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Using the tube test to measure social hierarchy in mice

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Investigation of the neural mechanisms underlying social hierarchy requires a reliable and effective behavioral test. The tube test is a simple and robust behavioral assay that we recently validated as a reliable measure of social hierarchy in mice. The test was demonstrated to produce results largely consistent with the results seen when using other dominance measures, including the warm spot test, territory urine marking or the courtship ultrasound vocalization test. Here, we describe a step-by-step procedure to use the tube test to measure dominance within a cage of four male C57/BL6 mice as an example application. The procedure comprises three stages: habituation, training to pass through the tube, and the tube test itself. The social rank of each mouse is determined by the number of wins it gains when competing against the other three cagemates. A stable rank is derived when all mice maintain the same ranking for 4 consecutive days. The time required to acquire a stable rank usually varies from 4 to 14 d. An additional 5 d is required for habituation and training.

Introduction

Social hierarchy is a fundamental self-organizing scheme in most animal societies by which group members maintain relatively dominant or subordinate statuses to determine access to resources[1–4]. As one of the most robust forms of animal behavior, social hierarchy has a profound impact on individuals' survival, reproductive success, health and other behaviors[3,5–8]. Indeed, dominance, together with the level of arousal, and positive versus negative affect, is considered to be one of the three major dimensions that describes all emotions[9–11]. A stable hierarchy is also essential in minimizing unnecessary fights among group members[12]. Despite its importance to societies and individuals, understanding of the mechanisms underlying social hierarchy at the neural, behavioral and cognitive level is limited. To investigate the mechanisms underlying social hierarchy, a robust and reliable behavioral assay is essential.

Social dominance is operationally defined as consistent winning in social conflicts when incompatible motivational priorities exist among individuals[13–15]. On the basis of this definition, several behavioral measures have been developed in the laboratory setting to measure the social dominance relationship. One such assay, the tube test, is based on an ecologically relevant resource, use of space. It models a right-of-way type of confrontation, which is common in the wild. After being trained to walk forward through a narrow tube, two mice are set to meet in the middle of the tube[16,17]. Because the tube allows only one mouse to pass at a time, this creates incompatible motivational priorities, and one of these two mice has to give way to the other. This protocol describes how to use this assay to measure social dominance.

Other behavioral assays are also available. In the food competition test, which has been used in rodents[18,19], chickens[20] and pigs[21], dominance is indicated by food consumed or weight change when highly appetitive food is provided or when food is restricted[22,23]. In the water competition test, a pair of water-deprived rats[19,23] or mice[24] competes for a single and limited source of water[23,25]. In the recently developed warm spot test[26,27], which utilizes animals' natural desire to stay warm, four cagemate mice compete for a small warm corner in an ice-cold cage. Moreover, territory urine marking[28,29], allogrooming[30–33], ultrasonic courtship vocalization[34–36], and offensive and defensive behaviors in a large vivarium[37] have also been used to determine dominance.

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Development and overview of tube test

The tube test was first established to evaluate dominance tendencies among different mouse strains half a century ago[16] and was afterward used to score the phenotype of genetically modified mouse lines[38–40]. In the tube test, two mice enter a narrow tube from opposite ends and meet in the middle. The mouse that forces the opponent out of its way is designated as the 'winner' and dominant. The one that retreats out of the tube first is designated as the 'loser' and subordinate.

We recently applied the tube test to cagemates for the first time and validated the tube test as a reliable measure of social hierarchy in C57BL/6J cagemate mice on the basis of its transitivity, stability and consistency with several other dominance measures[17]. Specifically, for transitivity, we found that if mouse A beats mouse B and mouse B beats mouse C, then during 95% of tests ($n = 264$) mouse A will beat mouse C. For stability, we found that 86% ($n = 162$) of any two mice maintain their relative tube test rank on 2 consecutive days, and for any four-mouse cage, 59% ($n = 216$) of cages maintain identical ranks to those seen on the previous day. For consistency with other dominance measures, we found that dominant mice in the tube test also tend to show less weight reduction in the visible burrow system, display more agonistic behavior in a new cage, make more urine marks and produce more ultrasonic vocalization toward females[17]. Among the seven cages showing a barber-like phenomenon, six barber mice had the top rank in the tube test[17]. Other studies have also reported the correlation of tube test dominance with barber behavior[31,32,40], urine marking[41] and warm spot competition[26]. The tube test can also be subjected to video analysis of detailed behaviors. By quantifying voluntary behavior (e.g., push initiation) and coping response (pushback, resistance and retreat), one can gain insights into the potential internal states of mice. Thus, the tube test appears to be simple, robust and reliable, permitting detailed quantification of dominance-related behaviors[42].

