between tests (**Figure 1A**). For the NL test, two identical objects were placed in the open field during the familiarization session (F1), and on the next day, one object was moved to a new location. In the NO test, one object was replaced with a new one during the testing session. For the PS test, the two familiarization sessions (F1, F2) introduced combinations of different floor grid patterns and objects. Then, on the test day, one object from each familiarization session was placed on the narrow grid floor pattern, making one object novel in the context of the narrow grid floor pattern (**Figure 1B**). The open field box can be placed on a desk directly under a

charge-coupled device camera and surrounded by a black curtain to avoid unnecessary visual cues (**Figure 2A**). The sample objects were easy to clean materials of a similar size or slightly larger than a mouse (**Figure 2B**). The object combinations needed to be prescreened to confirm that there was no significant preference between the two objects presented together (**Figure 2C**). The floor plates with different patterns were placed in the open field box to provide additional experimental cues in the PS test (**Figure 2B**). Once a mouse was introduced into the open field box, a video tracking system tracked its trajectory to analyze its total locomotion distance (**Figure 2D**). Six weeks after a pilocarpine injection, the epileptic mice showed a significant reduction in the discrimination ratio in the NL test, demonstrating spatial memory impairment (**Figure 3**). Moreover, in the NO test, which is a test for object recognition memory, epileptic mice showed impaired memory function compared to sham controls. When dentate newborn neuronal function was evaluated with the PS test, the epileptic mice had difficulty recognizing the novel object in a context with multiple cues. As control experiments, locomotor activity and latency to reach the exploration criteria during the familiarization session were assessed (**Figure 3**). The measurement of locomotor activity showed a significant increase in epileptic animals (**Figure 3C**), in line with previous reports[16,17], whereas the motivation to explore the objects was comparable between sham and epileptic animals (**Figure 3D**). Our dropout rates failing the exploration criteria in the familiarization session were 17.4%, 18.2%, 0% for the NL, NO, and PS test, respectively, suggesting that animals became accustomed to the experimental environments during the series of behavioral trials. Finally, we evaluated hippocampal cell death after pilocarpine-induced status epilepticus using cresyl violet staining to confirm seizure-induced neuronal damage (**Figure 4**). The pilocarpine-treated animals demonstrated pyknotic cells in the hilus and the CA3 subfield of the hippocampus, unlike the sham controls (**Figure 4**).

FIGURE LEGENDS:

Figure 1: Schematic presentation of the behavioral test battery. (**A**) A schematic drawing of the behavioral schedule for the novel object location (NL), novel object recognition (NO), and pattern separation (PS) tests for sham and epileptic mice. (**B**) Representative images of the object and floor plate arrangements for the NL, NO, and PS tests.

Figure 2: Behavioral apparatus for the evaluation of cognitive function. (**A**) A general overview of the behavioral setting. A camera was placed directly above the open field box, which was surrounded by the curtain to avoid unnecessary cues. (**B**) Sample objects for the novel object location (NL), novel object recognition (NO), and pattern separation (PS) test. For the PS test, a floor plate with different patterns, i.e., wide and narrow grids, was inserted into the open field box to provide additional cues. (**C**) Graphs showing the time exploring each object presented together during the NO and PS testing session (n = 7), respectively. Note that there was no significant difference in the preference between the two objects assessed by the Mann-Whitney U test (for NO test) and the Student's unpaired t-test (for PS test). (**D**) An image showing that a video tracking system detected the experimental mouse in the open field box. The red square indicates the preset zone for tracking the mouse's trajectory.

Figure 3: Impaired spatial memory and pattern separation in epileptic mice. (**A**) A schematic presentation of the novel object location (NL), novel object recognition (NO), and pattern

separation (PS) tests. A novel object is indicated as a red circle. (**B**) Graphs showing the discrimination ratio in the NL, NO, and PS tests between sham (n = 8) and epileptic mice (n = 10). Note that the epileptic mice demonstrated significant impairments in the NL, NO, and PS tests, which test memory function for places, objects, and contexts, respectively. *p < 0.05 by Mann-Whitney U test for the NL test. *p < 0.05 by Student's unpaired t-test for the NO tests. *p < 0.05 by Student's unpaired t-test with Welch's correction for the PS test. (**C**) A graph showing the locomotor activity of sham (n = 8) and epileptic mice (n = 10). Note that the epileptic mice demonstrated increased locomotion, in line with previous reports. *p < 0.05 by Student's unpaired t-test. (**D**) Graphs showing latency to 30 s criteria in the familiarization session of the NL, NO, and PS tests. Note that there were no differences in the motivation for exploring the objects between sham (n = 8) and epileptic mice (n = 10). The data are presented as mean ± standard error of mean (SEM). SE = status epilepticus.

Figure 4: Neuronal death in the hippocampus after pilocarpine-induced status epilepticus (SE). Representative images from the (**A**) sham and (**B**) epileptic groups 58 days after pilocarpine injection. Magnified images show the hilus (**a, d**), CA1 subfield (**b, e**), and CA3 subfield (**c, f**) of the hippocampus, which are indicated as white squares in the images with low magnification. Note the pyknotic cells in the hilus and CA3 subfield of the hippocampus. Scale bar in the far-left image = 200 µm, also valid for the bottom image; scale bar in **a**, **b**, **c** = 40 µm, also valid for **d**, **e**, **f**, respectively.

