Journal of Visualized Experiments

A behavioral test battery for repeated assessment of motor, mood and cognition in mice

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58973R2
Full Title:	A behavioral test battery for repeated assessment of motor, mood and cognition in mice
Keywords:	Neurodegeneration, Motor, Social interaction, Anxiety, Depression, Cognition
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Hong Kong

August 20, 2018

Dr. Ronald Myers, Senior Science Editor,
Journal of Visualized Experiment

Dear Dr. Myers,

Subj.: Submission of manuscript

I am writing to submit our manuscript entitled “A behavioral test battery for repeated assessment of motor, mood and cognition in mice” for your consideration to be published in *Journal of Visualized Experiment*. Behavioral analysis in mice is one of the most critical step in research in the neurodegeneration field. The complexity of the behavioral symptoms during the progression of neurodegeneration requires comprehensive analysis of the behaviors in research. We designed a behavioral test battery, which contained at least two well-accepted tests in motor, mood and cognition, all of which could be susceptible to neurodegeneration, to assess the overall behavioral changes in mice. The uniqueness of our method is the compatibility within the battery, which means the tests are arranged to achieve the maximum habituation for the mice and the minimum interference. In addition, this battery can be repeatedly tested in a longitudinal study, such as in long-term toxicological and/or pharmacological evaluation.

We believe that this behavioral test battery is important for the field of neurodegeneration, behavioral analysis, toxicology and pharmacology because it is proved to provide stable, convincing, comprehensive and repeated behavioral analysis in neurodegeneration-related study.

This submission was on inquiry from your editorial board. This method and part

of the representative data has been applied in a published research in Particles and Fibre Toxicology (2018 **15**:28 https://doi.org/10.1186/s12989-018-0263-3l), one of the open access BioMed Central's journals. The open access articles are made available under the Creative Commons Attribution (CC-BY) license, which means they are accessible online without any restrictions and can be re-used in any way, subject only to proper attribution (which, in an academic context, usually means citation). Therefore, I would like to declare on behalf of my co-authors that this manuscript is a brand-new paper with the authorization of Springer to use the published materials and data with proper citation. The entire paper is not under consideration for publication elsewhere. All the authors have agreed to submit this manuscript.

We highly appreciate your consideration of our manuscript, and look forward to receiving comments from the reviewers. Please do not hesitate to contact me if you have any queries.

Sincerely yours,

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TITLE:

A Behavioral Test Battery for the Repeated Assessment of Motor Skills, Mood, and Cognition in Mice

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KEYWORDS:

Neurodegeneration, motor, social interaction, anxiety, depression, cognition

SUMMARY:

A comprehensive behavioral test battery of motor skills, mood—including social interaction, depression, and anxiety—and cognition is designed for the repeated assessment of neurodegeneration-related behavioral changes in mice.

ABSTRACT:

Pharmacological and toxicological studies in neurodegeneration require comprehensive behavioral analysis in mice because motor dysfunctions and dysfunctions in mood and cognition are common and often shared symptoms in neurodegenerative diseases. Shown here is a behavioral test battery for motor, mood, and cognition, which can be repeatedly tested in a longitudinal study. This battery assesses the overall behavioral phenotype in mice by examining each domain of behavior with at least two independent well-accepted tests (*i.e.*, open-field test and rotarod test for motor function, social interaction test, elevated plus maze test, and forced swim test for emotional function, and Morris water maze test and novel object recognition test for cognitive function). Therefore, this sensitive and comprehensive test battery is a powerful tool for the study of behavioral alternation in neurodegeneration.

INTRODUCTION:

Neurodegenerative diseases featured devastating behavioral symptoms, including cognitive impairment, mood dysfunctions such as anxiety and depression, or motor dysfunction[1]. The pathogenesis of various kinds of neurodegenerative diseases is unclear[2]. Accumulative studies

indicate that genetic and environmental factors might both contribute to the pathogenesis of neurodegenerative diseases. Identifying the risk factor of neurodegeneration requires behavioral analysis. Although each type of neurodegenerative disease has its signature behavioral symptom (*e.g.*, Alzheimer's disease [AD] is featured with cognitive impairment and Parkinson's disease [PD] with motor dysfunction). With the progression of the disease, the patients manifest comorbidity of different behavioral abnormalities[3]. For example, AD patients show symptoms of mood dysfunction in the advanced stage[4,5]. PD patients may progress into PD-related dementia and develop cognitive impairment[6]. Based on these features, the behavioral analysis in neurodegeneration models is usually comprehensive and repeated.

To achieve this goal, a battery which contains classical and widely used behavioral tests with excellent validity was designed for behavioral analyses in motor, mood, and cognition. The motor function can be tested by the open-field test[7,8] and the accelerating rotarod test. Mood dysfunction, including social dysfunction, depression, and anxiety, are most commonly seen in neurodegenerative diseases[5]. Hence, this battery includes a social interaction test for sociability[9], the elevated plus maze test for anxiety[10], and the forced swim test for depression[11]. Cognitive impairment is one of the most characteristic symptoms in neurodegenerative diseases such as AD and frontotemporal lobar dementia[12]. Cognitive domains, including short-term memory and episodic memory, are susceptible to neurodegeneration[13-15]. Therefore, the Morris water maze test for spatial learning and memory[16] and the novel object recognition test for short-term memory[17] are included in the battery. These tests are compatible with each other. The order of the tests was designed to maximize habituation and to minimize interference, to further increase the compatibility within the battery. Since each function is tested by at least two independent tests that are different in principle and method, the results of each test can be further validated. Moreover, the protocols of some tests are highlighted for repeated testing, facilitating the longitudinal study of the development of neurodegenerative diseases. Therefore, this behavior test battery studies different subdomains of behavioral changes seen in various stages of neurodegeneration while costing a minimal number of animals. This battery has been used in a longitudinal study which evaluated the behavioral changes in young adult (3-month-old) male C57BL/6N mice after respiratory exposure to silica nanoparticles, an occupational hazard that is a potential risk factor of neurodegeneration[18]. However, other strains or models, such as aged mice and genetically manipulated mice, may behave differently than young C57BL/6N mice. Therefore, caution may be required when using this battery in these mice.

PROTOCOL:

All methods described here have been approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), the University of Hong Kong.

1. General protocol

NOTE: This section is based on Deacon[19].

1.1. Behavioral room setup

1.1.1. Get rid of unrelated stimulation/distraction, including direct bright light on the experimental apparatus, odor, noise, and other irrelevant animals, in the behavioral room (which should be about 10 m^2 with adjustable lighting and, preferably, have an anteroom).

NOTE: Since the mouse is a nocturnal animal, lighting under 15 lux in the open-field test, novel object recognition test, and social interaction test could minimize the interference/stress from the light and help the mouse to focus on the test.

1.1.2. Set the camera for video recording at least 1.5 m above the floor, ensuring that it is out of the sight of the testing mouse.

1.2. Housing and habituation

1.2.1. Group-house the mice in an animal unit under observation (*e.g.*, group-housing no more than four adult mice).

NOTE: Here, 3-month-old male C57BL/6N mice were used and housed in a 1144B cage. Rule out sick, injured, or severely stressed mice. Experiences of starvation, thirst, or being bullied may affect the performance of the mouse.

1.2.2. Arrange for the same animal handler to conduct all the behavioral tests, to diminish variability. Perform the transportation, handling, and experiment during the light cycle from 7:00 a.m. to 7:00 p.m. If possible, arrange all the other handling, such as the administration of any drug/toxin (*e.g.*, intranasal instillation of silica nanoparticles) or cage-cleaning, after the test.

1.2.3. Relabel the cages with random numbers to blind the experimenter before each experiment. Habituate the mice to the experimental environment in the behavioral room for 15 to 30 min in their home cages. Keep the home cages in the behavioral room during the entire experiment.

1.2.4. Before starting the experiment, put a nonexperimental C57BL/6N mouse in the apparatus so that the experimental condition for the first mouse is the same as the rest. Then, clean the apparatus as follows: remove the urine and feces with a clean paper towel, clean the experimental device with tap water, and then, cover the odor left by the mouse by wiping the apparatus with a paper towel lightly sprayed with 70% ethanol.

1.2.5. To minimize the distraction caused by the experimenter, ask the experimenter to leave the behavioral room during video recording, or stay behind the curtain during the Morris water maze test.

1.3. Behavioral test arrangement

1.3.1. Arrange the behavioral test in the order as shown in **Figure 1A**. Plan to perform the tests spaced out by 24 h, except for the elevated plus maze test.

NOTE: Since the entire procedure requires up to 2 weeks, simplify the battery by choosing among similar tests before application in an acute or short-term study.

CAUTION: The 2 day open-field test ensures proper habituation for the novel object recognition test. Test the forced swimming test last as it may cause stress in C57BL/6N mice.

2. Behavioral test protocol

2.1. Open-field test[8,18-20]

2.1.1. Perform the open-field test in a 60 cm (length [L]) x 60 cm (width [W]) x 40 cm (height [H]) nontransparent white plastic arena.

NOTE: Using multiple arenas can increase the throughput of the test.

2.1.2. Start the camera recording and gently put the mouse next to the middle of a wall of the arena, facing that same wall. Record the behavior of the mouse for 10 min before returning it to the home cage. Clean the apparatus as described in step 1.2.4.

NOTE: The start point in the open-field test can also be the center of the arena. Be consistent among the mice.

2.1.3. Repeat until all the mice finish the protocol. Counterbalance the testing order between groups.

2.1.4. Perform the data analysis as follows.

2.1.4.1. Divide the arena into four squares by four squares (imaginary grid) on the computer screen. To assess locomotor function, count the number of lines crossed by the mouse in the arena[19].

NOTE: The definition of “crossing a line” is when both hind limbs cross it. This definition also applies to the elevated plus maze test.

2.1.4.2. Measure the time spent in the central area as an indicator of anxiety. The central area is the four squares in the center of the arena.

NOTE: Additional parameters, such as rearing (both front paws off the ground, with front paws against a wall or standing), latency to the first rear, and grooming and freezing indicate the emotionality of the mouse.

2.1.4.3. Alternatively, use a tracking software, as described in detail by Seibenhener and Wooten[8], to measure the distance traveled, the speed, and the time spent in the center area.

2.2. Accelerating rotarod test[18]

2.2.1. Perform the 3 day training of the accelerating rotarod test before any treatment of drugs or toxin/modeling/onset of disease and test the motor function for 1 day, as planned in **Figure 1A**.

NOTE: The mouse receives three trials per day during the training and testing. Each trial starts with the rotation of the rod and ends with the drop of the mouse.

