WILEY

Online Proofing System

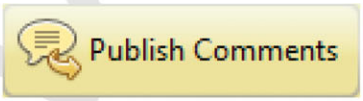
Enabling the Adobe PDF Viewer

In order to proof your article Adobe Reader or Adobe Acrobat needs to be your browser's default PDF viewer. See how to set this up for Internet Explorer, Firefox, and Safari at https://helpx.adobe.com/acrobat/using/display-pdf-in-browser.html

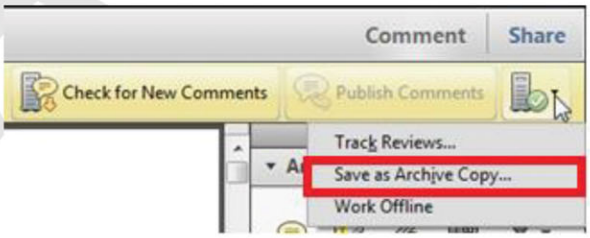
Google Chrome and Microsoft Edge do not support Adobe Reader or Adobe Acrobat as a PDF Viewer. We recommend using Internet Explorer, Firefox, or Safari.

1. Mark your corrections, changes, and query responses using the Annotation Tools outlined on the next 2 pages.

2. Save your proof corrections by clicking the "Publish Comments" button in the yellow banner above. Corrections don't have to be marked in one sitting. You can publish comments and log back in at a later time to add and publish more comments before you click the "Complete Proof Review" button.

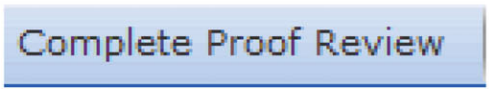


3. When your proof review is complete we recommend you download a copy of your annotated proof for reference in any future correspondence concerning the article before publication. You can do this by clicking on the icon to the right of the 'Publish Comments' button and selecting 'Save as Archive Copy…'.



IMPORTANT: Did you reply to all author queries found on the last page of your proof?
IMPORTANT: Did you click the "Publish Comments" button to save all your corrections? Any unpublished comments will be lost.
IMPORTANT: Once you click "Complete Proof Review" you will not be able to add or publish additional corrections.

4. When your proof review is complete and all corrections have been published to the server by clicking the "Publish Comments" button, please click the "Complete Proof Review" button appearing above the proof in your web browser window.



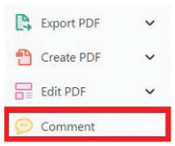
WILEY

USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 11 or above). (Note that this document uses screenshots from Adobe Reader DC.)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab (right-hand panel or under the Tools menu).

This will open up a ribbon panel at the top of the document. Using a tool will place a comment in the right-hand panel. The tools you will use for annotating your proof are shown below:



1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it:

- Highlight a word or sentence.
- Click on .
- Type the replacement text into the blue box that appears.

2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it:

- Highlight a word or sentence.
- Click on ..
- The text will be struck out in red.

experimental data if available. For ORFs to be had to meet all of the following criteria:

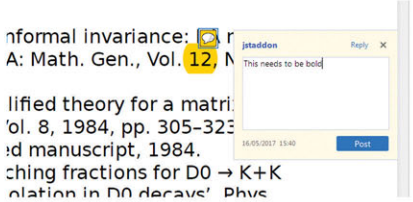
1. Small size (~~3~~5–250 amino acids).
2. Absence of similarity to known proteins.
3. Absence of functional data which could n the real overlapping gene.
4. Greater than 25% overlap at the N-termir terminus with another coding feature; ove both ends; or ORF containing a tRNA.

3. Commenting Tool – for highlighting a section to be changed to bold or italic or for general comments.

Use these 2 tools to highlight the text where a comment is then made.

How to use it:

- Click on .
- Click and drag over the text you need to highlight for the comment you will add.
- Click on .
- Click close to the text you just highlighted.
- Type any instructions regarding the text to be altered into the box that appears.

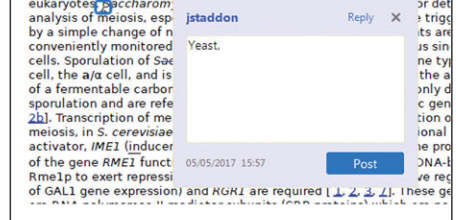


4. Insert Tool – for inserting missing text at specific points in the text.

Marks an insertion point in the text and opens up a text box where comments can be entered.