In an older version of the tube test[16], the apparatus and procedure are relatively complicated. The earlier apparatus included one tube, two goal boxes attached at both ends of the tube and three doors, two at the entrances connecting to the goal boxes and one in the middle of the tube. Before the tube test, mice were deprived of food to maintain 85% of normal weight. In the training and testing stages, mice were given a food reward in the goal box after passing through the tube and forcing the opponent out[16]. We simplified the apparatus, using only a transparent plastic tube without goal boxes, doors or dividers (unless these were required for a specific application such as calcium imaging, see 'Modifications of the tube test')[17]. We compared tube test results in the presence and absence of food deprivation and food reward and found no substantial difference in transitivity or stability. We therefore simplified the procedure, removing the food-deprivation and reward steps, and thus eliminating food reward as a confounder in the behavioral outcome[17].

Advantages and limitations of the tube test

The tube test has several advantages over other methods measuring social dominance. First, compared with assays based on scoring offensive and defensive behaviors, or food and water competition[23,37], which often require a large and complicated vivarium, hours of videotaping, animal tracking and behavior quantification, the tube test uses easy procedures and scoring, and requires only simple equipment (a plastic tube). Second, unlike the social defeat, resident intruder or other aggression-based assays, the tube test does not involve vicious attacks and minimizes physical injuries. Third, tube test results are robust and highly penetrant: almost every mouse can obtain a tube test rank. This is in contrast to the barber test, in which the occurrence of a barber is low, or the ultrasound vocalization test, in which not every mouse would produce ultrasound.

Clearly, few animal models can fully model the situation seen in the wild or in human society, and all have some limitations and caveats. The tube test also has its limitations. First, in wild-type mice, many factors, such as age, weight and basal stress level, can affect the results of the tube test. Indeed, the effect of stress when undertaking the tube test is itself a topic that is worth studying[43,44]. To minimize contributions from these confounding factors, mouse age and weight should be matched, and acute stress should be minimized through habituation and training procedures. Second, in mutant or manipulated mice, defects in social recognition, social memory and locomotion can also affect the tube test results. Necessary control experiments must be performed before conclusions about social dominance can be made. Given that each individual behavioral assay can be affected by multiple factors, we strongly suggest using more than two dominance measures, for example, by also carrying out the warm spot test, or territory urine marking or ultrasonic courtship vocalization assays,

in addition to the tube test. The additional assay used should be based on different sensory or motor properties to increase confidence in the conclusions reached regarding social dominance. Third, care must be taken when extending the tube test as a dominance measure to other mouse strains or rodent species. Although a good correlation between the tube test and other dominance measures has been established in C57/BL6 mice[17,26,31–33,35,40,41] and Lister Hooded rats[22], when comparing dominance tendencies among different mouse or rat strains, some earlier studies reported that the strains that advance in the tube test do not tend to dominate in tests of food competition or fighting[45,46]. In these studies, dominance relationships were compared across different strains of mice or rats. It may be debatable whether it is more appropriate to test dominance among individuals within the same strain, which naturally form a social structure. Nevertheless, when using the tube test for a new strain, gender or rodent species, it is highly recommended that correlation between the results of tube test and other dominance measures be examined first. Fourth, attention also needs to be paid to how animals win in the tube test, in addition to the simple outcome of winning or losing. An earlier study showed that administration of marijuana extract Δ^9-trans-tetrahydrocannabinol (THC) to rats increased winning in the tube test[47,48], but that the THC-treated rat tended to 'freeze' in the tube until its opponent eventually retreated. Quantification of push, resistance and retreat behaviors should be used to exclude this kind of 'winning'.

Applications of the tube test

As a simple and robust assay for social dominance, the tube test allows the investigation of the neural mechanisms underlying social hierarchy, including the neural circuit basis[17,43,49], hormonal regulation[50], the dynamics of hierarchical structure[26,51], and the contribution from internal states and external factors[26]. Furthermore, because dominance is one of the strongest factors affecting animal behaviors, the tube test also provides an opportunity to examine the relationship between social status and various behaviors such as reward motivation, addiction[52], learning and memory, territorial behavior[41], social interaction[53,54] and vulnerability to depression[43]. Finally, the tube test has also been used to investigate social dominance in mouse models of neuropsychiatric disorders such as schizophrenia[40], fragile X syndrome[55], major depression[56] and autism spectrum disorder[57]. In summary, the tube test provides a unique opportunity for researchers who are interested in exploring the neural mechanisms of social hierarchy and studying various behaviors and phenomena related to social hierarchy.

So far, the tube test has been applied to several different species. We have conducted tube tests in the adult (8–14 weeks old) male C57BL/6J strain and found good correlations with other dominance measures[17]. Other studies have used adult (10–12 weeks old, 30–35 g) male mice of the ICR strain[50], adult[58] and juvenile[59] male rats of the Sprague–Dawley strain, and adult (11–13.5 weeks old) prairie voles of both sexes[52]. Some of these studies have demonstrated a correlation between the results of the tube test and other dominance measures such as the resource competition task among outbred male Lister Hooded rats[22].