2.2.2. Place the rotarod apparatus on the bench in the behavioral room. Avoid direct lighting to the equipment. Program the equipment as starting from 4 rpm and accelerating to 40 rpm within 5 min.

2.2.3. In each trial, put the mouse on the static rod, facing the wall of the machine. Start the device when the mouse is settled. Stop the device once the mouse drops and record the time the mouse spent on the rod. Immediately repeat for another two trials before returning the mouse back to the home cage.

2.2.4. Repeat the procedure on the other mice.

2.2.5. Measure the average time spent on the rod of three trials during testing to estimate motor function.

NOTE: The average time on the rod during the third day of training is the baseline of motor function.

2.3. Social interaction test[18]

2.3.1. For the social interaction test, use an open-field arena with two identical transparent chambers (8 cm [L] x 6 cm [W] x 12 cm [H]) with holes on the surface—and a novel mouse (helper) which is a same-sex juvenile conspecific that has had no previous contact with the subject mouse. **Figure 1B** shows the scheme of the procedure. Clean the arena and the chambers as described in step 1.2.4.

NOTE: The novel mouse cannot be a littermate or cage mate of the subject mouse. It is group-housed and healthy. Habituate the novel mouse to the behavioral room for 15 to 30 min as described in step 1.2.3.

2.3.2. Separately place the two chambers in the middle of two opposite walls of the arena. Introduce the subject mouse into the arena as described in step 2.1.2 and shown in **Figure 1B**, for a 3 min exploration. Return the subject mouse to the home cage and remove any urine or feces in the arena.

2.3.3. Put the helper in one of the chambers. Reintroduce the subject mouse to the arena and record for 3 min. Afterward, return both mice to each of their own home cages. Repeat the procedures with other subject mice as described above.

NOTE: Counterbalance the side of the helper or randomly assign it within the group.

2.3.4. From the video, estimate the parameter describing the social interaction activity of the mice as t_{helper}/t_{empty}, which means the ratio of time interacting with the helper chamber (t_{helper}) and the empty one (t_{empty}), or use the recognition index $t_{helper}/(t_{helper} + t_{empty})$.

NOTE: An interaction between the subject mouse and the chamber is defined as when the mouse's nose is within 2 cm of the chamber and pointing toward it.

2.4. Elevated plus maze test[10]

2.4.1. Conduct the elevated plus maze test on the same day after all mice are tested in the open-field test. Clean the apparatus as described in step 1.2.4.

NOTE: The configuration of the elevated plus maze is a "+"-shape. It has two open arms (30 cm x 5 cm x 0.5 cm) across from each other and perpendicular to two closed arms (30 x 5 x 16 cm) with a center platform (5 cm x 5 cm x 0.5 cm). The maze is elevated 40 cm from the ground.

2.4.2. Place the mouse at the junction of the open and closed arms, facing the open arm that is opposite to the experimenter (**Figure 1C**). Record the behavior for 5 min before returning the mouse to the home cage. Repeat till all mice are tested.

NOTE: Entering the maze with its face to the open arm could increase the mouse's exploration of the open arm.

2.4.3. Measure the time the mouse spent in the open arms (t_{open}) and in the closed arms (t_{close}) based on the video: t_{open}/t_{close} indicates the level of anxiety.

2.5. Forced swim test[11]

2.5.1. The apparatus of the forced swim test is a cylindrical tank that is 30 cm high and 20 cm in diameter. Fill the tank up to 15 cm high with tap water at room temperature (23 - 25 °C).

NOTE: Use fresh water for each mouse.

2.5.2. Start the video recording and gently put the mouse in the water, in the center of the apparatus. Record the video for 6 min before putting the mouse back in its home cage under infrared light.

NOTE: Do not disturb the mouse by drowning it or twisting its tail.

2.5.3. Measure the immobility time in the last 5 min of the recorded video. Mobility means any movements other than those required to balance the body and to keep the head above the water.

2.6. Novel object recognition test[17,18]

2.6.1. Set up the novel object recognition test to include 2 days of habituation, 1 day of familiarization, and 1 day of testing (**Figure 1D**); each session is 10 min per mouse, and the intersession interval is 24 h.

NOTE: The habituation using the open-field test is performed as described in section 2.1. The mouse interacts with two identical objects (old objects) in familiarization. In the test, the mouse interacts with one of the old objects and a new object, both placed in the same place as the objects in familiarization. The apparatus of the novel object recognition test includes an open-field arena and two sets of objects. Each set contains two identical objects (objects A and A and objects B and B). Objects A and B are similar in size but different in texture (glass/plastic/paper), shape (round/cubic), and color (bright/dark). The objects should be odor-free and big enough for the mouse to explore within 10 min. The appropriate size for adult C57BL/6N mice is 8 cm tall and 5 cm wide/in diameter.

2.6.2. Mark the positions of the two objects in familiarization and test, which are 5 cm away from the side and 7 cm away from the top of the arena.

NOTE: Mark the position on the evening before the familiarization to avoid the smell of the marker.

2.6.3. In the familiarization, the mouse interacts with one set of identical objects. Clean the arena and objects as described in step 1.2.4 before placing the mouse in the arena, facing the middle of the wall as shown in **Figure 1D**. Record for 10 min before returning the mouse to the home cage. Repeat until all the mice are finished and return all the cages to the animal unit.

NOTE: Counterbalance the objects used in familiarization within the group to diminish bias (*e.g.*, mice No. 1 and 2 explore objects A and A, and mice No. 3 and 4 explore objects B and B; in this way, the novel object is object B for mice No. 1 and 2 and object A for mice No. 3 and 4).

2.6.4. Perform the test 24 h after the familiarization. Use the same procedure as for the familiarization, except replace one of the objects with one from another set (**Figure 1D**). Repeat until all the mice have performed the test and, afterward, return all the cages to the animal unit.

NOTE: Counterbalance the side of the new object within the group to diminish bias (*e.g.*, introduce mice No. 1 and 3 to objects A and B, and mice No. 2 and 4 to objects B and A). In this way, the novel object shows at the right side for mice No. 1 and 4, and at the left side for mice No. 2 and 3. Here, the left side is the left side of the experimenter when facing the arena.

2.6.5. Measure the time that each mouse interacts with the new object (t_{new}) and the old object (t_{old}) separately, from the video footage in the test phase. An interaction between the animal and the object is described in the note following step 2.3.4. Calculate the memory of the mouse as the preference to the novel object = t_{new}/t_{old}; or $t_{new}/(t_{new} + t_{old})$.

NOTE: t_{new}/t_{old} equals to 1 or $t_{new}/(t_{new} + t_{old})$ equals to 0.5 means the mouse has no preference for the novel object (*i.e.*, memory impairment). The time interacting with objects in the familiarization can serve as a control of the experiment. The total time indicates the exploration activity of the mouse, and t_{left}/t_{right} suggests spatial bias.

2.7. Morris water maze test[16]

2.7.1. Set up the apparatus as follows.

2.7.1.1. Put the water maze, a circular pool (of 120 cm in diameter and 60 cm deep), in the center of a behavioral room, and mark the position of the maze to ensure the position remains the same during the entire experiment.

2.7.1.2. Divide the maze into four equal imaginary quadrants. Hang the visual cues (*e.g.*, circle, square, triangle, and pentagon) in the center of each quadrant, 130 cm above the floor and 53 cm away from the wall of the maze.

NOTE: The maze and the cue must stay in the same position during the entire test, so the mouse can form accurate spatial memory.

2.7.1.3. Place a platform 25 cm away from the wall, in the center of the fourth quadrant, and mark the position. The platform for the mouse is 10 cm in diameter.

NOTE: The position and diameter of the platform determine the difficulty of the task. The nearer it is to the wall of the maze, or the bigger the platform, the easier the task.

2.7.1.4. Fill the water maze with water (with a temperature of 23 to 25 °C, colored into white and made opaque by milk powder/food whitening powder) until the water level is 1 cm higher than the platform. Bring the mice into the behavioral room for 15 to 30 min of habituation as shown in step 1.2.3 and turn on the infrared light above the cages, which will be used to dry the mice.

NOTE: Cover the top of the platform with white cloth and net so that the mouse can easily climb onto it. Make sure there is no direct lighting above the water.

2.7.2. Conduct the training phase as follows.

2.7.2.1. The training phase takes 5 days, four trials per day. Semi-randomly arrange the starting points on each day as demonstrated in literature[16]. This effort prevents the mouse from establishing associative memory, which is the most common way to “cheat” in the test.

2.7.2.2. At the beginning of each trial, start the video recording and gently put the mouse into the maze.

NOTE: Do not drop the mouse into the tank or twist its tail, which may cause extra stress and disorientation.

2.7.2.3. Ask the experimenter to stay out of sight of the mouse and to return to take the mouse back to its home cage when any of the following happens: (i) the mouse cannot locate the platform within 60 s; (ii) the mouse finds the platform within 60 s and stays on it for 10 s. In circumstance (i), ask the experimenter to place the mouse on the platform and let it stay there for 10 s.

NOTE: Point (ii) means the mouse successfully located the platform.

2.7.2.4. Stop the video and put the mouse back in the home cage under infrared light.

NOTE: Maintaining the mice's body temperature is critical for their performance because hypothermia stresses mice and may affect the following tests.

2.7.2.5. Repeat the procedure with another mouse.

2.7.2.6. Based on the video, record the escape latency, which is the duration of the period the mouse spends in the maze, from entering the maze till the moment it successfully locates the platform. If the mouse cannot find the platform or stays there for less than 10 s in 60 s, the escape latency counts as 60 s. Plot the learning curve against the training days with the average escape latency per day.

NOTE: The escape latency does not include the 10 s spend on the platform.

2.7.3. Perform the probe phase as follows.

2.7.3.1. On the sixth day of the Morris water maze test, set up the apparatus as described in step 2.7.1., take a picture of the maze to record the position of the platform, and then remove the platform from the tank.

2.7.3.2. Start the video recording, and gently put the mouse into the maze in the quadrant diagonally opposite to the target quadrant.

2.7.3.3. Ask the experimenter to stay out of sight of the mouse during the 1 min video recording. Afterward, have the experimenter take the mouse out of the maze and put it back in the home cage.

2.7.3.4. Use the image taken at step 2.7.3.1. as a reference to measure the duration of any platform crossing. Time the duration the mouse stays in the target quadrant (t_{target}), according to the video. The total time is t_{total}. Measure the preference to the target quadrant as t_{target} /t_{total}.