How to use it:

- Click on .
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the box that appears.



USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

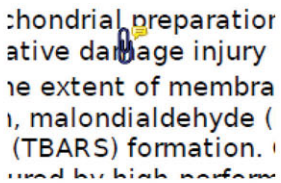
5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it:

- Click on .
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

The attachment appears in the right-hand panel.

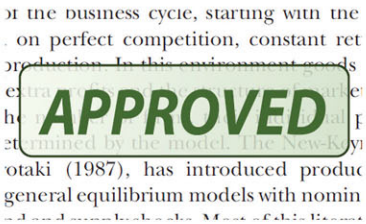


6. Add stamp Tool – for approving a proof if no corrections are required.

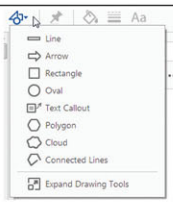
Inserts a selected stamp onto an appropriate place in the proof.

How to use it:

- Click on .
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears. Others are shown under *Dynamic*, *Sign Here*, *Standard Business*).
- Fill in any details and then click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

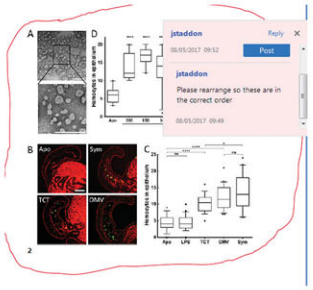


Drawing tools available on comment ribbon



7. Drawing Markups Tools – for drawing shapes, lines, and freeform annotations on proofs and commenting on these marks.

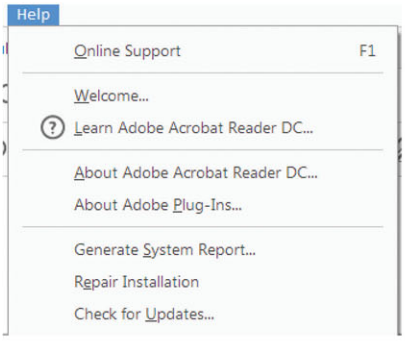
Allows shapes, lines, and freeform annotations to be drawn on proofs and for comments to be made on these marks.



How to use it:

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, right-click on shape and select *Open Pop-up Note*.
- Type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:



Author/Editor Queries

Unit: CPMO 170207 Date: October 23, 2017
Author: Sabine M. Hölter Copyeditor: Krystina Neuman Page 1 of 1

1. AUTHOR NAMES AND AFFILIATIONS: Please make sure that all author names are spelled correctly and all contributing authors are listed. In addition, review all affiliations and confirm that department, institution, and location information are correct. Once this article is submitted to PubMed for indexing, it is rarely possible to get this information corrected.
2. FUNDING: Please make sure that the appropriate funding information appears at the end of the article in the Acknowledgments section before the Literature Cited section. If the information is not present, you should supply it so the copyeditor can have it added to the article when final pages are created.
3. MATERIALS LIST ANNOTATIONS: Please note that for all Materials lists, annotations regarding materials were moved into the steps in which they are first used in order to conform with Current Protocols style (e.g., Basic Protocol 1, step 2). Please review and further edit as desired.
4. BASIC PROTOCOL 2, STEP 4: Current Protocols style generally uses no more than two levels of steps (i.e., numbered steps and lettered substeps). As such, step 4 was simplified and slightly edited to better match house style. Please review and further edit as desired.
5. BASIC PROTOCOL 2, DATA COLLECTION AND ANALYSIS: As in Query 4, steps were slightly edited and renumbered to better match house style. Please review and further edit as desired.
6. BASIC PROTOCOL 2, STEP 17 (Submitted step 15c): The parenthetical phrase regarding the 5-10 sec addition was moved into a step annotation to allow for further explanation. Please review annotation to ensure correctness and further edit as desired.
7. BASIC PROTOCOL 2, STEP 19 (Submitted step 16): Equations were reset using an equation editor. Please confirm correctness.
8. ALTERNATE PROTOCOL 2, STEP 8: Should the wire cages be placed in the testing apparatus at this time?
9. ALTERNATE PROTOCOL 2, STEP 17: Equation was reset using an equation editor. Please confirm correctness.
10. SUPPORT PROTOCOL 1, STEP 8: Equation was reset using an equation editor. Please confirm correctness.