DISCUSSION:

This work describes experimental procedures for evaluating cognitive function in mice with chronic epilepsy. Many different behavioral test paradigms are used to assess learning and memory functions in mice[18]. The Morris water maze, radial arm maze, Y-maze, contextual fear conditioning, and object-based tests are the most frequently used behavioral tests and provide reliable results. Among them, the NL, NO, and PS tests are efficient, simple methods for evaluating learning and memory in epileptic mice[8,10]. Because epileptic mice can have unexpected spontaneous seizures during behavior sessions, it is better to use behavioral tests based on the animals' natural inclination for exploring novelty without adding other positive or negative reinforcements, such as those used in aversive–motivated tasks such as fear conditioning, mild starvation, or forced swimming to stay afloat, which can trigger recurrent seizures[19,20]. Moreover, compared with other behavioral tests, the novelty-based tests are less stressful for the animals because extensive training sessions are not required. Further, the novelty-based behavioral tests can be easily modified to assess different types of memory (i.e., spatial memory, recognition memory, or episodic memory) by simply changing the object location, presenting a novel object, or combining additional stimuli. Taken together, novelty-based tests such as the NL, NO, and PS tests have versatile advantages for evaluating cognitive functions in epileptic mice.

Although the NL, NO, and PS tests are quick and useful experimental models for investigating learning and memory function in epileptic mice, several factors must be considered when using them. It is well-known that chronic epileptic mice show heightened anxiety from pilocarpine injections[7], leading to a marked decrease in object exploration during the familiarization sessions. This lack of exploration can cause misinterpretation of the test results.

Therefore, it is important to include enough habituation to the open field for the mice to get used to the environment before the familiarization session. Depending on the strains, the mice may still fail to explore the objects for 30 s within the 20 min of the familiarization session, even after 3 sessions of habituation. In that case, adding another habituation session with extra pairs of the objects in the open field box could help to reduce the anxiety of the mouse toward the objects. Curtains surrounding the open field box can minimize external room cues, allowing the experimental mice to focus on the objects in the open field. In addition, the exploration criteria should be strict enough to exclude behaviors in which the animal's snout does not point toward the object, such as sitting on the object, passing by the object, or resting with its hind end pointing at the object. Finally, although it may be very rare, seizure events can occur during the behavioral tasks. In this case, it is recommended that those animals be removed from further assessments as this can be a possible source of confounding bias for the evaluation of memory function.

As the NL, NO, and PS tests are very sensitive experiments relying on the animals' natural curiosity for novel stimuli, subtle changes may affect the exploratory behavior of mice, resulting in inconclusive discrimination ratios[21,22]. For example, harsh handling of the mouse, a bedding change right before the behavioral tasks, inconsistent timing of the test, and insufficient acclimation to the testing room can all elevate the stress levels of the animals, causing equivocal test results. Moreover, altered testing environments, such as inconsistent presentations of asymmetric objects at each session, placement of home cages near the experimental arena, or switching the olfactory signature of the experimenter should be carefully considered to avoid additional factors. At the stage of data analysis, assessments by multiple experimenters may contribute to increased variabilities in the behavioral outcomes due to different criteria of rodent exploratory behaviors or stopwatch usages. Collectively, these aspects should be also kept in mind for successful implementation of the NL, NO, and PS tests.

The hippocampus and parahippocampal region are known to play unique roles in memory processing[23,24]. It is widely accepted that spatial memory largely depends on the function of the hippocampus, which can be easily assessed by the NL test[23,24]. On the other hand, object recognition memory seems to involve multiple brain regions, including the perirhinal cortex, insular cortex, and ventromedial prefrontal cortex, in addition to the hippocampus[25–38]. Pilocarpine-treated epileptic mice have consistently demonstrated behavioral impairments in spatial memory testing with extensive hippocampal neuronal damage[39–42], whereas object recognition memory tests have produced controversial outcomes, with variable neuronal degeneration in the parahippocampal brain regions[10,41–44]. These data imply that object recognition might require sophisticated network connections among multiple brain regions, unlike spatial memory in which the hippocampus can play a central role. When the specific hippocampal subfields are closely assessed, the CA1 and CA3/dentate gyrus regions are found to process different information. Specifically, CA1 neurons are thought to be activated by exposure to similar items, whereas CA3 and the dentate gyrus are involved in discriminating similar objects[23,45]. Consistent with that hypothesis, emerging evidence suggests that dentate newborn neurons can contribute to pattern separation performance[45–47]. Given that aberrant hippocampal neurogenesis can be induced during epileptogenesis[10], epileptic mice can

demonstrate impaired performance in discriminating analogous experiences due to the disrupted integration of newborn neurons in chronic epilepsy.

In conclusion, we describe how to evaluate memory impairments in epileptic mice. Specifically, we provide experimental protocols for three behavioral tests, the NL, NO, and PS tests, which test memory for places, objects, and contexts, respectively. Among the many cognitive test paradigms available for mice, the NL, NO, and PS tests are quite simple, short assays that minimally stress the animals, which makes them optimal for evaluating memory function in epileptic animals without triggering recurrent seizures.