REPRESENTATIVE RESULTS:

This behavioral test battery was designed for the comprehensive and valid behavioral analysis of motor, mood, and cognition, which are commonly affected in neurodegeneration[5]. We have applied this battery to study the behavioral changes in young adult C57BL/6N mice after respiratory exposure to silica nanoparticles for 1 month and 2 months[18]. The results revealed that C57BL/6N mice exposed to silica nanoparticles showed various behavioral changes after different exposure times[18]. Briefly, results in the open-field test (**Figure 2A**) and the accelerating rotarod test (**Figure 2B**) demonstrated that silica nanoparticles exposure did not affect the locomotor or motor function in mice, indicating a full capability of accomplishing the other tests. Social interaction activity was affected after a 1 month exposure to silica nanoparticles (**Figure 2C**). Considering anxiety or depression would also decrease sociability, we analyzed data of the open-field test, elevated plus maze test (**Figure 2D,E**), and the forced swim test (**Figure 2F**), which did not indicate any comorbidity of anxiety nor depression at the 1 month time point. A 2 month exposure to silica nanoparticles resulted in anxiety according to the results in the elevated plus maze test (**Figure 2E**). A similar trend was shown in the central area duration in the open-field test (**Figure 2D**). Cognitive impairment was also detected in the Morris water maze test and novel object recognition test after a 2 month exposure (**Figure 3**). It should be noted that the protocol was slightly different in the two trials of the Morris water maze test. An additional lamp was added in the second trial, so all the mice always stayed under the lamp to keep warm. Hence, no nonperformer was shown in the second trial, whilst two out of eight mice became nonperformers in the probe test in the first trial.

We adjusted the protocol so that most of the tests in the battery can be repeatedly tested. The key is to maintain the motivation of the tests. Tests like social interaction test, novel object recognition test, and elevated plus maze test are motivated by novelty (*i.e.*, a novel juvenile helper, novel objects, and a novel environment, respectively). By maintaining the novelty in the protocol, the young adult C57BL/6N mice showed a consistent performance when tested again after 1 month. According to our data, when introduced to two different helpers in the two trials, mice consistently showed a preference greater than ten-fold to the helper than to the empty chamber in the social interaction test (**Figure 4A**). In the novel object recognition test, the normal mice consistently preferred the novel object to the old object (**Figure 4B**). However, in the elevated plus maze test, when tested again in the same environment after 1 month, the exploration dropped by half (**Figure 4C**)[10]. Theoretically, young adult C57BL/6 mice can be tested repeatedly in these tests as long as the experimental condition, including the novelty and the status of the mice, remains the same. We have repeated these tests every month for up to three times in our lab. Noteworthy, the Morris water maze test cannot be tested repeatedly in the same group of young adult C57BL/6N mice as the experience significantly interferes with the performance when repeatedly tested. According to our data, the mice still remembered the platform even after 1 month, showing correct and long-term spatial memory. When changing the

position of the platform, the experienced mice learned faster than naïve mice, as they had learned the rules and searching strategy from the prior training (**Figure 4D**).

FIGURE LEGENDS:

Figure 1: Schematics. (**A**) Arrangement of the behavioral test battery and the schematic plots of (**B**) the social interaction test, (**C**) the elevated plus maze test, and (**D**) the novel object recognition test. Abbreviations: R = accelerating rotarod test; OF = open-field test; EPM = elevated plus maze test; NOR = novel object recognition test; SI = social interaction test; MWM = Morris water maze test; FST = forced swimming test. The starting point of the mouse in the test is shown by the mouse in the scheme.

Figure 2: Changes in motor and mood in mice exposed to silica nanoparticles for 1 month or 2 months, detected by the behavioral test battery. Mice were tested in (**A** and **D**) the open field test, (**B**) the rotarod test, (**C**) the social interaction test, (**E**) the elevated plus maze test, and (**F**) the forced swimming test comprised in the battery. *N* = 8, 12, or 20, which means each group had 8, 12, or 20 mice, respectively, as demonstrated in each figure. *N* = 17 - 20 means each group had 20 mice, except for a control group at 1 month, which consisted of 17 mice. In panels **A**, **D**, and **E**, data were first normalized to control at each time point and, then, were analyzed with two-tailed Student's *t*-test. The data in panel **B** were analyzed by repeated measures two-way ANOVA. The data in panels **C** and **F** were analyzed with two-tailed Student's *t*-test. All data is shown as mean ±S.E.M. * and **** mean $p < 0.05$ and 0.0001, respectively. These data have been published previously by You *et al.*[18].

Figure 3: Changes in cognition after being exposed to silica nanoparticles for 1 month or 2 months. Changes in the mice's cognition after being exposed to silica nanoparticles for (**A**) 1 month or (**E**) 2 months, detected by the novel object recognition test. Changes in the mice's cognition after being exposed to silica nanoparticles for (**B** - **D**) 1 month or (**F** - **H**) 2 months, detected by the Morris water maze test. Mice were repeatedly tested in the novel object recognition test. A different batch of mice was tested in the Morris water maze test at different time points. *N* = 6, 8, 12, or 20, which means each group had 6, 8, 12, or 20 mice, respectively, as demonstrated in each figure. In panels **A**, **C**, **D**, **E**, **G**, and **H**, data were analyzed with two-tailed Student's *t*-test. The data in panels **B** and **F** were analyzed by repeated measures two-way ANOVA. All data are shown as mean ±S.E.M. * means $p < 0.05$. These data have been published previously by You *et al.*[18].

Figure 4: Representative data in tests. Representative data in tests, including (**A**) the social interaction test, (**B**) the novel object recognition test, and (**C**) the elevated plus maze test, tested in naïve mice (trial 1) and repeatedly tested in the same batch of mice (trial 2). (**D**) Representative data of the Morris water maze test when repeatedly tested. This figure has been modified from You *et al.*[18]. All data are shown as mean ±S.E.M. and analyzed with unpaired Student's *t*-test. $P < 0.001$, compared to trial 1.

DISCUSSION:

Behavioral analysis of mice is critical for neurodegeneration research. While cognitive function is often the most susceptible domain of behavior affected in neurodegenerative diseases, mood dysfunction, such as depression and anxiety, is often comorbid. Moreover, motor function often affects the interpretation of the results in some tests, such as the novel object recognition test, the elevated plus maze test, and the social interaction test. Based on these thoughts, a comprehensive behavioral test battery is required for an overall and precise assessment of the behaviors.

The first step is choosing the proper tests. We included well-accepted and classical tests, namely the open-field test and the rotarod test for motor function, the elevated plus maze test for anxiety, the forced swim test for depression, the social interaction test for sociability, and the Morris water maze test and the novel object recognition test for cognition. There are three classical tests for anxiety, namely the open-field test, the elevated plus test, and the dark/light box test. These tests all exploit the conflict between the innate curiosity about the novel environment and the aversion to the open, elevated, or brightly illuminated field. Hence, mice are not conditioned by noxious stimuli, such as electric shock, predator odor, and so on. However, abundant studies have revealed that these tests have poor intertest reliability, even within a single laboratory[21]. We used the open-field test and elevated plus maze test to analyze the anxiety in mice in the shortest possible time. The open-field test examines anxiety-like behavior in addition to locomotor function and is also the habituation for the novel object recognition test and social interaction test. We chose the elevated plus maze test because it is a well-established paradigm. It has excellent face validity (phenomenological similarity between the behavior in mice and the symptoms in patients), construct validity (the degree to which the test reflects the underlying theoretical assumptions), and predictive validity (the accuracy of the test results when translating them to humans)[10]. If anxiety was the priority, researchers might consider including the dark/light box test and comprehensively analyzing the behavior in these tests with more parameters, such as rearing frequency, stretch-attend behavior, and defecation[21]. Secondly, results of these tests can be easily interpreted because the motivations of the tests are clear, which are either the innate curiosity to novelty or water, to perform a simple task in a controllable condition. Unlike starvation and pretraining, which may cause different levels of motivation in mice, these kinds of motivation are strong enough for most of the C57 mouse to perform the task. Thirdly, these tests have a good compatibility with each other, because the motivation is not too stressful to cause long-term or even permanent stress, such as electric shock or food/water deprivation may cause. Despair tests, including the forced swim test and the tail suspension test, utilize the principle that the mouse gives up struggling when trapped in a desperate environment. By measuring the “despair time,” the tests tell how depressed the mouse is. C57BL/6N mice usually show 180 s of immobility out of 300 s in these tests[11,22]. We chose the forced swim test over the tail suspension test because it is comparatively less stressful when the mouse has experience in water maze training. In this way, they are adapted to water and know how to prevent hypothermia after getting wet. In contrast, we observed around 2 g of body weight loss overnight after the tail suspension test, indicating great stress. Nevertheless, repeated testing of the forced swim test should be done cautiously, and the mice need to recover for a longer time than 24 h before any further experiments/sacrifices. Alternatively, scientists may consider including a sucrose preference test, a paradigm extensively used in stress-induced

depression-like anhedonia, in the battery. However, the protocol of this test requires days of habituation to individual housing[23], which may be a stimulus that affects the outcome of other tests. Lastly, the tests done here have reasonable throughput. All the tests can finish within 10 min/mouse. When increasing the amount of equipment, such as the open-field arena, the channels in the rotarod test, the tanks in the forced swimming test, and the lanes in the tail suspension test, the throughput can also be increased. Although the Morris water maze test makes it difficult to test multiple mice at the same time, each trial only takes ~60 - 75 s.

The most critical concern while applying the behavioral test battery is the interference among the tests and the stress from frequent handling. The adverse effects of these issues can be minimized by further optimizing the order and the interval of testing. While it is common sense that the order of the behavioral tests should be from the least to the most stressful to the mouse, some tests in this battery can be used as acclimation for the following tests and improve the stability of the performance. For example, performing the elevated plus maze test following the open-field test increases the exploration of the open arm[10]. Besides, when the mice are tested in the order of open-field test, novel object recognition test, and social interaction test, they gradually habituate to the environment and the task, including the behavioral room, the open field, and the objects. Hence the mice are unlikely to manifest neophobia[7,24,25], which means showing an unusually low interaction with the novel object/individual, and are all adequately focused on the task. This arrangement decreases the fluctuation in data of tests that are motivated by innate curiosity. The Morris water maze test and the forced swim test have a stronger motivation. Hence, the mice are unlikely to be affected by the experience of the tests. Frequent transport and handling during the entire procedure is another stress source, thus requiring proper animal handling and sufficient habituation. It is recommended that the testing interval in the battery should be at least 24 h, so the mice can recover from the stress of these stressors[26-28]. However, there are other studies that tested mice in multiple tests per day[29].