Experimental design

Our protocol for measuring social hierarchy consists of three main stages: habituation (3 d), training (2 d) and testing (at least 4 d). In the habituation stage, mice are handled to reduce stress. In the training stage, mice are trained to walk forward through the tube from both ends of the tube. In the testing stage, mice are tested in a pair-wise fashion in the tube. Tests are conducted among cagemates using a round-robin design, and mouse rank is assessed by the number of times a particular mouse wins[17]. The rank is considered stable when all mice maintain the same ranking for 4 consecutive days.

Habituation (Step 1)

The habituation stage aims to reduce stress and anxiety when mice are handled and exposed to the tube. In our lab, mice are imported from external providers and travel time is ~6 h on the road. We recommend that mice be group housed and allowed to rest for at least 7 d in the animal facility before habituation starts. Handling helps to familiarize mice with the experimenters, which takes 1–2 min per mouse per day. For pre-exposure, we put a short habituation tube into the home cage to familiarize mice with the tube. Handling and placement of the habituation tube can start 3 d before the tube test.

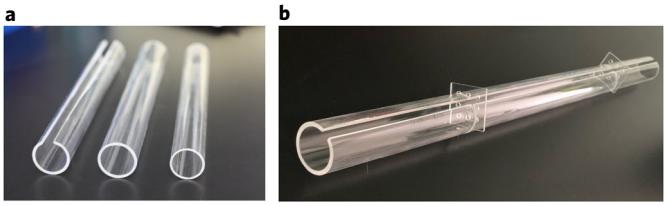

Fig. 1 | Tubes. a, A tube with a 15-mm slit for optogenetics and tetrode recording experiments (left); a tube with an inside diameter of 30 mm, for testing adult C57BL/6J males (middle); and a tube with an inside diameter of 26 mm, for testing adult C57BL/6J females (right). The tubes mentioned above are 30 cm long. **b**, A 60-cm-long tube for calcium imaging experiments; it has a slit opening at the top and two perforated sliding gates near the entrances. All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at Zhejiang University.

Training (Steps 2–11)

The training stage (Supplementary Video 1) aims to familiarize the mice with the test procedure and environment. It is important for the mice to know the consequence of walking through the tube and that the other end of the tube is safe. All mice should go through the tube ten times per day, five times from each side. Training takes 2 d, 10–15 min per mouse each day.

Testing (Steps 12–19)

In the testing stage (Supplementary Video 2), mice are tested in a pair-wise fashion in the tube for at least 4 d. Each trial of the tube test usually takes <30 s. In rare cases, neither mouse retreats within 2 min; in such cases, the tube test should be stopped (see Troubleshooting section). After each trial, the mice are put back into the home cage and left to rest with their cagemates for 2 min before starting the next trial, in order to reduce the potential immediate impact of recent winning or losing. A round-robin design is used to randomize the test order. It takes ~20 min to conduct tube tests for a cage of four mice each day. The mouse rank is assessed by the number of wins against the other three cagemates. We consider a rank stable if all four cagemate mice maintain the same ranking for 4 consecutive days, based on our following observation: 98.4% (124 out of 126 pairs from 21 cages) of mouse pairs showed the same rank on the 5th day after maintaining a stable ranking for 4 consecutive days.

Video analysis of behavioral details (Step 20)

A video device can be used to record the whole test procedure (for example, Supplementary Video 3) and precisely capture specific behavioral epochs in the tube test. By undertaking frame-by-frame analysis, it is possible to classify behaviors into push initiation, pushback, resistance, retreat or stillness (see definition of each behavior in the Procedure). For example, after analyzing videos of multiple tube test trials, Zhou et al.[26] found that natural winner mice, or mice receiving optogenetic stimulation in the dorsal medial prefrontal cortex (dmPFC), are more likely to initiate pushes with longer duration and generate more pushbacks and resistance, demonstrating more effortful behaviors than loser mice or unstimulated mice.

Modifications of the tube test

The tube test can be modified. First, the size of the tube can be modified. The standard size is ~30 cm in length, with a 3-cm internal diameter (Fig. 1a), which is sufficient to permit only one male adult C57BL/6J mouse to pass through smoothly without reversing direction or crawling over the opponent mouse. Tubes with suitable internal diameter should be chosen on the basis of body size: 3 cm for testing adult C57BL/6J males; 2.6 cm for adult C57BL/6J females, which are smaller than male mice; and 4 cm for CD1 adult male mice, which are larger than C57BL/6J mice (Fig. 1a). If needed (e.g., to increase behavioral epochs per trial for imaging or recording purposes), the length of the tube can be increased to 60 cm, which may allow more and longer pushes and retreats. When conducting the tube test on rats, some groups used a tube 1.5 m in length, with an inside diameter of 60 cm, for adult male rats[58]; others used a tube 45 cm in length, with an inside diameter of 4 cm, for juveniles[59].