Assessing Sociability, Social Memory, and Pup Retrieval in Mice

Annemarie Zimprich,[1,2] Jörn Niessing,[3] Lior Cohen,[4] Lillian Garrett,[1] Jan Einicke,[1] Bettina Sperling,[1] Mathias V. Schmidt,[5] and Sabine M. Hölter[1,2]

[1]Institute of Developmental Genetics, Helmholtz Zentrum München, Neuherberg, Germany
[2]Developmental Genetics, Technische Universität München-Weihenstephan, Helmholtz Zentrum München, Neuherberg, Germany
[3]Department of ~~Stress Neurobiology and Neurogenetics~~, Max Planck Institute of Neurobiology, Munich, Germany
[4]Sagol Department of Neurobiology, University of Haifa, Haifa, Israel
[5]Department of Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Munich, Germany

Adaptive social behavior is important in mammals, both for the well-being of the individual and for the thriving of the species. Dysfunctions in social behavior occur in many neurodevelopmental and psychiatric diseases, and research into the genetic components of disease-relevant social deficits can open up new avenues for understanding the underlying biological mechanisms and therapeutic interventions. Genetically modified mouse models are particularly useful in this respect, and robust experimental protocols are needed to reliably assess relevant social behavior phenotypes. Here we describe in detail three protocols to quantitatively measure sociability, one of the most frequently investigated social behavior phenotypes in mice, using a three-chamber sociability test. These protocols can be extended to also assess social memory. In addition, we provide a detailed protocol on pup retrieval, which is a particularly robust maternal behavior amenable to various scientific questions. © 2017 by John Wiley & Sons, Inc.

Keywords: social behavior • pup retrieval • three-chamber sociability cage • social memory • mice

How to cite this article:
Zimprich, A., Niessing, J., Cohen, L., Garrett, L., Einicke, J., Sperling, B., Schmidt, M. V., & Hölter, S. M. (2017). Assessing sociability, social memory, and pup retrieval in mice. *Current Protocols in Mouse Biology*, *7*, 1–20. doi: 10.1002/cpmo.36

INTRODUCTION

Social interactions are fundamental to many different species, including mice. Under control conditions mice prefer to spend time investigating another mouse, thus demonstrating active sociability behavior (Hartmann et al., 2012; Masana et al., 2014). Appropriate social behavior is necessary in social animals, not only for building and maintaining hierarchies, but also for mate choice and parental actions. Altered social behaviors can be seen in different psychiatric diseases such as autism spectrum disorders, anxiety disorders, depression, bipolar disorders, and schizophrenia.

Another important naturally occurring behavior in mammals is parental care, because it ensures the survival of the offspring (Krasnegor & Bridges, 1990; Numan & Insel, 2003). Several studies, including studies with genetically modified mouse lines, have highlighted the relevance of particular signaling pathways for the development of maternal behavior (Brooks, Le, Chung, & Tsai, 2012; Sheleg et al., 2017; Umemura, Imai, Mimura,

Current Protocols in Mouse Biology 7:1–20, December 2017
Published online December 2017 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmo.36

Fujiwara, & Ebihara, 2015), and a particularly robust maternal behavior is elicited by the emission of ultrasonic vocalizations (USVs) from rodent pups, which trigger the mother to search for the pup and retrieve it back into the nest (Noirot, 1966; Sewell, 1970). Because of the robustness, biological relevance, and amenability of pup retrieval behavior to different scientific questions, we describe here a detailed experimental protocol for this assay (Basic Protocol 1).

Sociability, also called social affinity or social approach behavior, is probably the most frequently assessed social behavior in genetic mouse models because of its relevance to autism spectrum disorder and related neurodevelopmental disorders with strong genetic components. A widely used test paradigm to quantitatively measure this behavior is the three-chamber sociability test (Moy et al., 2004; Nadler et al., 2004), but simpler versions of the test also exist where the focus of investigation lies on social avoidance behavior after social defeat stress (Berton et al., 2006; Golden, Covington, Berton, & Russo, 2011). The main difference between both approaches is that the latter takes place in an undivided open field arena, and the presentation of an inanimate object and a living conspecific takes place sequentially. The advantage of the three-chamber over a single-chamber test lies in the fact that the mouse displays a real choice by entering one of the three chambers, thus allowing a direct measurement of sociability. A second advantage is the option to extend the test for measurements of social memory. Of note, if the influences of the circadian alterations in corticosterone secretion need to be avoided, the test should be run during the first 6 hr after lights on.