Another concern about the repeated testing of the behaviors is the influence of the previous experience. The open-field test and the accelerating rotarod test can be repeatedly used in motor function analysis. Novelty-motivated tests, including the elevated plus maze test, the novel object recognition test, and the social interaction test, can be utilized repeatedly when the motivation is novel in each trial, which means a new experimental environment, pairs of objects, or helper, respectively. As shown in the representative data, C57BL/6N mice cannot be trained repeatedly in the classical protocol of the Morris water maze test as they remembered the searching strategy from experience.

This battery assesses multiple domains of behavior. In this way, the results in some tests can be a reference for the data interpretation of others. In the representative data in **Figure 1**, when the mice exposed to silica nanoparticle for 1 month showed a reduction in the social interaction activity, it could be the phenotype of depression or anxiety or the consequence of a motor function deficit. However, the results of the motor function and anxiety tests indicated that the decrease in social interaction was primary, not subsequent. Also, this battery contains different tests for the same domain of behaviors, with different sensitivity and usage. Consistent trend/results in these tests increase the reliability of the battery. However, this arrangement

takes extra time for the entire procedure. The short-term experiment that takes less than 1 month should use a simplified version of this battery.

This behavioral test battery is designed to screen the behavioral phenotype shown in different stages after exposure to the genetic or environmental risk factor of neurodegeneration. Therefore, this protocol only lists the essential readouts of each test. It is noteworthy that each behavioral test can provide loads of information; hence, the user can expand the protocol for further investigation. For example, spontaneous activity such as grooming, rearing, defecation, and thigmotaxis in the open field reveals emotionality[8]. These behavior traits in the elevated plus maze test also can be indicators of anxiety[10,21]. The rotarod test studies motor learning the mice are trained after modeling/disease onset/exposure to environmental risk factors[30]. The social interaction test can also study social memory by introducing a second novel helper after the sociability test[9]. Therefore, expansion of the battery can be customized to fit different priorities of study. However, this battery has only been tested in young adult C57BL/6N mice due to time limitations. The baseline performance of other strains or aged C57BL/6 mice may be different. Moreover, transgenic neurodegenerative mice models may exhibit behavioral deficits such as hypo- or hyperactivity. Hence, they may not be suitable for tests with a low motivation, such as the novel object recognition test. Therefore, further optimization should be required for the behavioral assessment of these mice.

In conclusion, this battery allows a convincing and comprehensive behavioral analysis of neurodegeneration in the C57 mouse strain. It is most suitable for neurodegeneration-related longitudinal studies of the toxicity of potential risk factors/neurotoxin or drug development, which often features long-term administration and repeated testing.

ACKNOWLEDGMENTS:
The authors thank Dr. Cora SW Lai from the School of Biomedical Sciences, the University of Hong Kong, for lending the elevated plus maze test, and the Department of Anesthesiology from the University of Hong Kong for lending the rotarod test apparatus.

DISCLOSURES:
The authors have nothing to disclose.