In addition, a slit can be opened on the tube to allow optogenetic manipulation or in vivo electrophysiology recording or calcium imaging during the tube test in real time. The width of the slit can

Box 1 | Optogenetics application ● Timing 3d

Procedure

▲ **CRITICAL** The tube test can also be modified to accommodate optogenetic experiments.

1 After a viral injection of channelrhodopsin-2 and fiber implantation into the brain region of interest, let the mice recover for 3–4 weeks. This time delay enables channelrhodopsin-2 to be fully expressed.

2 Use a tube with a 15-mm slit opening at the top of the tube to allow mice with optic fibers to pass through the tube smoothly. Set up the recording device when needed.

3 Following the same procedure as for a regular tube test, train the mice for 2 d and conduct the test once daily until ranks are stable for at least 4 consecutive test days.

4 To accommodate mice performing the tests with optic fibers, test mice with a dummy fiber attached to the head for 1–2 d. Ensure that the ranks remain unchanged.
? TROUBLESHOOTING

5 For the test, attach an optic fiber to the mouse chosen for manipulation. Attach dummy fibers to the other three mice. Allow the mice to accommodate to their surroundings for several minutes in their home cage. Prevent mice from biting the fibers attached to other mice. Conduct the six-trial tube tests once without light to ensure that the ranks remain the same.

6 Repeat the test with light stimulation delivered to the chosen mouse right before it enters the tube, and observe potential behavioral changes.
▲ **CRITICAL STEP** Choose rank 1–4 mice in the same cage to compete with each other in random order. Each manipulated mouse should be allowed to compete against cagemates with the closest rank first and then against those with more distant ranks. For each trial, we use minimum light stimulation at the beginning and gradually dial up to maximum intensity if the rank does not change. Once the rank changes, we switch the mice to the other end of the tube and repeat the process. Mice have to win or lose two out of three tube tests from both sides of the tube. Only then can we conclude that light stimulation of this intensity successfully induces tube test rank change. If needed, repeat the tube test 24, 48 and 72 h later to determine whether light stimulation has a long-term effect on rank.

be adjusted on the basis of the size of the optic connector or cannula on the mouse's head (Fig. 1a). However, it should not be too wide, because if it is, it could allow a mouse to escape. In our laboratory, the width of the slit is <15 mm for adult male mice and <12 mm for adult female mice.

Gates can also be added to the tube when needed. For example, for calcium imaging during the tube test, we use a slit tube with two perforated sliding gates near the entrances. Mice are trained to wait at the gate for a delaying period of 5 s, during which the calcium signal baseline becomes stable (Fig. 1b).

Other groups have reported several modified versions of the tube test. For example, a sophisticated automated version has been designed with doors, air valves, an IR tracking system and attached chambers[51]. The IR tracking system automatically tracks the position of the mouse and controls the opening of doors and activation of air valves. Another group encourages mice to enter the tube by encasing the tube in a dark box and gently poking the mice with a 25-ml pipette to encourage them to walk through the tube[60]. In addition, a modified 'hierarchy corridor test' uses a 1-m-long corridor with two antechambers and three gates to replace the tube[49]. These modifications may have their own advantages and disadvantages. For example, the automated tube test has the advantage of minimizing variabilities introduced by different experimenters, but it may introduce other confounders, such as air puffs, into the test and is more complicated to operate.

Controls

When conducting tube tests in mutant or optogenetically/pharmacogenetically manipulated mice, proper controls are needed. It is essential to exclude whether the genetic mutation or manipulation causes changes in tube test rank through non-specific factors such as defects in social recognition, social memory, anxiety level, locomotion or muscle strength[26]. For optogenetic and pharmacogenetic manipulation experiments, control experiments conducted on a GFP-expressing group can exclude artifacts induced by light, drugs or viral injection.

Optogenetic manipulation in the tube test

For optogenetic manipulations, a tube with a width-adjustable slit is used for mice implanted with optic connectors (Box 1). For example, we have successfully increased tube test rank by optogenetic activation of the dmPFC[26]. After recovery from the surgeries and expression of AAV-CAG-ChR2(H134R) for at least 2 weeks, mice were handled and trained to walk through the tube for 2 days, and then were tested in the tube test. Once a stable rank was acquired, mice were habituated with dummy optic fibers and tube tests were conducted for 2 d to ensure that the rank was

still stable. On the day of photostimulation, we measured the rank without light stimulation first, then switched on 473-nm light stimulation just before mice entered the tube. Upon optogenetic activation of the dmPFC, eight of ten mice increased their rank in the tube test[26]. Further details of how to implement this assay are described in the Procedure, and an example is shown in Supplementary Video 4.

Materials

Biological materials

Mice. We use adult (8–14 weeks old) male or female mice of the C57BL/6J strain from the Shanghai SLAC Laboratory Animal Center. Age and body weight are matched within the same experiment. **! CAUTION** Experiments using rodents must conform to local and national regulations. Our study using the tube test was approved by the Animal Care and Use Committee of the animal facility at Zhejiang University.