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGEMENTS:

We thank Dr. Jae-Min Lee for his technical support. This work was supported by the National Research Foundation of Korea (NRF) grants funded by the Korean government (NRF-2019R1A2C1003958, NRF-2019K2A9A2A08000167).

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672

Figure 1

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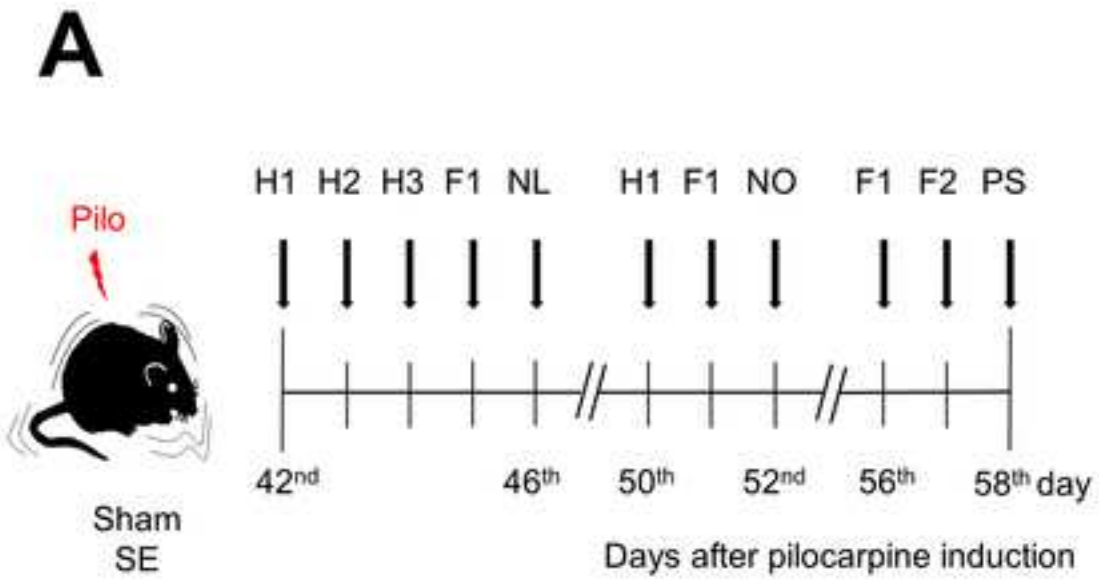