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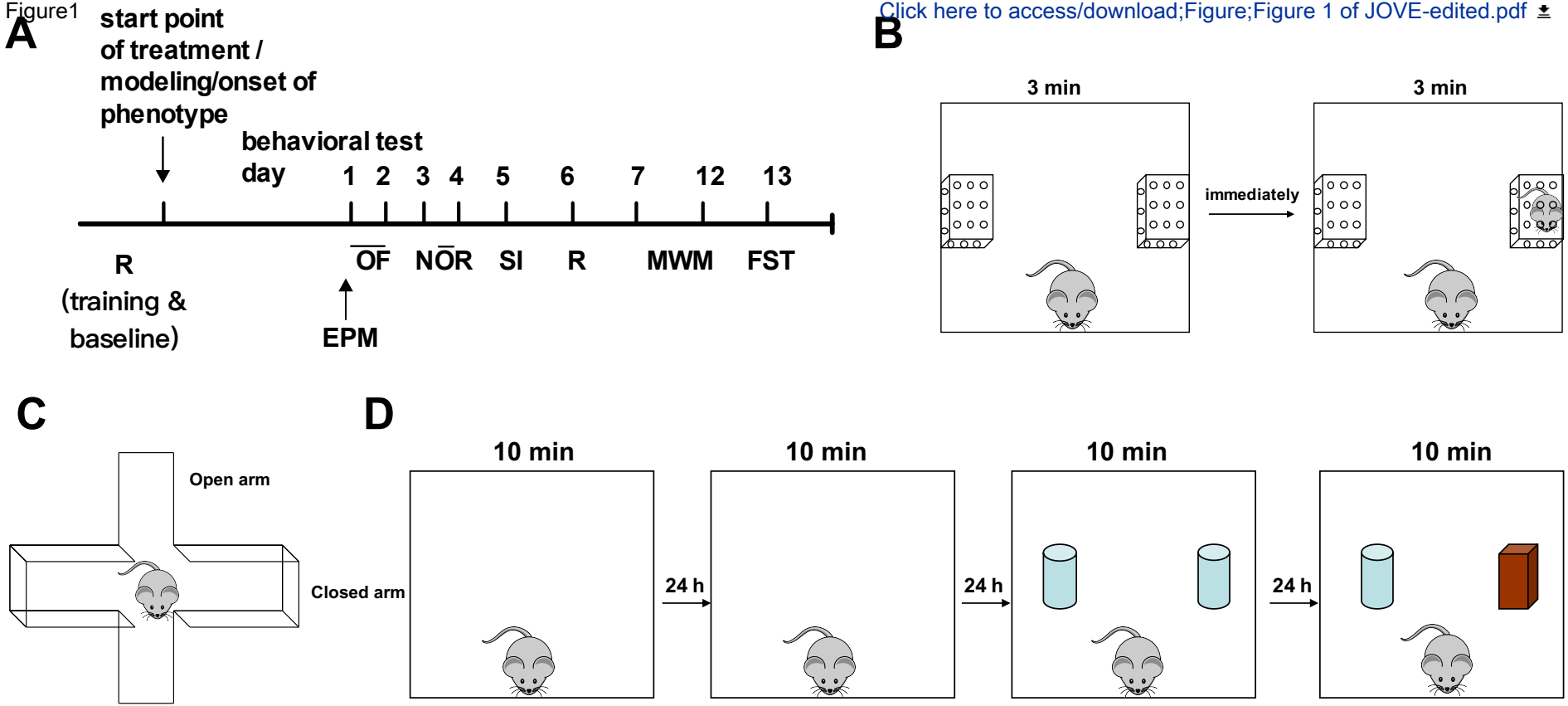
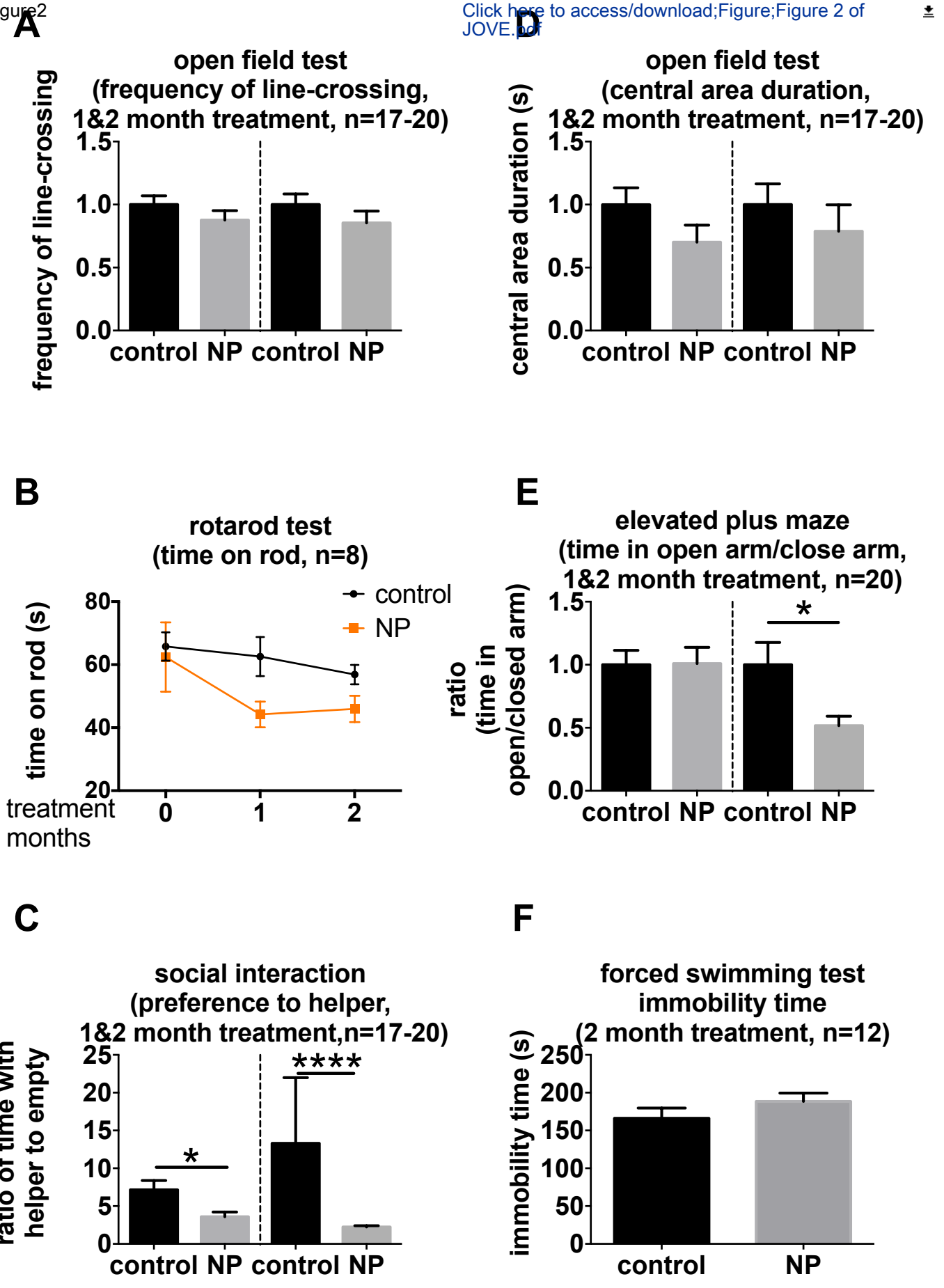
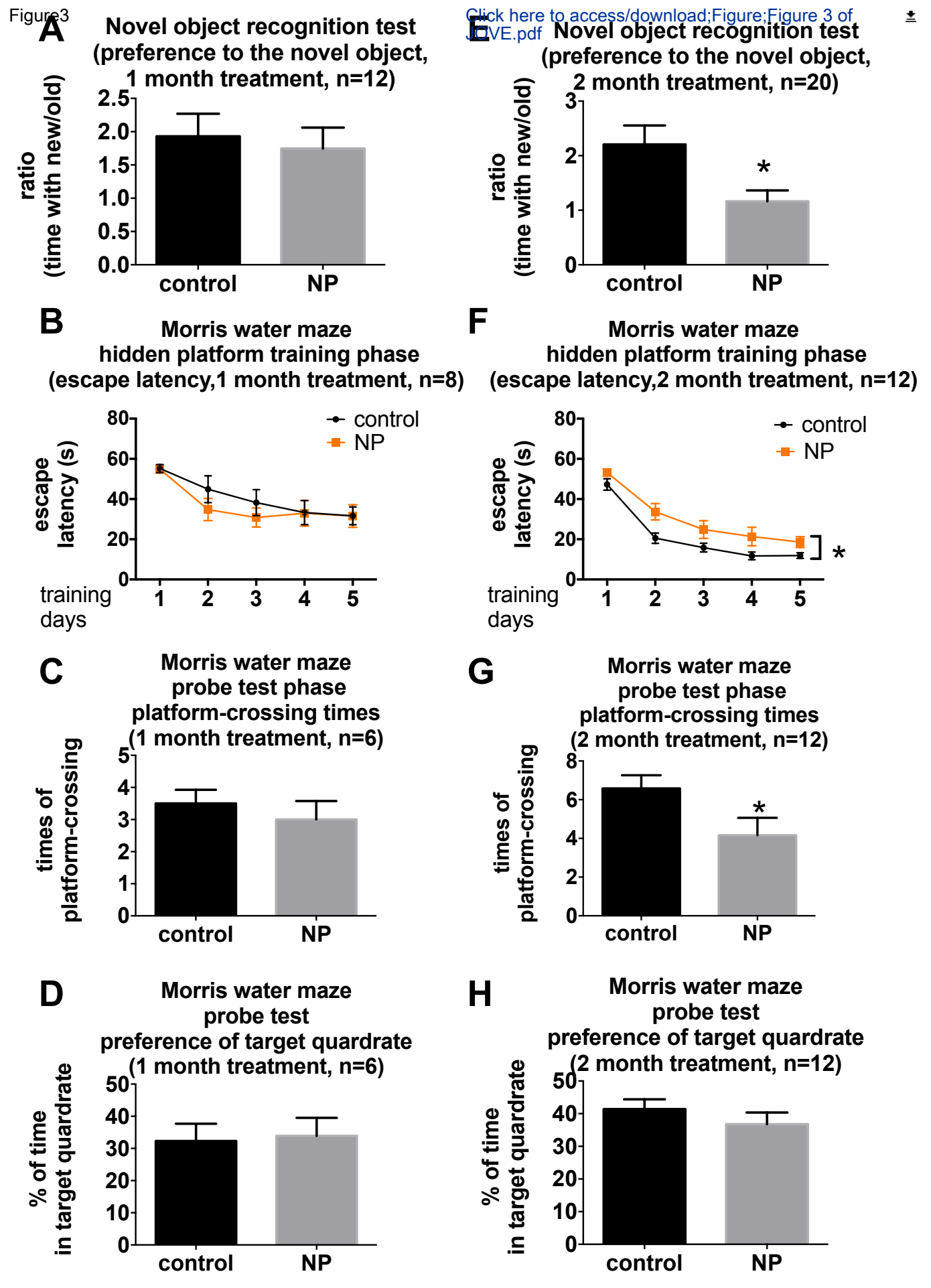
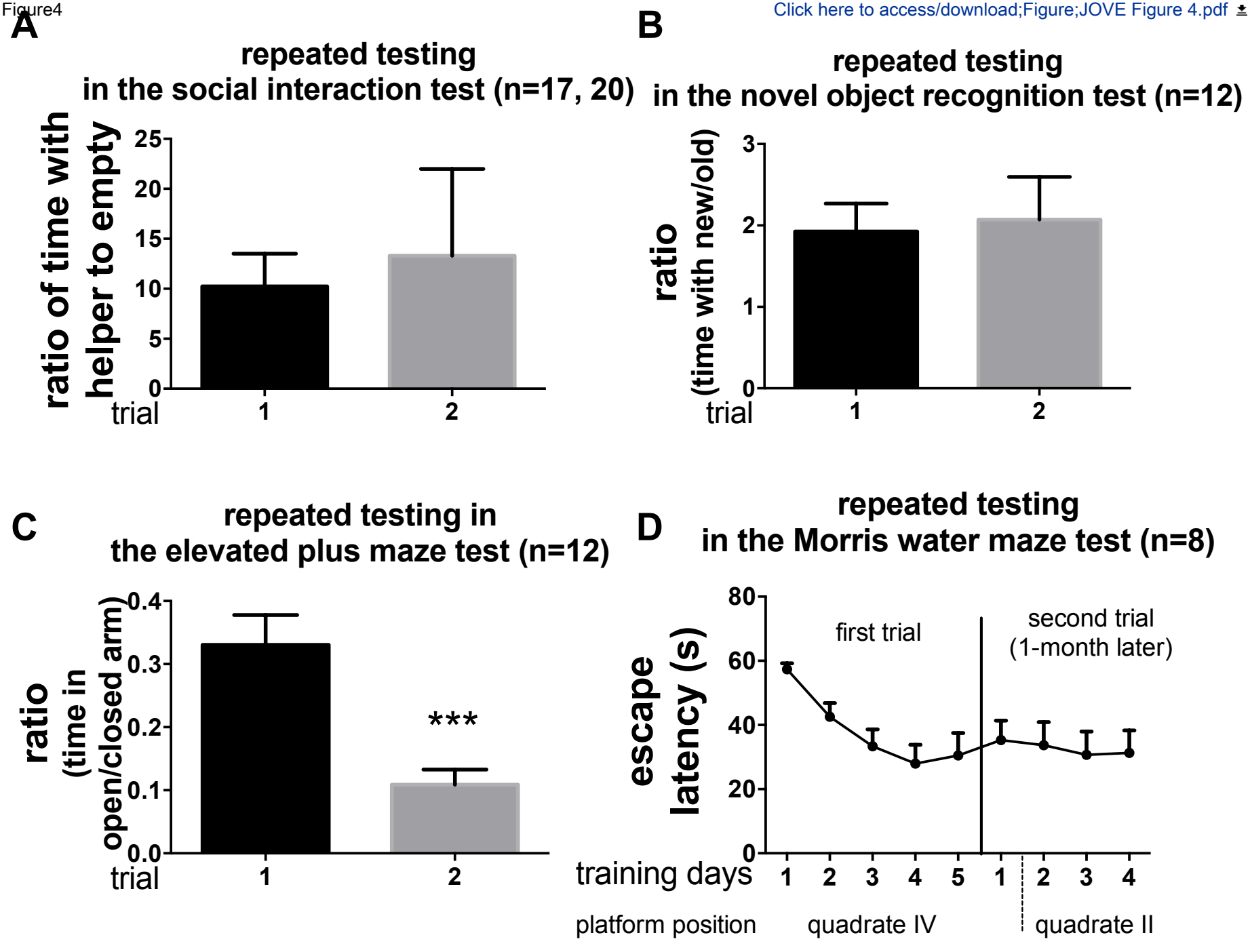
Figure1









Name of Material/ Equipment	Company
chambers in social interaction test	home made
cylindrical tanks used in forced swimming te	home made
elevated plus maze	home made
IITC Roto-Rod Apparatus	IITC life science Inc.
open field arena	home made
water maze	home made

Catalog Number	Comments/Description
	(8 cm (L) x 6 cm (W) x 12 cm (H)), transp
	30 cm height, 20 cm diameters, glass
	open arms (30 x 5 x 0.5 cm) ,closed arms
755, series 8	
	60 cm (L) x 60 cm (W) x 40 cm (H), plasti
	120 cm in diameter, 60 cm deep, steel

arant with holes, plastic

s (30 x 5 x 16 cm), center platform (5 x 5 x 0.5 cm), 40 cm tall. Plastic, nontransparant

c, nontransparant

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Line 1: The manuscript will benefit from thorough language revision as there numerous grammatical errors throughout. Please thoroughly review the manuscript and edit any errors preferably by a proficient English speaker.

We sincerely appreciate your efforts in editing our manuscript. We have revised the parts in the social interaction, the rotarod test and the novel object recognition test in response to your comments. Please be noted that the line number may be different with the trace of revision. We apologize for any potential inconvenience.

Line 76: What is the exact age and sex?

We have stated in the manuscript that the mice used were 3-month-old male C57BL/6N mice in line 76 – 77.

Line 105: Guidance of what?

We have stated in the manuscript that it was the guidance of the local animal unit.

Line 114: Administration of what?

We have stated in the manuscript that the administration of drug/toxin (e.g. intranasal instillation to silica nanoparticles).

Line 165: Basically a cross?

4 x 4 grid means a grid composed by 4 squares * 4 squares.

Line 171: What is the definition of central area here? Unclear.

The central area is the four squares in the center of the arena.

Line 178: Please remove or replace the commercial name. You can add it to the table of materials.

Thanks for the instruction. The commercial name has been removed.

Line 183 – 184: Treatment with what?

We have revised this part for clarity.

Line 187: Unclear what happens in each trial. Please describe. How long is each trial?

Thanks for pointing out this issue. Each trial starts with the rotation of the rod and ends with the drop of the mouse.

Line 194: Is this training or testing?

The protocol is the same on each day of training and on the day of testing.

Line 196: How long of a rest do you allow during repeats? Where is the mouse placed during the rest?

There is no rest between trials. The mouse stays on the static rod between trials.

Line 211: Was this mouse experienced in the other tests? How was the novel mouse handled before the social interaction test? Are they cage-mates? Are they litter-mates? Are they from the same group?

We have added this part from line 210 – 219. The novel mouse means it is novel to the subject mice. hence it is not a litter-mate nor cage-mate of the subject mice. It is not from the same group of the subject mice.

Line 223: I cannot understand this sentence, please revise.

We have edited this part (line 223 – 227).

Line 230: Different home cages?

Yes, in different home cages. We are sorry for the ambiguous expression and has revised it.