Reagents

- Bleach (Bluemoon, cat. no.1038227)
- Ethanol (Oyeah, cat. no.3098331)

Equipment

- A transparent Plexiglas tube, 30 cm in length, with a 3-cm internal diameter (Fig. 1a) (Zhiyuan, cat. no. 520732022123)
- A short habituation tube, 15 cm in length, with a 3-cm internal diameter (for habituation in the home cage; Zhiyuan, cat. no. 520732022123)
- Video camera: we use a Logitech C930e camera (Logitech, cat. no. 1140630) connected to a computer for regular experiments. This camera captures sufficiently clear images under dim light and allows accurate detailed behavioral analysis (Supplementary Videos 3 and 4); another camera (Panasonic, model no. HC-X920M) was used to shoot higher-resolution videos for illustration purposes (Supplementary Videos 1 and 2).
- A plastic stick (for cleaning the tube and manual intervention in behavioral training; Zhiyuan, cat. no. 520732022123)
- Stopwatch (to time the tube test trial; Tianfu, cat. no. TF307)
- Integrated laser (for optogenetics; Newdoon, cat. no. NEWDOON-470-180212)
- Super Glue (Lanmei, cat. no. 39060278588)
- Double-sided adhesive tape (3M, cat. no. 6516527)

Biological materials setup

Animal housing

The mice we used were 6–8 weeks old upon purchase and were group-housed (four in a cage under standard housing conditions). Mice should be kept in a temperature-controlled room with a 12 h/12 h light/dark cycle (lights on at 7 AM/lights off at 7 PM).

Equipment setup

Tube

We use a transparent Plexiglas tube with a 30-cm length and a 3-cm internal diameter. The exact size should be just sufficient to permit only one adult mouse to comfortably pass through without turning around (Fig. 1a). For mice of smaller or larger size, the tube size should be adjusted accordingly. In the experimental room, attach the tube to the table with double-sided adhesive tape and make sure it does not move during tests (Supplementary Video 1). Alternatively, the tube can be secured using other methods such as with Super Glue or joists and brackets. Before each trial, use 5% (vol/vol) bleach and 75% (vol/vol) ethanol sequentially to clean the tube.

Tube for optogenetics or in vivo recording

Use a tube with a 15-mm slit opening at the top to allow mice wearing an optic fiber or tetrode to pass through the tube smoothly (Fig. 1a).

Tube for calcium imaging

Use a tube with a slit opening at the top and two perforated sliding gates near the entrances and train the mice to wait at the gate for a delay period of 5 s during which the calcium signal baseline becomes stable (Fig. 1b).

Procedure

Habituation ● Timing 3 d

1 House four mice of similar weight and age in one cage. Place a habituation tube into the home cage so the mice become used to the tube. 3 d before the tube test starts, begin handling the mice daily (1–2 min/mouse) to reduce their stress during experiments. At the end of the habituation period, mark the tail of each mouse, using prominent colors and distinguishable patterns. When marking tails, gently hold the tail and allow the animal to move freely. Periodically check the mark and re-mark when necessary to prevent fading. Alternatively, mark the mice with ear tags.

! CAUTION Experiments using rodents must conform to local and national regulations. All animal studies and experimental procedures presented here were approved by the Animal Care and Use Committee of the animal facility at Zhejiang University.

▲ CRITICAL STEP The age and weight of the mice can influence rank in the tube test. Therefore, choose mice of a similar age and with weight differences of <15%.

Training mice to go through the tube ● Timing 2d, ~15 min per mouse on day 1 and ~10 min per mouse on day 2

2 Remove the mice from the animal facility and habituate them in the behavioral test room for at least 20 min.

▲ CRITICAL STEP The behavioral test room should be quiet, temperature-controlled and uniformly illuminated by dim light.

3 Before training, prepare a tube, a plastic stick, 5% (vol/vol) bleach, 75% (vol/vol) ethanol, paper towels, a recording sheet, a pen and a timer. Clean the table, tube and plastic stick with 5% bleach and 75% ethanol sequentially to reduce odor cues and ensure proper disinfection. Attach the tube to the middle of the table with double-sided adhesive tape.

4 Remove the cage lid and let the mice freely explore in the cage for about a minute to become accustomed to the cage with the open roof.

5 Gently lift a mouse briefly by the tail, hold it in your hand and then place it on the table. Let it explore freely for about a minute to reduce its stress. Then place the mouse at one end of the tube.

▲ CRITICAL STEP When picking up the mouse by the tail, try to be swift and gentle, and do not keep the mouse hanging in the air for too long. Let its paws touch the table as soon as possible to reduce stress.

6 Release the tail once the mouse enters the tube. Let the mouse walk through the tube. Use a plastic stick to follow the mouse and gently touch its tail when it retreats or stops moving for a long time (Supplementary Video 1).

▲ CRITICAL STEP The purpose of this step is to train the mouse to walk forward in the tube and prevent it from backing out. This is done without bias to each mouse.