Because many different protocol versions can be found in the literature differing in equipment and data acquisition methods, as well as the kind of stimulus used, we account for this by describing three different versions in detail. Basic Protocol 2 utilizes a commercially available transparent test apparatus and video tracking system for data acquisition, using ovariectomized (OVX) females as stimulus animals. Alternate Protocol 1 differs from Basic Protocol 2 only in the data acquisition method (i.e., a human observer manually recording the animal's behavior using a handheld device). In contrast, Alternate Protocol 2 makes use of a similar-sized, but workshop-made grey test apparatus and a juvenile and a dummy as stimulus animals. Support Protocol 1 explains how to use the three-chamber sociability test to assess social memory as a second step after measuring sociability.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must use officially approved procedures for the care and use of laboratory animals.

BASIC PROTOCOL 1

PUP RETRIEVAL ASSAY TO TEST FOR PARENTAL RESPONSIVENESS

When rodent pups are isolated from the nest, their body temperature drops, and they start to emit USVs. USVs trigger an instinctive behavior in the mother to look for the isolated pup and retrieve it back into the nest (Noirot, 1966; Sewell, 1970). Since this behavior is very simple and robust, it makes a useful tool to probe animals for parental responsiveness in various contexts. Mouse pups are able to self-maintain their body temperature in the second week of their life and as a consequence stop emitting USVs (Noirot, 1972). Thus, the assay is restricted to mouse pups in the first week of life. Since many mouse strains suffer from hearing loss (both early onset and progressive later onset), the adult retrieving animal should be at an age before the onset of hearing loss for the tested strain (Zheng, Johnson, & Erway, 1999).

The behavioral test is performed in a standard, clean mouse cage with fresh woodchip or paper bedding. The cage should provide a "safe zone" for the animals in the form of red transparent plastic housing (mock nest) in the corner of the cage. The adult animal should

be placed in the cage 20 to 30 min before the behavioral test starts to let it habituate to the new environment. Then 5 pups are placed subsequently into different areas of the cage at 30-sec intervals. The experiment is finished after retrieval of all 5 pups or after 5 min. The number of pups and retrieval latencies are analyzed subsequently from video recordings (see Video 1: Pup retrieval assay).

Materials

Adult female mice of interest
5 pups for testing (postnatal day 3 to 7 [P3–P7])

Standard mouse cage with fresh woodchip or paper bedding (e.g., 26 cm × 42 cm)
Clean red transparent plastic house (mock nest; e.g., Plexx BV)
Sound-attenuated testing room with adjustable light source
Lux meter
Video camera installed for top view
Timer
Ultrasound microphone for monitoring USV emission (optional)

1. Place the behavioral cage in a quiet and dimmed (30 to 50 lux) room, and install the video camera above the cage.

   Place the cage low enough to be able to watch the experiment without leaning above the cage, or watch the live video on the computer screen instead. Looking into the cage from above will scare the animals and likely affect the experiment.

2. Put the mouse of interest in the behavioral cage, and let it habituate 20 to 30 min prior the actual behavioral test.

   Appropriate mice include mothers, experienced virgins, naïve virgins, naïve males, and sires. All animals should be around week 12 to 15 and age matched. See protocol introduction for comments regarding hearing loss.

   The time needed for habituation might differ between individuals. Wait until animal seems to be calm. Anxiety or nervousness of the animal might interfere with the behavior.

3. Start the video camera and timer to measure the time intervals between pup placements.

4. Place the first pup into the cage. Choose a distant spot to the mock nest.

   Ideally wait until the adult animal is located in the mock nest before placing the first pup. Since the animal cannot see through the mock nest this minimizes the interference of the experimenter on the task.

5. Place the remaining 4 pups subsequently in different distant places across the cage at 30-sec intervals.

6. Terminate the experiment when all pups are retrieved. If none or only a few of the pups are retrieved, end the experiment after 5 min.

7. From the video recordings determine the number of pups retrieved and the latency for retrieval.

   The first latency value is measured from introduction of the first pup to the arena until the first retrieval of any pup (sometimes the first pup introduced is not the first pup retrieved). The next latency value is measured from retrieval of the first pup until retrieval of the second pup and so on until all pups are retrieved.

   For example data see Cohen, Rothschild, & Mizrahi (2011).