Line 240: There is no step 2.3.8, please revise.

We have revised this part in line 239 – 241.

Line 250: Unclear what IS meant.

We have revised the description of the elevated plus maze in line 248 -250.

Line 259 – 260: Define these terms please.

Thanks for the instruction. We have done so in the revised manuscript.

Line 269 -270: In the water?

Yes, in the water. We have clarified this point in this revision.

Line 275: Define this.

We have defined the mobility in the forced swim test in line 276 – 277.

Line 287: Unclear what is meant. Later you say the familiarization involves placing the novel objects in the field. Unclear what the difference between habituation and familiarization is. If the mice are familiar with the objects, how can you call them "novel objects"?

We have revised the expression in the protocol of the novel object recognition test for clarity. The mouse interacts with two identical objects (old objects) in familiarization. In the test, the mouse interacts with one of the old objects and a new object.

Line 303: Unclear which wall.

We have stated this point in the manuscript (line 303).

Line 312: What is meant here? How do the objects differ between the sets?

The difference of the two objects are described in the Note of 2.6.1..

Line 316: This terminology is not clear because it has not yet been defined clearly. What do A and B represent?

A and B represents two objects. Each set of objects has two identical ones, which is A & A and B & B. The mouse interacts with A & A in the familiarization so object A is the old object for this mouse. In the test, the mouse interacts with A & B so B is the novel object. We wanted to emphasize here that the choice of novel objects between A & B and the side where the novel objects appears should be counterbalanced within the group.

Line 324: Define tnew and told.

We have done so in line 319 – 320.

Line 329: Define the terms clearly. Which is the right and left side of the arena?

The right and left side is the right and left side of the experimenter when facing the arena in our study (line 317 - 318).

We have revised the typo in the protocol of Morris water maze test.

Line 353: Is it made opaque or just translucent?

We turned the water opaque with milk powder.

Line 354: What is done to habituate the mouse?

As described in 1.2.3., the mouse are habituated to the behavioral room for 15 to 30 min once done set-up.

Line 355: Is the infrared light in the maze? Why does the mouse need drying prior to swimming? Please clarify.

The infrared light is above the cages, hence is outside the maze. We turned on the light beforehand to habituate the mice to the lighting.

Line 376: How? Unclear what the experimenter does to guide the mouse.

As described in line 376 – 377, the experimenter places the mouse to stay on the platform to stay for 10 s.

Line 386 – 387: Does escape latency include the 10s spent on the platform?

The escape latency does not include the 10s on the platform.

Line 389 : What is escape latency plotted against?

We have clarified this point in line 389.

Line 396: 6^{th} day relative to what time point? 6^{th} day of MWM?

Yes, it was the 6^{th} day of MWM.

Line 399: Diagonally opposite?

Yes.

Line 406: As in 2.7.2.6?

In the probe test phase of the MWM, the parameter is the times of platform-crossing. So it is not the same in 2.7.2.6.

Line 408: Is this quantified? If so, how?

We have added this part in line 407 – 408.

Line 456: Please add a figure title here.

We have added the figure title accordingly.

Line 457: Please edit “Close arm” to “Closed arm” in the figure. Please edit “24h” to “24 h” in 3 instances in the figure.

Thanks for the instruction. The figure has been edited and uploaded.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread and please be noted that the entire manuscript has been extensively edited according to the reviewer's comments.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

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3. Please provide an email address for each author.

We are sorry for the negligence. The email address for co-authors has been added in line 19 – 21.

4. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

The Introduction section has now been expanded as required.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

The protocol has been revised as required.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

1.1: Please specify the size and setup of the behavioral room.

This issue has been addressed in Line 95 – 100 in the revised manuscript (1.1.1).

1.2.1: Where is the camera located?

This issue has been addressed in Line 102 in the revised manuscript (1.1.2).

1.3.1: Please specify the age, gender and strain of mouse. How many mice are kept in one unit/cage?

This issue has been addressed in Line 108 (1.2.1) in the revised manuscript.

1.3.4: What is used to clean?

The cleaning procedure is in 1.2.4., line 124 – 126.

2.1.6.2: Please describe how to divide the arena. Is this achieved by software? Please add more specific details (e.g. button clicks for software actions).

This part has been revised as advised in line 156 – 168 (2.1.4.).

2.2.1: A scheme showing the elevated plus maze and the starting position of the mouse would be helpful with the protocol.

The scheme is now in Figure 1C.

2.3.1, 2.4.1: Similarly, a scheme showing the test/chamber set up and positions of mouse and two objects would be helpful.

The scheme for the social interaction test and novel object recognition test is now in Figure 1 B & D, respectively.

2.5.2: Please specify the apparatus. Where is it placed?

The apparatus for rotarod test was a commercially available one, IITC Roto-Rod Apparatus. It is placed on the bench without direct bright lighting in the behavioral room. (section 2.2.2., line 176 – 178)

2.7.2: Where is the mouse placed in the apparatus, in the center or at the edge?

The mouse is put in the center of the tank for forced swim test (section 2.5.2., line 233).

7. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Thanks for the instruction. We have revised the protocol as required.

8. Please include single-line spaces between all paragraphs, headings, steps, etc.

Thanks for the instruction. We have revised the protocol as required.

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the steps for filming in the revised protocol.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Thanks for the reminding. We have highlighted as required.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Thanks for the reminding. We have highlighted as required.

12. Figure 2: Please split the figure into two figures. Please describe different panels in the figure legend.

Thanks for the instruction. We have made the revision as required.

13. Figures 2 and 3: Please define all error bars and asterisk symbols in the figure legend.

We are sorry for missing this information in the first edition of the manuscript. These information has been added in the revised manuscript.

14. Figure 3: What do the labels 1 and 2 refer to? Please explain. What do numbers 17 and 20 in panel A represent?

As shown in the figure, 1 and 2 means trial 1 and 2, which are the first trial in the naïve mice and when repeated tested in the experienced mice. We have explained it in the figure legend.

15. While the results present data of mice exposed to silica nanoparticles, the protocol

does not mention about this at all. Please consider including a brief description of exposing mice to silica nanoparticles in the protocol.

We appreciate your suggestion., we mentioned in 1.2.2. to perform administration, such as intranasal instillation of silica nanoparticles, post test in the revised manuscript.

16. Discussion: Please discuss any limitations of the technique.

We have added this part in the discussion, line 512 – 527.

17. References: Please do not abbreviate journal titles.

Thanks for the reminding. This issue has been taken care of.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

You and colleagues suggest a series of behavioral tests to measure behavioral changes in neurodegeneration.

Although they mention tests commonly used in Alzheimer's and Parkinson's studies, a better detailed discussion regarding other tests such as the Dark-Light box test to

measure anxiety or Sucrose Preference Test to measure anhedonic behavior are necessary. Particularly because these test are less invasive.

A point that catches the attention is that they indicate that the tests can be repeated. However, as one reads the manuscript, it realizes that there are aspects that the authors go through. For example, there are tests that are not as repeatable as the elevated plus maze, since the mice would lose the motivation for novelty (Figure 3C). Moreover, the authors make a comparison of the repeatability of the tests (FIG.3). And in 3 of the 4 tests (Fig. 3A, B and D) they do not find statistically significant differences. On the other hand the example that they use, the C57 mouse, apparently is not so good model for the Morris water maze because it is a rodent that is able to remember the location of the platform 1 month later (Fig. 3D).

Thanks for the insightful comments.

We have discussed and explained why choosing open field test and elevated plus maze test over dark/light box test in line 433 - 448. We used open field test and elevated plus maze test to analyze anxiety in mice. Open field test was initially used to describe emotionality in mice. The central area duration reveals the level of anxiety-like behavior. In addition, the open field test is also the habituation for the following tests in the battery. Elevated plus maze test is a well-established test for anxiety in mice. This test has excellent validity. Therefore, results in these two tests could be reference for each other. The principle of dark/light box test is similar to these tests, which is the

conflict between the curiosity to novel environment and the avoidance to aversive environment such as the open, elevated and / or illuminated environment in these tests. To increase the efficiency of the battery, we did not initially include the dark/light box test. Sucrose preference test is a well-known test for studying anhedonia in mice after expose to stress, such as social defeat, or unpredictable stress. Its protocol often requires days of habituation to individually housing, which may also be a stress stimulus. Adding sucrose preference test in the battery may further extend the experimental period. This part has been included in the discussion Line 464 – 467.

We highlighted the key points that can increase the repeatability of the test in the young adult C57Bl/6 mice. Open field test and rotarod test measure the motor function. When repeatedly tested every month, the performance of the normal young adult mice was quite stable. Social interaction test, novel object recognition test and the elevated plus maze test were motivated by novelty. Maintaining the novelty, which means using novel helper in the social interaction test, new pairs of objects in the novel object recognition test and novel novel behavioral room in the elevated plus maze test, greatly helped to increase the repeatability of the test. Figure 3A and B demonstrated stable performance in these tests when maintaining novelty; in contrary, Figure 3C showed significantly decreased exploration to the open field when performing the experiment in the same behavioral room even after 1 month.

Figure 3D showed that C57 mice cannot be repeatedly tested in the Morris water maze. Therefore, we used naïve mice each time when repeatedly tested.

Major Concerns:

Line 55-65. Authors referred to elevated plus maze test for anxiety, why they did not consider the dark-light box test.

We have addressed this issue in line 433 – 448 in the Discussion part and in the response to the reviewer's comments above.

If mice are nocturnal animal why the experiments were made during light cycle? The authors do not believe that this can influence behavioral tests. In what time of the day behavioral tests were conducted.

Thanks for bringing up this point. We performed all the handling and behavioral tests from 7 am to 7 pm. This arrangement was made according to Deacon. Nature Protocols. 2006. Doi:10.1038/nprot.2006.120., which recommended to conduct behavioral tests during the light cycle. This is because mice are easily roused during the day, especially when hungry or in novel environment (apparatus or novel social partner). If left unattended, they could go back to a light sleep. This feature allows the mice to accomplish the tests, hence the author stated that it was unnecessary to inverting the light-dark cycle during most of the behavioral tests. Considering tests in our battery all contains novel environment / apparatus / partner, or have motivation, we complied with the suggestion in this protocol.