? TROUBLESHOOTING

7 Place the mouse on the other side of the tube and repeat Step 6.

8 Repeat Steps 6 and 7 four more times so the same mouse goes through the tube for a total of ten times, from alternating ends of the tube.

9 Return the mouse to the cage. Clean the tube with 5% bleach and 75% ethanol to remove odor, urine and feces. Ensure that the tube is clean, dry and without residual odor of bleach and ethanol.

10 Repeat Steps 5–9 on the other three mice. Train each mouse to pass through the tube five times from each end.

11 Repeat training steps 5–10 on the second day. It becomes quicker and easier for the mice to go through the tube.

▲ CRITICAL STEP All mice should go through the tube ten times in total on day 2, five times from each side.

Perform the tube test until the ranks become stable ● Timing at least 4 d, requiring ~20 min to complete six trials each day

▲ CRITICAL The time to acquire a stable rank may be affected by stress levels and usually varies from 4 d to 14 d for male mice.

12 Remove the mice from the animal facility and habituate them in the behavior room for at least 20 min. If videotaping is needed, place a camera to take a lateral view of the whole tube. Find a proper distance between the camera and the tube to capture an image of the whole tube and make sure the image is sharp and clear. We use a distance of 40 cm. A video rate of 20 frames per s is needed for detailed frame-by-frame behavioral analysis.

13 Remove the cage lid and let the mice freely explore in the cage for ~1 min.

14 Before the test, again train each mouse to pass through the tube once from each end. Draw a middle line on the tube.

▲ CRITICAL STEP This step greatly reduces the likelihood that mice will refuse to enter the tube, even after repeated losses.

15 During the test trial, grasp two mice by the tail briefly, without restricting their movement in the home cage, and hold them in your two hands. Transfer the mice from the home cage to the opposite ends of the tube. Continue holding the mice gently by the tail until they enter the tube and meet in the middle, and then simultaneously release them and start the timer (Supplementary Video 2).

? TROUBLESHOOTING

16 The mouse that pushes the other mouse out of the tube is designated as the 'winner'. The mouse that retreats from the tube first is designated as the 'loser'. Stop the timer when all four paws of the loser are out of the tube. Use a plastic stick to follow the winner to prevent it from retreating or returning to the tube after it exits. Let both mice explore the table freely for about a minute before placing them back into their home cage (Supplementary Video 2). A normal tube test takes <1 min.

? TROUBLESHOOTING

17 Record the test results and time spent in the tube for each trial. Between trials, clean the tube with 5% bleach and 75% ethanol.

18 From trial to trial, the same mouse should enter the tube from each end alternately. Rank mice by their number of wins, which should vary from 0 to 3. The order of the two mice in each trial should be randomized. We use the following round-robin design to randomize the test order (A, B, C and D are mouse IDs):

Day 1 AB CD BC DA BD AC; Day 2 AD CB DC BA AC DB; Day 3 DB CA AD BC CD AB; Day 4 DC AB BD AC DA CB; Day 5 CA BD DC AB BC DA; Day 6 BC AD CA DB BA CD. To reduce the impact of the most recent win or loss, we let the mice rest in their covered home cage for 2 min before starting the next trial. For example, after testing AB and CD on day 1, we let all four mice rest before proceeding to test BC.

19 Repeat Steps 12–18 on each following test day.

▲ CRITICAL STEP We consider a stable rank to have been obtained if all mice maintain the same ranking for 4 consecutive days.

? TROUBLESHOOTING

Video analysis ● Timing 1 d

20 Manually analyze the video frame by frame. The behavior in the tube can be categorized as push initiation, push back, resistance, retreat and stillness. We mark these behavioral epochs (using BORIS[61]) for further analysis.