MEASURING SOCIABILITY IN THE THREE-CHAMBER SOCIABILITY CAGE WITH VIDEO TRACKING AND OVARIECTOMIZED FEMALES AS STIMULUS MICE

Generally social animals, such as mice, prefer to spend time with a conspecific. In the three-chamber sociability cage, the test animal has the free choice to spend time in the chamber where a conspecific is placed within a cylinder. Sociability is then measured as the time the test mouse spends within that chamber. By confining the stimulus mouse to a cylinder and having three chambers, the test mouse can freely explore, the approach tendencies of the test mouse is maximized, and the contribution of the stimulus mouse is minimized.

The mice are tested for their sociability in the three-camber sociability cage at an illumination level of 200 lux in the testing room, thereby generating comparable results to the publications of Moy et al. (2004) and Nadler et al. (2004), who designed the test for detecting autism-like behaviors. The procedure consists of two phases: the habituation phase and the sociability phase, each lasting 10 min. In the habituation phase the test animal is familiarized with the empty sociability cage arena. During this phase the side preference can be measured and used to verify that there are no underlying preferences for a chamber. During the sociability phase the test mouse can distribute its behavior between the central chamber, the "non-social" chamber to investigate an empty cylinder, and the "social" chamber to investigate a cylinder with an unfamiliar OVX mouse inside. The cylinders allow nose contact but prevent fighting. The amount of time spent within the chambers is recorded and subsequently analyzed. The recording of the animal's behavior can either be done with commercially available video tracking software, such as ANY-maze (Stoelting) or EthoVision (Noldus), or manually by a human observer (see Alternate Protocol 1). In Basic Protocol 2 we describe the recording by EthoVision, tracking the mouse's center of gravity using a commercially available sociability cage (Noldus) and OVX mice as stimulus animals. In order to measure closer contact to the stimulus animal, a second zone (separate from the whole "chamber") can be included (i.e., a narrow zone around the cylinder; in the following protocol called the "ring").

Materials

Experimental animals
Stimulus animals (e.g., OVX females purchased from Charles River)
Disinfectant solution (e.g., Pursept-A Xpress)

Data sheet
Sound-attenuated testing room with adjustable light source
Three-chamber sociability cage (e.g., Noldus)
Video camera
Video tracking system (e.g., Noldus EthoVision)
Video recording program (optional; e.g., Noldus MPEG Recorder)
Lux meter

Initial setup

1. House mice in groups of 4 to 5 separated by sex in individually ventilated cages in a temperature- and humidity-controlled environment (22°C to 24°C and 50% to 60%) on a 12/12 hr light/dark cycle for at least 2 weeks prior to and during the experiments. Do not handle mice other than described within this protocol for the experiment and for caretaking.

   The choice of the experimental mice depends on the experimental design. These can, in principle, be of any strain (e.g., C57BL/6N) and males or females. The age of the mice should be older than 8 weeks. As the test depends on olfactory abilities, it must be

Table 1 Example Datasheet for Tracking Individual Mice

									Habituation		Sociability phase			
Cage	Mouse identification	Ear mark	Sex	Date of birth	Age at testing (weeks)	Genotype or treatment	Strain	Date of testing	Time	File number	File number	OVX ID	OVX side	Comments
50	102	1	M	18.12.2016	11	WT	C57BL/6N	6.03.2017	10:00	1	2	711	Left	
50	103	2	M	18.12.2016	11	WT	C57BL/6N	6.03.2017	10:25	3	4	711	Right	
50	104	3	M	18.12.2016	11	WT	C57BL/6N	6.03.2017	10:50	5	6	712	Left	
50	106	4	M	18.12.2016	11	WT	C57BL/6N	6.03.2017	11:15	7	8	712	Right	
50	110	10	M	18.12.2016	11	WT	C57BL/6N	6.03.2017	11:40	9	10	713	Left	
68	111	12	M	18.12.2016	11	WT	C57BL/6N	7.03.2017	9:40	11	12	714	Right	
90	112	36	M	18.12.2016	11	WT	C57BL/6N	7.03.2017	10:05	13	14	714	Left	

OVX, ovariectomized; WT, wild-type.