Line 126: time in the central area of OF was an indicator of anxiety. Can the authors please give a more detailed explanation of why considered this point? Why they did not consider time in the corners as an indicator of anxiety

We used central area duration as an indicator of anxiety, following the protocol by Deacon. Nature Protocols. 2006. Open field test was initially developed to study emotionality of mice. Similar as other tests for anxiety, such as elevated plus maze test and dark/light box test, open field test exploits the motivational conflict between exploration of a novel area and aversion to open space. Anxious mice reduce exploration to the central zone and stay longer in the corners and near the walls. Noteworthy, studying anxiety with open field test relies on the locomotor, because measuring anxiety is confounded by activity of mouse. In our protocol, we introduce the mouse into the arena next to the wall. Therefore, staying in the central area had to be the consequence of movement / exploration. Based on this thought, we used central area duration to indicate anxiety-like behavior.

Why authors performed the Elevated plus maze test in the same day that the OF. Elevated plus maze measure anxiety, is it possible that animals are more anxious because they were submitted to OF. How long mice stay in the home cage after OF and before Elevated plus maze?

This arrangement was suggested in Walf A.A, et al,. Nat Protoc. 2013., which stated that performing elevated plus maze test after open field test could increase the exploration to the open arm. Therefore, we performed elevated plus maze test after all

the mice has finished open field test on the same day. The interval between these two tests ranges from 1 to 3 hours.

In the elevated plus maze test, the mouse faces the conflict between exploration of a novel area and aversion to an unknown, open and elevated space. The conflict is stronger than in the open field test, as the difference between open and closed space was much more drastic. As reviewed in literature (Walf A.A, et al,. Nat Protoc. 2013.), pre-exposure to a novel environment, such as open field, could be habituation of the elevated plus maze test, hence increase the likelihood of entering the open arms. The authors also conducted elevated plus maze test alone or following open field test, and did not find anxiogenic effect in the latter arrangement. Therefore, we also arranged the elevated plus maze test after open field test.

Why the authors no considered the Novel Local Recognition version as the second part of the Novel object recognition test (see Rivera et al. 2016, 2018).

Thanks for the suggestion. Morris water maze test and the novel object recognition test are included in the battery to study cognition. These two tests are distinct in the principle, protocol and motivation, which could test different cognitive domains. Novel local recognition test is the other version of the novel object recognition test. However, due to the limitation of time, we did not include novel local recognition in the test battery.

I think it is better calculated the preference of the novel object as tnew/(tnew + told).

See Rivera et al. 2016, 2018.

We agree that using tnew/(tnew+told) may be easier to be understood than discrimination index, whose calculation was shown in Leger M, Nat Protoc. 2013.. We have made the revision in line 267 – 269 accordingly.

In social interaction test, why authors used juvenile mouse as helper? Social interaction protocols indicate: for control mouse use a mouse of the same background, age, gender, and weight, without any prior contact (not littermates) with the subject mouse.

Thanks for the question. Social interaction test studies the sociability of the subject mouse by allowing it to freely interact with an imprisoned novel conspecific. The result can be easily interpreted as the social interaction is initiated by the subject mouse and the interaction is limited to odder and sound. Thus, this method prevents the aggressive behavior that may affect the social interaction behavior. Our protocol was the same as Poon DC, et al,. 2016; which are similar to Felix-Ortiz AC, et al,. 2014, J Neuroscience; Lin YT, et al,. 2018, J Neuroscience; Zhan Y, et al,. Nature Neuroscience. 2014. etc.. In these studies, the subject mouse was introduced to a novel juvenile conspecific of the same gender. Comparing to adult stranger, juvenile is smaller, which should be more attractive than adult same-sex conspecific. Moreover, juvenile is sexually immature. Therefore, the social interaction activity is unlikely to be affected by the sexual factor. Based on these thought, we used the protocol with juvenile helper.

When the authors explain the relevance results the explication of the tests was very

different from the order given in section 2. I suggest changing the order of behavioral test similar to the Representative result section. In addition, it was clearer if the cognition tests were explained together.

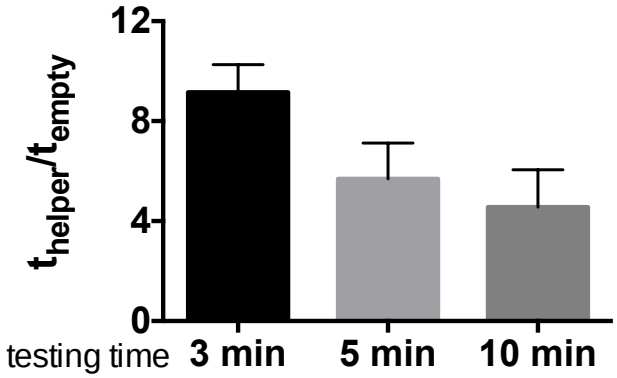
We sincerely appreciate your suggestion and have made the revision accordingly.

Social interaction test is a double test able to measure social affiliation and cognition (measured as short-term social memory). Why the authors did not consider measuring the social memory. In my opinion, measuring the social memory is biologically more relevant that measuring cognition with inanimate object such NOR

Thanks for the constructive suggestion. Social interaction test can be used to study sociability and social memory. We did not initially include social memory test to ensure our battery could cover different different behavioral domains with considerable throughput. Although social memory is only one step addition to the social interaction test, the results may be not as easy to interpret, as the behavior in the social memory test is likely affected by the sociability of the subject mouse. Moreover, this battery was designed for preliminary behavioral phenotyping, social memory test could be included in further analysis. Nevertheless, the biological importance of social memory is not ignorable, we have added your suggestion in the revised manuscript (line 518 – 519).

I do not understand why the protocol of social interaction test is shorter. Only 3 min. Please consider more time for interaction (10 to 20 min).

The protocol of social interaction test is adopted from Poon DC, et al,. 2015 and Felix-Ortiz AC, 2014, Journal of Neuroscience. Other studies, such as Lin TY, et al, 2018, Journal of Neuroscience; also used similar interaction time. According to our observation presented below, normal C57 mice showed higher preference to the helper at the beginning than the ending of the test (one-way ANOVA, $p < 0.05$). The explanation is quite simple, as the helper gradually losses novelty to the test mouse with the time goes by. Therefore, to increase the sensitivity of the test, we choose to use the protocols with less testing time. Nevertheless, we have included this point in the manuscript to thoroughly introduce social interaction test.



For the analysis of social interaction time, I suggest consider the Recognition index as the quotient of the time the mice spent with helper divided by the sum of the time spent with helper and the empty chamber. See Rivera et al. 2016 and 2018.

Thanks for the suggestion. We have made the revision in the manuscript (line 207 – 208) as required.

I strongly suggest that the authors review the protocol of the second part of social

interaction test (Rivera et al. 2016 and 2018 or https://www.jove.com/video/2473/assessment-of-social-interaction-behaviors).

Thanks for the constructive suggestion. We have added this part in the revised edition of our manuscript (line 512 – 521).

Fig. 2C there was a lot of variability in the control group during the 2sd month of treatment. An explanation could be that they used the same juvenile (line 176) and the control animals were no longer interested in interacting with a known animal?

Thanks for point it out. Indeed, the subject mouse may lose curiosity to the juvenile when met again. Being aware of this issue, we used different novel juvenile as helpers in trials at different time point. The same juvenile was used in the same trial as part of the parallel experimental condition and this is what we meant in line 176 (original manuscript). We have addressed this issue in the main text to avoid misunderstanding. The data variation was indeed quite a lot. However, this was due to individual difference as some mice in the control group was highly interested in the helper.

Fig 2D, why authors conclude that 2-months exposure to silica nanoparticles resulted in anxiety? I did not see any differences between control and NP groups.

We concluded that 2-months exposure to silica nanoparticles resulted in anxiety based on the results in elevated plus maze test (Figure 2E), because comparing to control mice, mice exposed to silica nanoparticles for 2 months showed significantly less exploration to the open arm in the elevated plus maze test. Figure 2D was the central

area duration in the open field test, which was used as an indicator of anxiety and showed similar trend of reduced central area exploration in the nanoparticle-exposed mice. We are sorry for the misleading expression and has made revision in the main content (line 352 – 354).

The authors give a quite detailed explanation of the repeatability of the tests, in figures 3A and 3B, there are no statistical differences. Then, I do not understand the relevance of these results.

Figure 3A and 3B showed that the performance of the same group of mice did not vary when repeatedly tested in social interaction test and the novel object recognition test, respectively, indicating these two tests can be repeatedly tested.

According to the explanation of the repeatability in the elevated plus maze, it is possible that the differences detected in fig. 2E are due to the animals losing the desire to explore an environment that they already know?

Thanks for the question. Figure 2E demonstrated decreased exploration to the open arm in the nanoparticles-exposed mice comparing to the control mice. Mice in both treatment groups were tested in parallel and the data was normalized to control group at each time point. Therefore, the difference was not likely due to the experimental protocol.

Legend of fig 2 means that for the Morris water maze the animals used during month 1

were different from animals used in month 2?

Yes, different animals were used in Morris water maze test in different time point.

Age, sex, species, and strain differences influence MWM performance, a more detailed explanation in the legend of the figures is necessary to understand the context of the experiment (perhaps in the legend of Fig. 1)

Thank you for the instruction. The information has been added in the revised manuscript (line 108).

Minor Concerns:

Line 74: ….is correct: ….in the open field arena in the open field?

Thanks for the correction. We have made the revision accordingly.

Line 87: 15 to 30 min for habituate animals to the experimental environment? There is some way to prove that the habituation in the experimental room of 15-30 min is enough?

When placed in a novel environment, mice tend to explore for a while, and then reduce the exploration. Such reduction of movement and exploration is defined as habituation. According to Deacon RMJ. Nature Protocols. 2006., habituation after transportation is recommended to be 5 to 30 min. We give it 15 to 30 min for habituation, which normally meaning that the experimenter leaves the room until all

mice are settled down in the home cage without climbing up and down.

Is it possible that authors calculate the speed and total distance traveled in the OF arena?

Thanks for the suggestion. We adopted the open field test from Deacon RMJ. Nature Protocols. 2006., which counted number of squares entered as total distance traveled. Nowadays, the total distance traveled and speed can be analyzed by several software. We have introduced SMART in the main text (2.1.4.3. line 167 – 168).

Line 133: What are the differences between point 2.1.2 and point 2.1.4?

Thanks for the reminder. We have revised these points.

Line 137: The authors wanted to say 2.1.2

Thanks for the reminder. We have made the correction accordingly.

In Novel object recognition test, why the authors did not consider measure familiarization time with the objects as a way to control the test

Thanks for the suggestion. Familiarization time is a useful parameter. The total time reveals the exploration of the mouse, the ratio between time exploring left and right objects reveals the spatial bias in the behavioral room. We have added this point in the protocol (line 271 - 272).

What happens with the negative values that the discrimination index can throw?