▲ CRITICAL STEP Five forms of behaviors can be unambiguously identified (Supplementary Video 3). Push initiation is when one mouse meets another mouse in the tube and initiates shoving its head under the opponent. Pushback is defined as a counter-push after being pushed by the other. Resistance is when both mice hold on to their own territories when being pushed, which is usually paired with one mouse's head being pushed up. Retreat is defined as going backwards after being pushed or voluntarily withdrawing, which is specifically characterized by bending its head down. Stillness is when the mice have no movement except for some sniffing.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
6	A mouse does not enter the tube during the training period	The mouse cannot find the tube entrance	Put a little piece of mouse food at the tube entrance to lead the mouse into the tube; habituation with a short tube in the home cage also improves this situation
	A mouse retreats or stops moving in the tube for an extended period of time during training	The mouse is stressed and unfamiliar with the other end of the tube	Use a plastic stick to follow the mouse, gently touch the tip of its tail (Supplementary Video 1) and let it voluntarily walk out. Do not catch it immediately, but let it explore outside the tube for 1 min. This is to minimize the stress associated with the tube and the uncertainty about the consequences of walking through the tube
	A mouse exits and returns to the tube immediately	The mouse feels unsafe being outside the tube	When the mouse exits, put a plastic stick into the tube from the entering side to prevent it from returning to the tube immediately. Let it explore freely for about a minute without catching it immediately, in order to minimize the stress associated with the outside environment
15	A mouse refuses to enter the tube after being well trained	The remaining odor cue or the smell of bleach or ethanol inside the tube may be repellent	Clean the tube carefully again with 5% bleach and 75% ethanol, and shake the tube to accelerate the volatilization of ethanol
16	Both mice are still in the tube during the test period, and neither mouse retreats within 2 min	The mice are stressed	The trial should be stopped and repeated after a break. Noise or smells in the environment should be minimized. Most well-trained mice usually spend <1 min in the tube
	Immediate voluntary retreat without any push when a mouse meets opponents in the tube during the early phase of the tube test	The mouse is not well trained	Train the mouse to push an object of its own weight through the tube. This additional training works only in the early but not the late phases of the tube tests. We find this training increases voluntary push while keeping ranks unchanged. When the mouse exits, let it explore freely for about a minute without catching it immediately, in order to minimize the stress
	Both mice back out of the tube. Sometimes, the winner backs out even after the loser has already retreated	The mice are not well trained and are stressed	Train the mice to run through the tube for 2 d. Use a plastic stick to follow each mouse, gently touch the tip of its tail (Supplementary Video 1) and let it voluntarily walk out. Do not catch it immediately, but let it explore outside the tube for 1 min
19	Cannot acquire a stable linear rank after conducting the tube test for >2 weeks	The mice may be under stress	Animals need to be well handled and trained before tube tests. Take care to minimize any stress for the mice, e.g., do not hold them by their tails for too long. Let their paws touch the table as quickly as possible to reduce stress. Try scooping the mice instead of picking up their tails when transferring them from cage to table. When the mouse walks out of the tube, let it explore the table freely for some time. Do not catch it immediately, in order to avoid the mouse associating exiting the tube with being caught
		Four mice adopt a nonlinear rank	Very occasionally, there may be loops in the tube test results (Fig. 2c). Try testing for more days until the rank becomes linear
			If the rank continues to be unstable (Fig. 2d) or nonlinear for >3 weeks, stop using this cage
Box 1, step 4	The ranks change after insertion of optic fibers into a mouse's head	The mice are not well accommodated to performing the tests with optic fibers	Test the mice for another day with dummy fibers inserted into their heads
		Mice may be under stress after insertion of fibers	Repeat the trials after a break

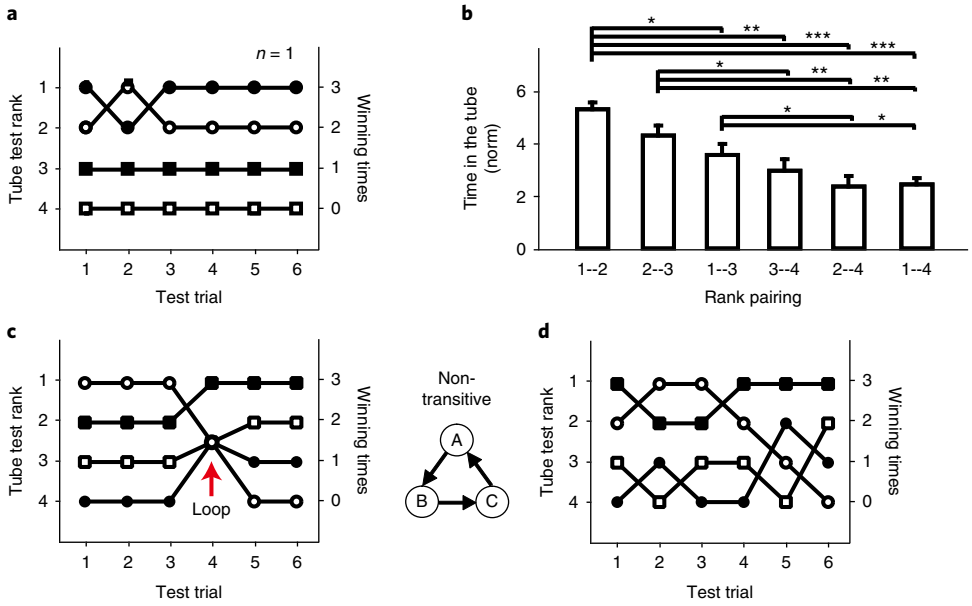

Fig. 2 | An example showing the stability of tube test rank and time spent in the tube for different ranked pairings. a, The tube test ranking of one cage of four mice tested daily over 6 d. **b**, Normalized time spent in the tube for the six pairings ($n = 10$ cages), e.g., '1--2' stands for rank 1 against rank 2. Wilcoxon rank-sum test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Error bars, s.e.m. **c**, An example (left) and an illustration (right) of a rare non-transitive loop relationship. **d**, An example of an unstable tube test ranking. **a**–**c** adapted with permission from Wang et al.[17], American Association for the Advancement of Science. All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at Zhejiang University.