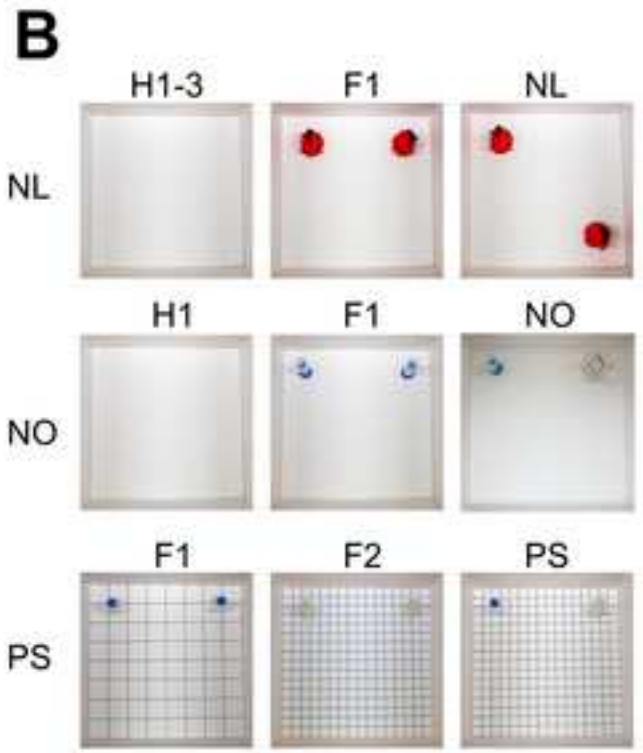

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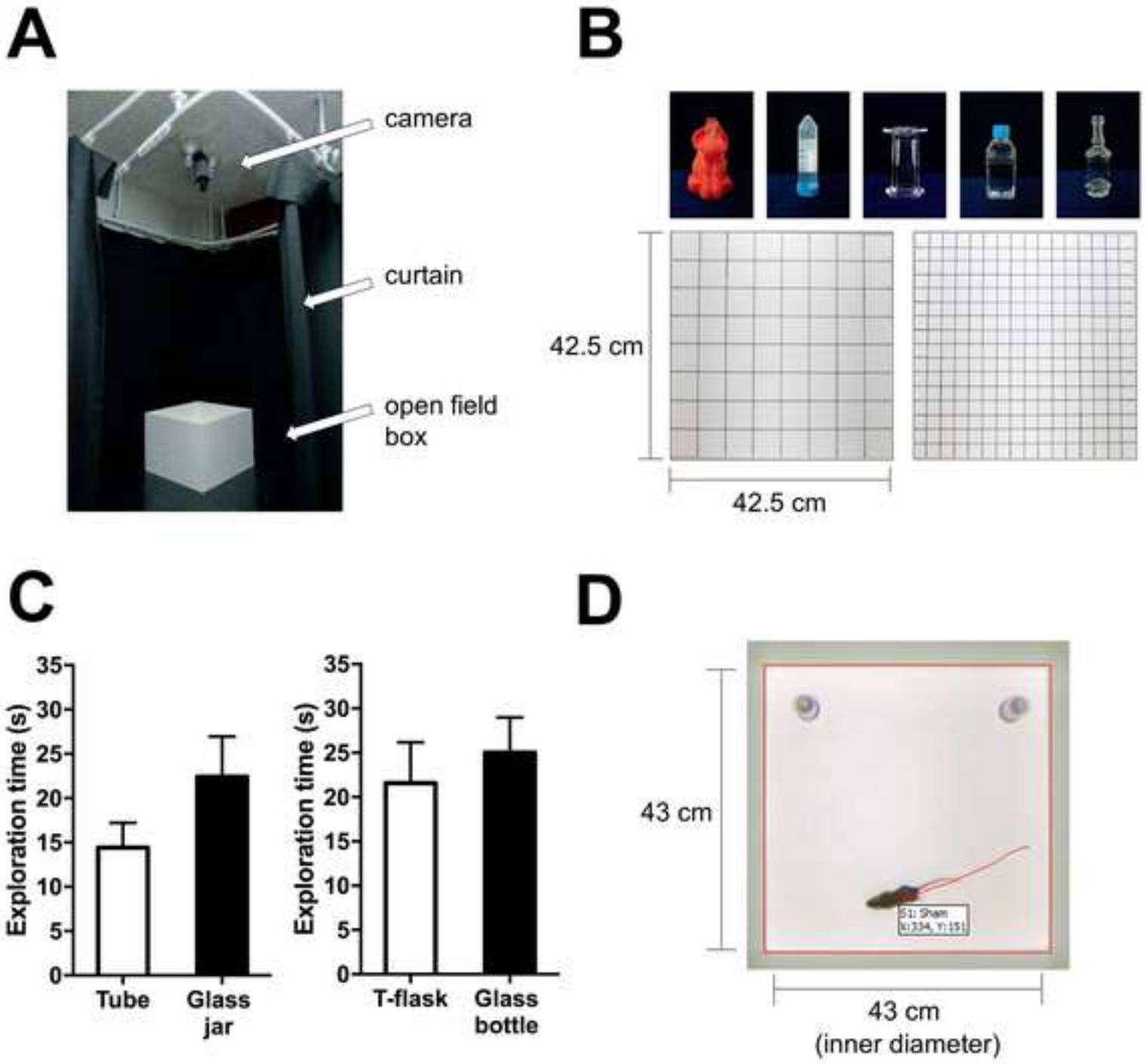

Figure 3

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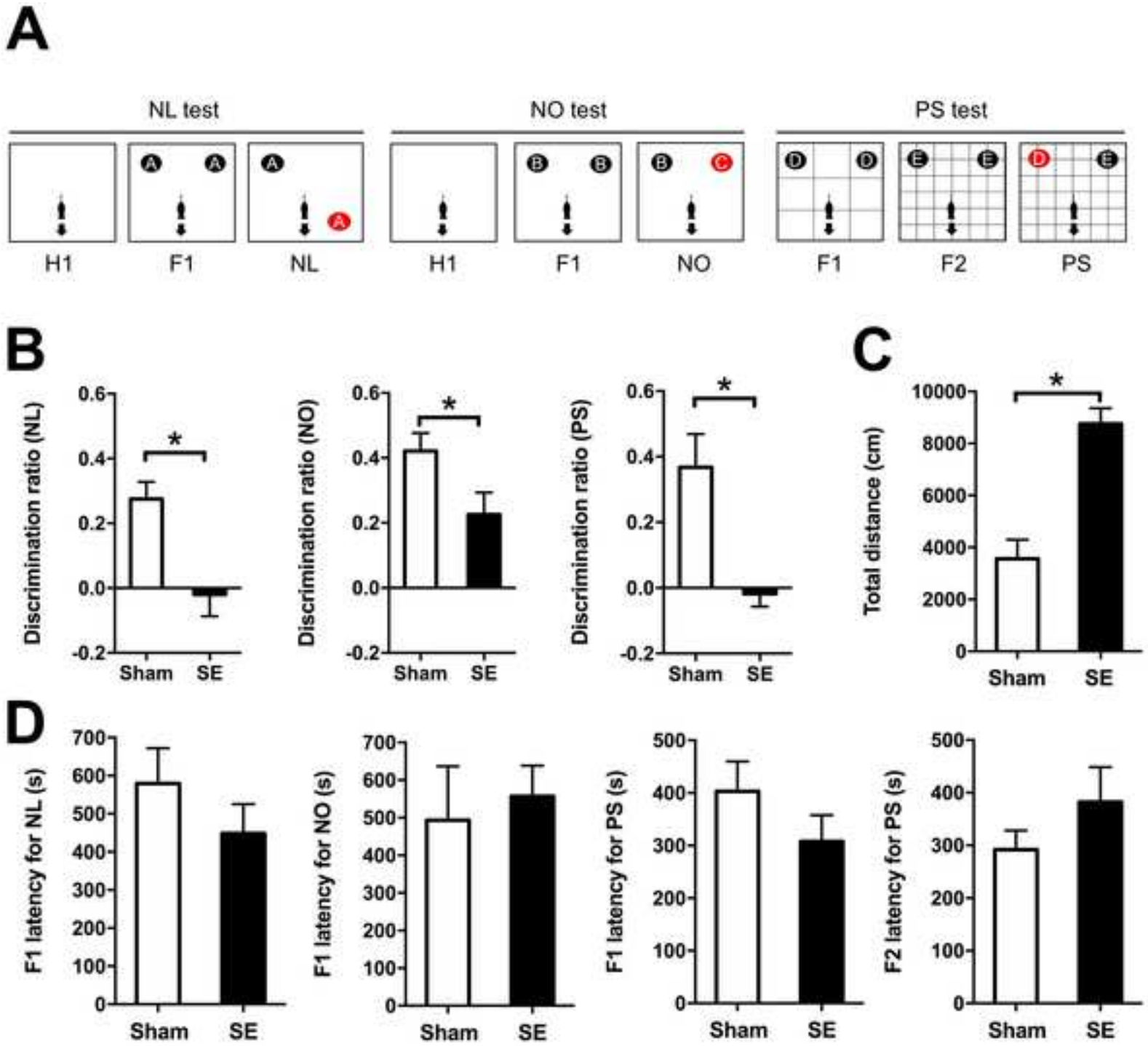