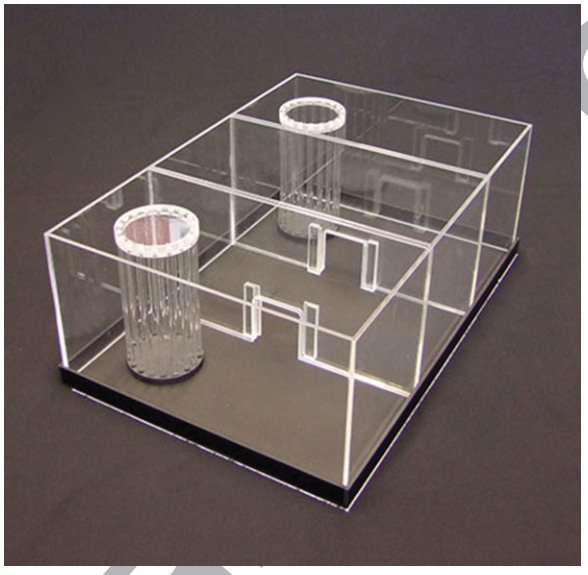


Figure 1 Three-chamber sociability cage by Noldus (image from http://www.noldus.com/content/sociability-cage; reprinted with permission).

excluded that the experimental groups vary in their olfactory sensitivities. This should be taken into consideration, especially when testing animals at older ages (i.e., over 6 months of age; see Troubleshooting).

2. Prepare a data sheet and assign stimulus animals (OVX females) to experimental animals, as well as the side/chamber into which the OVX females will be inserted. Make sure to alternate the position.

   The data sheet should contain a list of mouse identification, cage number, sex, genotype/treatment/ group, age, file number, side at which the OVX will be placed, and date of testing (see Table 1).

3. Set up the sociability cage and camera in the test location.

   The sociability cage should be 59 cm l × 39.5 cm w × 21.5 cm h with a chamber width of 18.5 cm, passage with sliding doors equal to 7.1 cm h × 6.5 cm w (see Fig. 1), and cylinders for holding the stimulus animal or "non-social" object.

We test with the commercially available sociability cage from Noldus. This has a removable black floor plate, which is infrared permeable. As we are mainly testing dark animals and do not use an infrared light source from below, we remove the floor plate and place the cage on a light-colored table, as with described light conditions black animals cannot be detected. When testing exclusively light-colored animals, the floor plate can be used. Also, if the sociability cage is placed above an infrared light source together with an infrared-sensitive camera, the floor plate can remain, and animals can be detected independent of their coat color.

Make sure the whole arena is detected by the camera. It must be ensured the mouse can be detected anywhere in the arena, especially when it is close to walls. Adjust the camera's height to diminish optical distortions. Alternatively, use two or more cameras to track the mouse and minimize blind angles.

4. Set up arena definitions according to the manufacturer's instructions (EthoVision Software):

   This can be done long before testing, but be aware that the sociability cage fits into the defined zones on the testing day. We prefer setting up the arena definition 1 day before testing and then not moving the sociability cage.

   a. Open the software.
   b. Add a new experiment.
   c. Set lower tab "Definitions:" Add labels (mouse identification, cage number, sex, genotype/treatment group). Include systems variables as well. Under "Properties" make sure that you can edit the entries before and after data acquisition.
   d. Set lower tab "Values:" Add tracks and enter in the mouse identification, cage number, sex and genotype/treatment group.
   e. Select the tab "Experiment," then "Arena Definition," and select the camera/video source.
   f. Select the tab "Arena," then "add Arena," and define the arena by outlining it. Make sure ends meet/overlap.
   g. Select lower tab "Acquisition Zone Definition," then the tab "Zone," and then select "add Zone." Define the different Zones by drawing lines around them (Fig. 2). Make sure ends meet/overlap; in this way create at least 3 Zones: center, left chamber, and right chamber. Make sure that zones are separated from each other.

      We also include so-called "rings" around the cylinders. These measure ~3 cm from the edge of the cylinder to the outer border. If you add these make sure to define "Cumulative Zones." Then, the new left chamber should consist of "left chamber" and "left ring," and the new right chamber should consist of "right chamber" and "right ring". The "ring" is used to measure if the experimental animal is in close proximity to the cylinders; if the software can track the mouse´s nose, the width of the "ring" can be narrowed.

   h. Select the lower tab "Calibration." Calibrate the arena by drawing a line from the inner sides of the walls from one side to the other, which should measure 58 cm; draw a line across the width of the arena, which should measure 38.5 cm.
   i. Select the tab "Experiment," and then "Acquire data."
   j. ~~Select the tab "Tracking," and then "Trial protocol." Set the following parameters:~~