Timing

Step 1, habituation: 3 d
Steps 2–11, training mice to go through the tube: 2 d, ~15 min per mouse on day 1 and ~10 min per mouse on day 2
Steps 12–19, once-daily tube test until ranks are stable for 4 d: at least 4 d, requiring ~20 min to complete six trials each day
Step 20, video analysis: 1 d
Box 1, optogenetics application: 3 d

Anticipated results

Tube test rank is determined by the total number of wins of each mouse against its three cagemates. As number of test trials increases, the ranks stabilize. Once all mice maintain the same ranking position for 4 consecutive test days, we consider it a stable rank and proceed to other manipulations or tests (Fig. 2a).

We averaged the time spent in the tube for each mouse pair. Time is significantly shorter when the lowest-ranked (rank 4) mouse is involved or as rank distance increases, demonstrating that competition is fiercer between mice with higher and closer ranks (Fig. 2b). Remarkably, in most cases the rank is linear and stable, but very occasionally a non-transitive (Fig. 2c) or unstable (Fig. 2d) rank may be observed.

For manipulation, we tested the function of the dmPFC in social hierarchy, using optogenetics as described in Box 1. We induced winning by optogenetically activating dmPFC neurons (Fig. 3, Supplementary Video 4). Our frame-by-frame video analysis revealed that photostimulation of the dmPFC increased pushing and resisting in the tube tests (Fig. 4). Under photostimulation, the originally subordinate mice pushed more (Fig. 4a,b), resisted against the opponent for a longer duration (Fig. 4c) and retreated less (Fig. 4d).

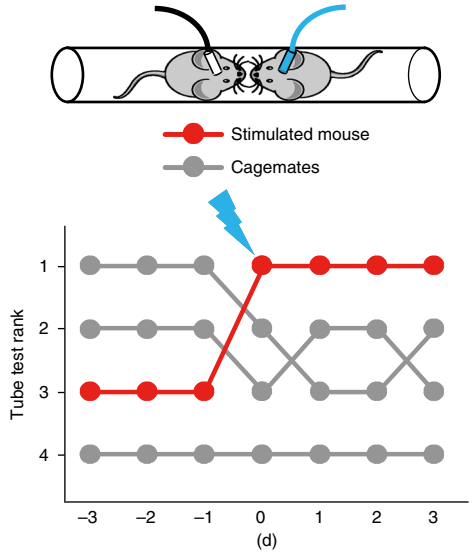

Fig. 3 | Example of daily tube test ranking of one cage of four mice injected with AAV-CAG-ChR2(H134R) virus before and after acute dmPFC photostimulation of the rank-3 mouse at day 0. Lightning bolt represents photostimulation. Adapted with permission from Zhou et al.[26], American Association for the Advancement of Science. All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at Zhejiang University.

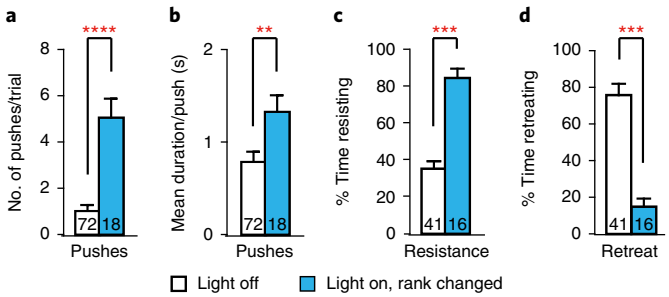

Fig. 4 | Comparison of the behavioral performances of photostimulated mice during light-off and light-on trials. **a**–**d**, Photostimulated mice increased both the number and mean duration of pushes (**a**,**b**), resisted against the opponent for a longer duration (**c**) and retreated less (**d**). Mann–Whitney *U*-test (***P* < 0.01; ****P* < 0.001). Number of trials are indicated inside the boxes. Error bars, s.e.m. **a**–**d**, Adapted with permission from Zhou et al.[26], American Association for the Advancement of Science. All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at Zhejiang University.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

T.Z., H.Z. and H.H. designed the experimental strategy. Z.F., T.Z. and H.Z. optimized experimental procedures. Z.F., S.W., Y.W. and H.H. wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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Study description	a detailed protocol of a behavioral assay
Research sample	male mice of the C57BL/6J strain
Sampling strategy	Required sample sizes were estimated based on our past experience performing similar experiments.
Data collection	Pen, paper and video camera connected to a recording device was used to record the data.
Timing	Data were collected from 2015.9.1 to 2018.8.1
Data exclusions	Values were excluded from the analyses if the tube test rank is unstable or nonlinear for more than 3 weeks.
Non-participation	No participants dropped out.
Randomization	Participants were not allocated into experimental groups.

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☒	☐ ChIP-seq
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Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	C57BL/6J strain mice, male, above 8 week
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve field-collected samples.
Ethics oversight	All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at Zhejiang University.

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