Figure 4

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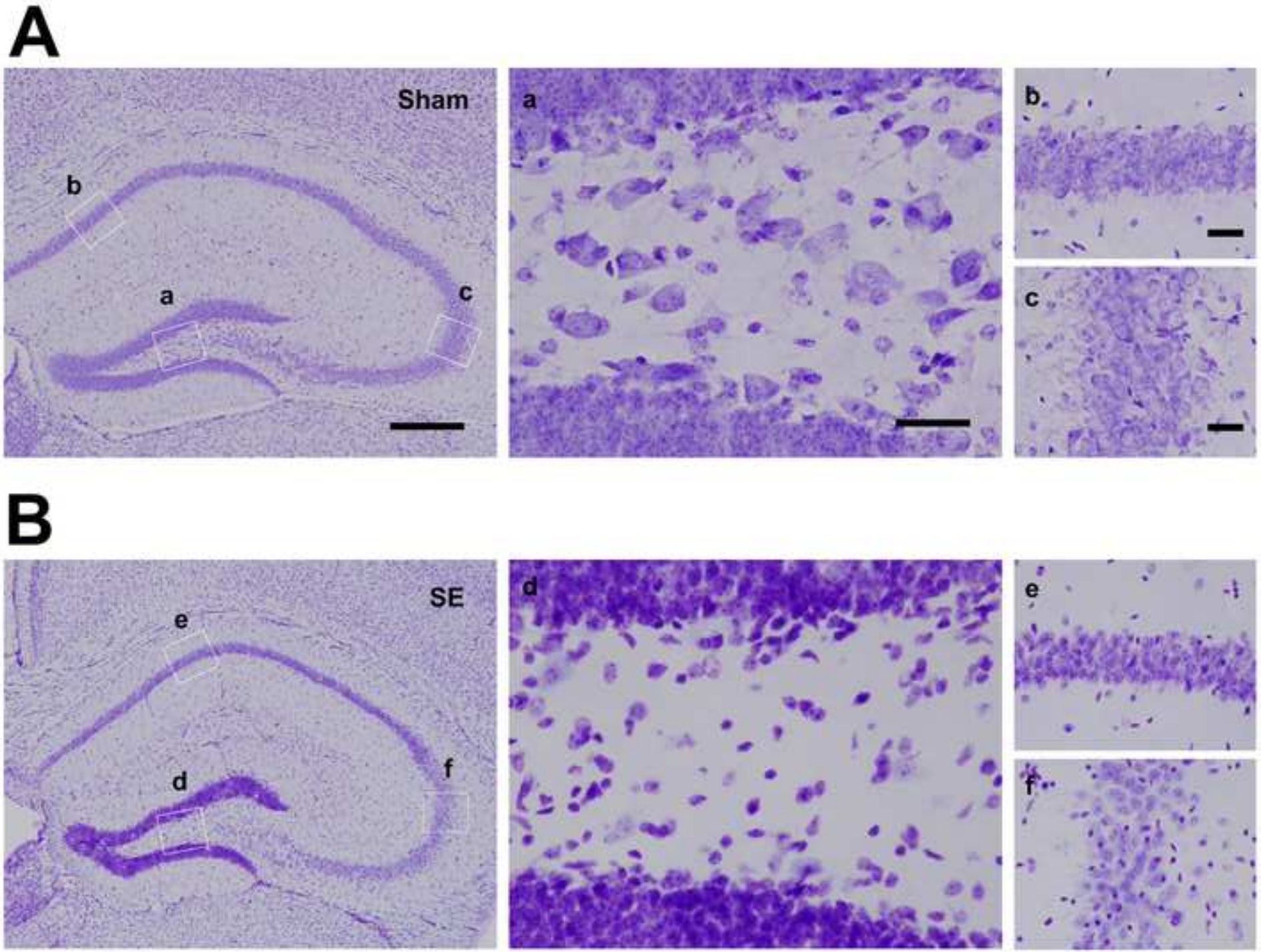