The negative discrimination index indicates a prone to the old object, which could be neophobia that comes with anxiety.

In line 191: what means before and after treatment?

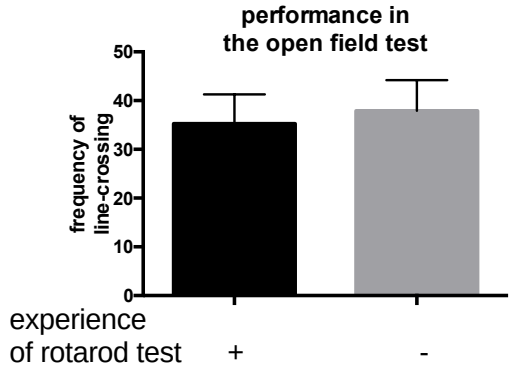
We apologize for the obscure expression. In our study published on Particle and Fibre Toxicology, we arranged the training of the rotarod test before the treatment of silica nanoparticles so that the baseline of the mice in the test was the same. If our reader wanted to test motor function with this battery, we suggest to arrange the rotarod training before the treatment/onset of disease/modeling as shown in the protocol.

In Figure 1 rotarod test was made before OF Is it correct? How the authors discard any possible bias in the open field if the rotarod test is done before?

Rotarod test contains motor learning and motor function test. Naïve mouse learns how to focus on the test, balance itself on the accelerating rod and stay on as long as possible. In the current battery, we aim to study motor function but not motor learning. Therefore, we suggested in Figure 1 to do the rotarod test training in the naïve mice before treatment / the onset of phenotype to ensure that all the mice have learnt the skill.

In our study about silica nanoparticles that published in *Particles and Fibre Toxicology*, we trained the mice in the rotarod test, start the exposure and then do the open field

test. Therefore, the interval between the rotarod test and the open field test was 3 weeks. We studied the performance of mice with or without experience in the rotarod test in the open field test. The results shown below indicated that the locomotor activity in the open field test was not affected by the rotarod test.



In line 200, can the authors explain better why the third day of training is taken as a baseline of motor function?

The first trial of rotarod test, which was conducted before treatment or onset of disease, trains the mice to acquire motor learning. Mice learnt the skill of staying on the rod for longer time in the first two days and the performance on the third day normally reach the plateau. Therefore, we used the third day as the baseline of the motor function.

Line 207: What kind of cues the authors used. Please explain

The cue was visual cues, which were circle, square, pentagon, and triangle (line 281).

Line 218: how long is the room habituation before begging the test?

As stated in 1.3.4., the room habituation is 15 to 30 min. We have specified in the revised manuscript (line 292).

How long after the other tests the Morris water maze is performed

Morris water maze test was performed 24 hours after other tests.

Line 233: how much time the animal rests between sessions in the same day. Only one trial per animal during probe phase for Morris water maze

The mice usually gets at least 30 min rest between sessions.

Authors proposed rotarod test only for Parkinson disease or also for AD, What is the logic to consider this test under a scenario of Alzheimer's disease?

When the animal model is known to manifest AD symptom, which is featured as cognitive impairment, cognitive tests such as Morris water maze test and novel object recognition test should be used in priority. However, when developing a new mice model or studying the neurotoxicity of a toxin that may potentially induce neurodegeneration, comprehensive behavioral analysis would provide more information. In this scenario, studying motor function with rotarod test can help the interpretation of tests that requires motor function.

Reviewer #2:

Manuscript Summary:

The manuscript describes a battery of tests to assess motor function, cognitive function and 'mood' in rodents. The investigators have put forth a behavioral assessment battery that has three key features to positively impact progress:

1. Well Supported. The tests used and methods detailed are well supported across a range of disciplines and their incorporation into a visualized experiment will be an important contribution to several scientific fields (neuroscience, neurobehavior, drug development and preclinical treatment screening, etc).

2. Translational / Clinically Meaningful. The selected metrics have face validity as comparative assays of several clinically relevant functional subdomains essential to maintain independence and quality of life in humans (see NIH Toolbox).

3. Economy of time and resources. These tests are not overly stressful, non-invasive, easily repeatable. This supports the feasibility and cost of time/effort for both animals and investigators.

Major Concerns:

1. Introduction implies test battery designed to detect impairments that accompany AD or PD and yet all example data and discussion context center only on short term treatment with nanoparticles in young C57BL6 mice. Test performance changes with age

and neurological conditions. This is acknowledged in discussion (pg 8 of 10, lines 384-390) with statement that "optimization is required for behavioral assessment in these models," but the introduction is misleading. Please revise.

Thank you very much for the instruction. We have made the correction accordingly in the Introduction part.

2. Statistics: the only statistical test mentioned are for unpaired t-tests. This a a major limitation. Repeat analysis requires use of 2-way Repeated Measures ANOVAs at minimum. The battery proposed multiple tests with multiple outcomes and hypotheses being tested simultaneously. This would involve multiple comparisons, and a correction factor should be applied, or multivariate analysis models (strongly suggested).

We used repeated measures 2-way ANOVA when analyzing the training of Morris water maze test and the rotarod test, as demonstrated in the figure legend. Utilizing ANOVA would be of great value when studying the time course of the behavioral changes in mice. Although we highlighted points that improves the stability of the performance of control mice in the tests, there are variants that cannot be controlled in each trial. Firstly, the status of the mice cannot be the same. Hence the data in the open field test and elevated plus maze test, both requires spontaneous locomotion, could only be compared with the control group tested at that time. Secondly, the outcome of other tests also depends on the experimental condition. For example, in social interaction test, each trial requires a novel helper. It would be difficult to control the individual difference of the helpers used in different trial. Based on these factors,

we did not compare changes in different time point in these tests.

3. Please include statement about animal handlers and training. Animal behavior is sensitive to investigator. When possible, the same animal handler or investigator should perform longitudinal behavioral assessments. At minimum, attention to intra- and inter-rater reliability during protocol training should be adhered to (Intraclass correlation coefficient, ICC, >0.80 between raters when possible)

Thank you for the reminding. We were aware that proper handling is critical for the outcome of behavioral tests. Hence the mice were handled by the same experimenter during the entire study. We have added this point in the protocol as advised (line 112).

4. Assessment battery designed for repeat testing, but proposed frequency of testing should be stated, and interpretation of change (or no change) over time warrants discussion. Authors acknowledge this in results section (lines 289-290: "Other tests cannot be repeatedly tested in the same group of mice, as the experience greatly interfere the performance."). Please provide a recommended testing frequency or limits (basement/ceiling effects) for each test in the assessment battery. Provide evidence when available.

Thanks for the instruction to improve our manuscript. We have made the suggestion as required in the Discussion section.

Minor Concerns:

1. Introduction: paragraph 1, line 41. "…largely unknown and associated with environmental factors or toxins." Recommend to strike extraneous text after "unknown."

Thanks for the advice. We have revised this sentence accordingly (line 51).

2. Introduction: paragraph 2, line 51-52 "Results of these two tests…" Sentence unclear and unnecessary introduction (but appropriately addressed in discussion). Please omit sentence from introduction.

Thanks for the suggestion. We have deleted this sentence from introduction in the revised manuscript.

3. Introduction: paragraph 2. Include references for open statements about symptoms and specific impairments in etiology and progression of disease (e.g. references needed for: "Cognitive domains including short-term memory and episodic memory are most susceptible to neurodegeneration.")

Thanks for the constructive advice. We have included the references as required.

4. Introduction, paragraph 2, lines 64-65. Last sentence can be omitted.

Thanks for the instruction. We have deleted this sentence as suggested.

5. Methods, 1.3.1, line 103. Omit "Do not delete for no good reason."

Thanks for the instruction. We have deleted this sentence as suggested.

6. Methods, 1.3.1, line 104-105. Add references or specific example to statement: "Tail suspension test is more stressful than forced swimming test."

Thanks for the instruction. We have made the correction in this sentence.

7. Methods, 2.1, Open Field. Camera recording should also detect total distance traveled, speed of movement, and time spent not moving (freezing behavior). These measures may be more sensitive to detect change over time and with intervention. Please expand this section and provide data to support use of 'line crossing' vs. these other standard open field test outcomes.

We appreciate your constructive advice. Open field test can be used to analyze locomotor function, anxiety, spontaneous activity, and even memory etc.. Observation in open field test is valuable when at the beginning of investigating a new treatment or mutation because the open field test can show whether behavior is within normal limits. A mouse that does not move for several minutes in the open field is unlikely to be worth testing in more complex behavioral tests. Therefore, locomotor is studied in the battery. Using 'line crossing' or 'squares entering' to indicate locomotor function is described in Deacon. Nature Protocols. 2006., which divided the open field arena into small squares and the number of square entering or line crossing reveals how much the mouse moves in the arena, i.e. the locomotor function. This method is important for some non-behavioral laboratory as analysis software is costly. Other parameters, such as freezing, is also important, hence is added in the revised protocol as suggested (line 156 – 168).

8. Open field and Elevated plus maze are tested on the same day. Please state time interval between tests.

The elevated plus maze test is tested after all the mice has been finished testing in the open field test (line 213 – 214). Depending on the number of the mice and arena, the interval between these two tests was 1 to 2 hours in my experience.

As the experimental set-up, the configuration of the apparatus, the lighting, and sometimes even behavioral room, is different among these two tests, it was unlikely that the elevated plus maze test could be interfered by the open field test. Moreover, exploration in the open field test has been suggested to increase the open arm exploration if elevated plus maze test is performed afterwards. (Walf AA, et al,. Nat Protoc. 2007)

9. Results, paragraph 2, pg 6 of 10, lines 285-284. Please revise sentence "The data showed that normal mice consistently had 2 folds of the preference to the novel object comparing to the old object (Figure 3B)." Statement meaning unclear.

Thanks for the instruction. We have revised this sentence (line 368 – 369).

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To whom it may concern,

I am an author who recently published my work in your journal "Particles and Fibre Toxicology", entitled " Silica nanoparticles

induce neurodegeneration-like changes in behavior, neuropathology, and affect synapse through MAPK activation".

I was approached by the editor of Journal of Visualized Experiment to write about the methods I used in this paper. I chose to introduce the behavioral tests as visualizing the procedure would be most helpful to the community to repeat. I am now writing to ask for your permission to allow me to present the behavioral test data in this brand new article. The data will be used as representative data to demonstrate the usage and efficacy of the method, and will be properly cited.

Please let me know what material should I prepare and how long it will take. I am looking forward to your response.

Thank you very much.

Best regards,
Ran You