Table of Materials

Click here to access/download;Table of Materials;material sheet_revised.xlsx

Name	Company
1 ml syringe	Sung-shim
70% Ethanol	Duksan
black curtain	
Cresyl violet	Sigma
cryotome	Leica
double-sided sticky tape	
DPX mounting medium	Sigma
ethanol series	Duksan
floor plate with narrow grid patterns	Leehyo-bio
floor plate with wide grid patterns	Leehyo-bio
illuminometer	TES Electrical Electronic Corp.
Intensive care unit	Thermocare
ketamine hydrochloride	Yuhan
LED lamp	Lungo
objects	
open field box	Leehyo-bio
paper towel	Yuhan-Kimberly
paraformaldehyde	Merck Millipore
pilocarpine hydrochloride	Sigma
ruler	
scopolamine methyl nitrate	Sigma
Smart system 3.0	Panlab
stopwatch	Junso
sucrose	Sigma
terbutaline hemisulfate salt	Sigma
video camera (CCD camera)	Vision
xylazine (Rompun)	Bayer korea
xylene	Duksan

Catalog Number
UN1170
C5042
E21040041
06522
UN1170
1334A
#W-1
7003
P13A-0422-WW-04
47201
104005
P6503
S2250
JS-307
S9378
T2528
VCE56HQ-12
KR10381
UN1307

Comments
Use with the 26 or 30 gauge needle
Spray to clean the box and objects
For avoiding unnecessary visual cues
For Cresyl violet staining
For tissue sectioning
For the firm placement of the objects
Make 100%, 95%, 90%, 80%, 70% ethanol solutions
Behavioral experiment equipment, plate size: 42.5 x 42.5 x 0.5 cm, grid size: 2.75 x 2.75 cm
Behavioral experiment equipment, plate size: 42.5 x 42.5 x 0.5 cm, grid size: 5.5 x 5.5 cm
For the measurement of the room lighting (60 Lux)
Use to anesthetize the mouse for transcardial perfusion
Lighting for the behavioral test room
Rubber doll, 50 ml plastic tube, glass Coplin jar, plastic T-flask, glass bottle
Behavioral experiment equipment, size: 44 x 44 x 31 cm
Use to dry open field box and objects
Make 4% solution
Use to locate the objects in the open field box
Make 10X stock
Video tracking system
For the measurement of explorative activities of mice
For cryoprotection of tissue sections
Make 10X stock
Place the camera directly overhead of the open field box
Use to anesthetize the mouse for transcardial perfusion
For Cresyl violet staining

Click here to access/download;Rebuttal Letter;rebuttal letter.docx

Please see section 1. I have remove all the redundancy and made the steps in order. Please have a look and work on the remaining sections accordingly.

We are grateful for the Editor's reorganization of our protocol. Based on the Editor's suggestion, we have rewritten the rest of the manuscript. Please refer to our revised manuscript.

Please ensure that the highlight is no more than 2.75 pages including headings and spacings. Also, ensure that if step 1. Is highlighted, then substep 1.1. showing the action should also be highlighted. Presently its around 4 pages.

We have reduced the highlighted parts to be approximately 2.75 pages.

Please ensure that no more than 2-3 actions are present per step.

We have checked that all the protocol steps have no more than 3 actions.

Please include a figure to show the placement of the object A, B,C, D, E.

It is already included in the Figure 1B in our original manuscript.

Here you are preparing epileptic mouse after pilocarpine injection?

The purpose of step 1 is to prepare epileptic mice that can be generated at 4-6 weeks after pilocarpine-induced acute seizure induction. As we have already cited our previous report describing how to generate epileptic mice, we believe that the reference to that protocol can reduce the redundancy and make our protocol concise.

(Page 2, line 80-81)

Acute seizures were induced by intraperitoneal pilocarpine injection, following the protocol detailed in our previous report[14].

Kim, J. E., Cho, K. O. The Pilocarpine Model of Temporal Lobe Epilepsy and EEG Monitoring Using Radiotelemetry System in Mice. J Vis Exp. (132), (2018).

Is this done for the second time to the mouse in question? Also, 1.1 can be made as section 1: generating epileptic mouse and NL test can be section 2.

The protocol introducing how to induce acute seizures by intraperitoneal pilocarpine injection is described in detail in our previous protocol, which is published in JoVE (see the reference #14). Basically, we induce acute seizures by injecting pilocarpine, and wait for 4-6 weeks until the mouse develops chronic spontaneous seizures, which is the chronic stage of epilepsy. Instead of making our new protocol lengthy and redundant by repeating the same steps, we believe that referring to our previous paper may be more efficient. If repeating the same protocol is required, we will add those steps.

Please include the step number.

We have included the familiarization step number.

(Page 3, line 140-142)

Then, release the mouse near the wall of the open field box that is the farthest from where the objects will be during the familiarization session (Step 1.9.3.).

Please ensure that you have included the software details in the table of materials.

We confirmed that the software details are included in the table of materials.

Smart system 3.0	Panlab		Video tracking system

How is this done in the software? If using manufacturer's instruction, please include this detail. Else include button clicks etc.

We have added information about how to analyze the total distance moved.

(Page 3, line 153-154)

1.7.4.1. Open the video tracking software and video clips. Then, click "analysis" to calculate total distance moved, based on the calibration of the open field box size.

This is confusing, here it says habituation is for 3 mins and step 1.14.2 says 10 mins. Please check and make the steps in the order of it being performed as done for the steps above.

Three min habituation is the brief rehabituation for alleviating anxiety-related stress of the mouse. We included 3-min rehabituation phase just before the familiarization (20 min) and testing session (10 min). We have the NL testing steps in the order of it being performed.

Please see page 4-5, line 211-266.

Please see section 1 and format the steps accordingly in order to bring out clarity.

We have reformatted the steps according to the way the Editor suggested.

Please see page 5-6, line 270-370.

Please check the new step numbers.

The step numbers are correct.

Please see section 1 and format the steps accordingly in order to bring out clarity.

We have reformatted the steps according to the way the Editor suggested.

Please see page 6-9, line 374-696.

Made the steps in order of it being performed. Please check.

We thank the Editor for the reorganization. It is written in the order of it being performed.

Please include a reference or detail the process.

We have added a reference describing how to do transcardial perfusion.

Gage, G. J., Kipke, D. R., Shain, W. Whole animal perfusion fixation for rodents. J Vis Exp. (65), (2012).

When was this done? Please include all the action in a stepwise manner.

Decapitation was performed after transcardial perfusion was completed. We have added detailed steps for the brain isolation, in addition to the citation of a relevant reference.

(Page 9, line 708-711)

After the transcardial perfusion is finished, remove the head with a pair of scissors15. Then, remove the skull using a pair of iris scissors to expose the brain. After the brain is isolated, postfix it in 4% paraformaldehyde overnight, followed by cryoprotection in 30% sucrose in 0.01 M phosphate-buffered saline.

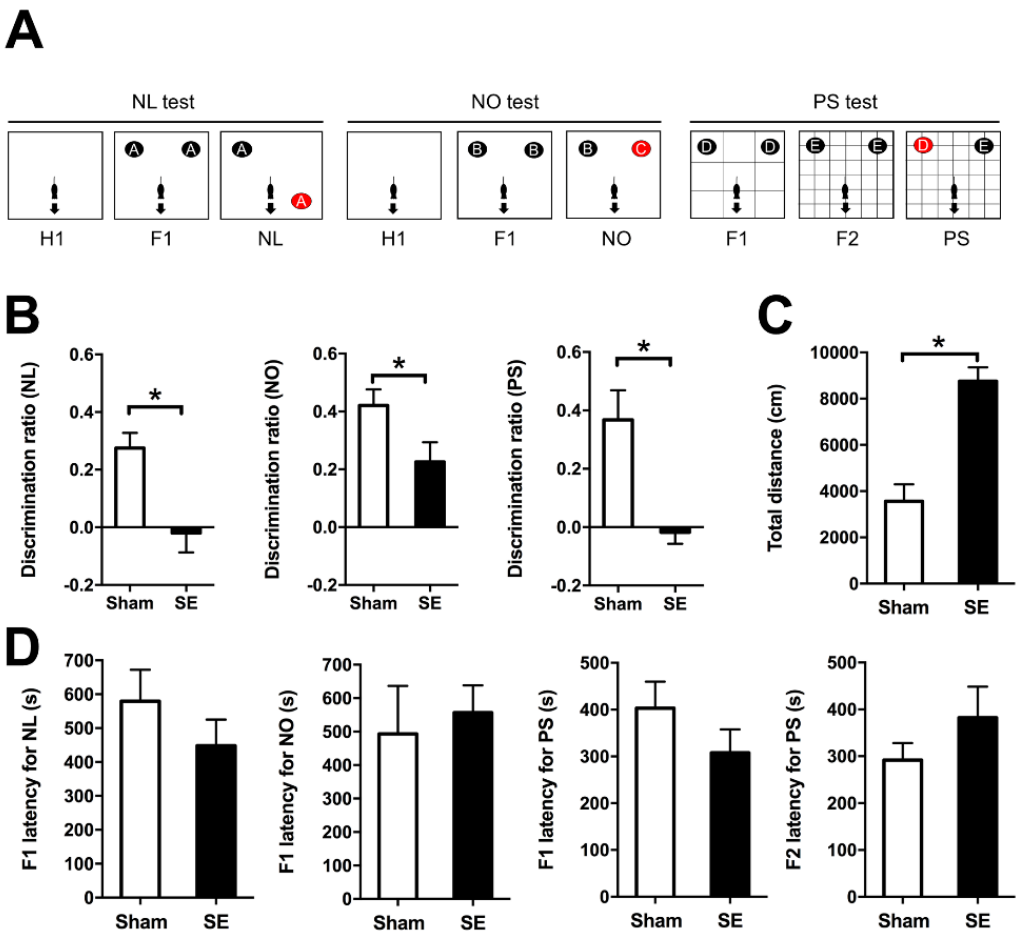
Gage, G. J., Kipke, D. R., Shain, W. Whole animal perfusion fixation for rodents. J Vis Exp. (65), (2012).

Please make the color code in the figure represent this.

We have indicated the novel object in red (See our new Figure 3).

(Page 11, line 800)

A novel object is indicated as a red circle.



Please include details for Figure 2d as well.

Details for Figure 2d have been already included in our original manuscript. To describe the results of Figure 2 in an alphabetical order, we have replaced figure 2d with 2c (see our new Figure 2).

(Page 10, line 744-747)

The example objects are materials that are easy to clean and similar or slightly larger than a mouse (Figure 2b), of which combinations need to be pre-screened to confirm that there is no significant preference between the two objects presented together (Figure 2c).

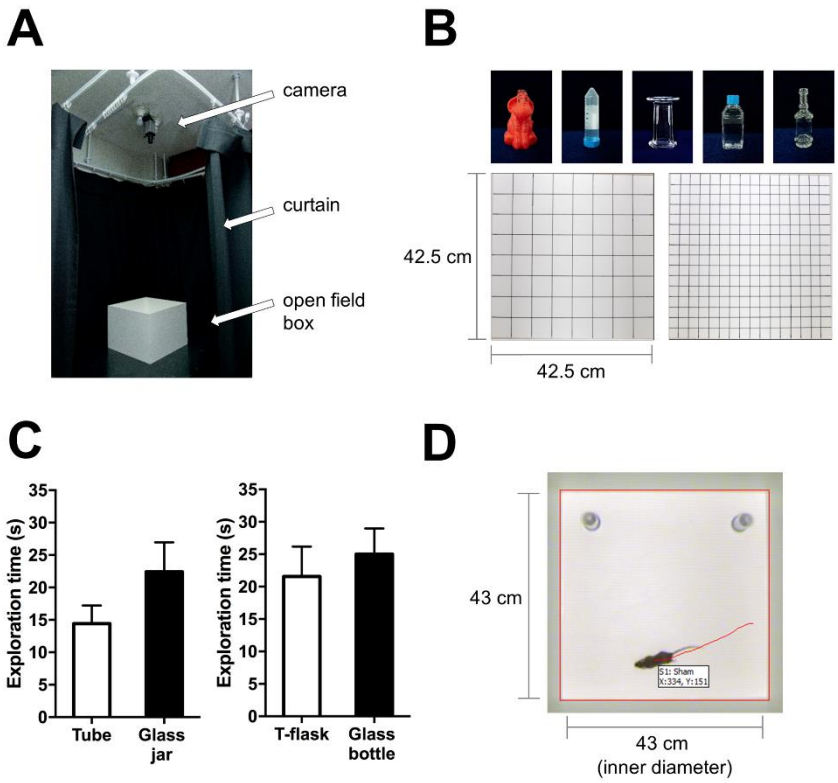
(Page 10, line 748-750)

Once a mouse has been introduced into the open field box, a video-tracking system can track its trajectory to analyze its total locomotion distance (Figure 2d).

(Page 10, line 779-784)

(C) Graphs showing the time exploring each object presented together during the NO and PS testing session (n = 7), respectively. Note that there is no significant difference in the preference between the two objects assessed by Mann-Whitney U test (for NO test) and Student's unpaired t-test (for PS test). (D) An image showing that a video tracking system detects the experimental mouse in the open field box. The red square indicates the preset

zone for tracking the mouse's trajectory.



N= what here?

We have tested object preference using 7 mice.

(Page 10, line 779-782)

(C) Graphs showing the time exploring each object presented together during the NO and PS testing session (n = 7), respectively. Note that there is no significant difference in the preference between the two objects assessed by Mann-Whitney U test (for NO test) and Student's unpaired t-test (for PS test).

N = ?

The number of animals we have tested in Figure 3 is 8 sham and 10 epileptic mice.

(Page 11, line 800-810)

(B) and epileptic mice (n = 10). Note that the epileptic mice demonstrated significant impairments in the NL, NO, and PS tests, which test memory function for places, objects, and contexts, respectively. *p < 0.05 by Mann-Whitney U test for the NL test. *p < 0.05 by Student's unpaired t-test for the NO tests. *p < 0.05 by Student's unpaired t-test with Welch's correction for the PS test. (C) A graph showing the locomotor activity of sham (n = 8) and epileptic mice (n = 10). Note that the epileptic mice demonstrated increased locomotion, in line with previous reports. *p < 0.05 by Student's unpaired t-test. (D) Graphs showing latency to 30 s criteria in

the familiarization session of the NL, NO, and PS tests. Note no differences in the motivation for exploring the objects between sham (n = 8) and epileptic mice (n = 10).

Please include all the references as mentioned by reviewer 1 and cite them accordingly in the text.

We have included all the references that the Reviewer #1 recommended and cite them in the discussion.

(Page 12, line 876-878)

On the other hand, object recognition memory seems to involve multiple brain regions, including the perirhinal cortex, insular cortex, and ventromedial prefrontal cortex, in addition to the hippocampus[25-38].

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