      ~~Recording Duration: 10 min plus 5 to 10 sec, depending on how fast you place the mouse into the arena after starting the trial.~~

      ~~Sample Rate: 12,500 samples/sec.~~

      ~~Processing, Detection Method: Select "Subtraction" and for Object intensity "Detect all Objects that differ from the background."~~

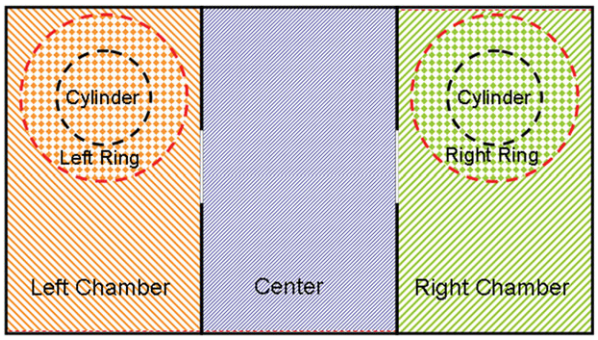


Figure 2 Scheme of the three-chamber sociability cage. Depicted are the different zones, which need to be defined within the EthoVision program. When inserting a "ring," make sure to include it into the cumulative zone of the "chamber" as well. The "cylinder" is not a defined zone; here it is used to depict the location of the cylinder when testing sociability.

~~Noise removal: Tick the box "Minimum Object size," and set the value to 45.~~

~~Update Detection Variables: Adjust the threshold so that an animal can be detected properly anywhere in the arena.~~

For this it is advisable to either have an extra mouse running over it while you observe if it is tracked properly or place an opaque object at different places of the arena to check its detection. Click on "update now" when the arena is empty to snap the reference image.

Testing day

5. Bring animals into the testing room at least 30 min prior to the start of the test to permit acclimatization.

   Test mice in the morning on a standard light/dark cycle, at least 1 hr after lights on.

   Avoid exposing mice to environmental stressors (such as loud noise) before and during the test. It may be reasonable to hang signs outside the room to inform others that testing is in progress inside the room.

6. Habituate stimulus animals to the cylinders before the actual test phase for at least for 10 min.

7. Using the light meter as a guide, adjust the light level to 200 lux (± 5 lux) in the center of the arena.

8. If the arena definitions were set up earlier, open the appropriate file and make sure that the arena and zones correspond between the software definition and the actual sociability cage.

   Record details for each individual animal, such as file number, mouse identification, genotype/treatment/group, and sex in the software prior to starting the test.

9. Ensure that the sociability cage is clean and dry before commencing the experiment.

Habituation phase

10. Start the testing procedure:

    a. Take a snapshot of the clean and empty arena as background image within the software to ensure proper detection of the animal.
    b. Start the video to record the animal's movement.

This is advisable in case you need to reanalyze with different settings afterwards or to capture the animal's movements for further analyses

c. Start the trial in the software.
d. ~~d.~~ Place the animal into the central chamber so it faces the back wall.

It is essential that there are no disturbances during the test and that noise is kept to a minimum.

Keep handling to the absolute minimum necessary to transfer the mice between the home cage and the testing apparatus.

The software tracks the mouse while it is freely exploring the whole arena during a 10-min period.

11. End the trial:

    a. Stop the video recording if this does not occur automatically.
    b. Save the acquired data.
    c. Remove animal from the arena.

To avoid conflicts and stress, do not put already-tested mice back with their cage mates that are to be tested later. Put them in a fresh home cage, and take care to maintain the original housing groups. Single-house the experimental animal for the interval between the habituation and the sociability phase.

Sociability phase

12. Start the testing procedure:

    a. Insert an empty cylinder and a cylinder including the assigned OVX female into the corresponding chambers of the sociability cage.
    b. When working with "rings," make sure that the actual cylinders are centered in the defined "ring" zone.
    c. Continue as in steps 10a to 11c.

13. Remove cylinders, fecal boli, and urine from the arena. Wipe the surface with disinfectant solution to remove any olfactory cues that may remain and to maintain hygiene standards and dry.

14. Test the next animal starting with step 10.

Data collection and analysis

15. Store a backup of the acquired raw data before you perform further processing of the data.

16. Select the tab "Experiment," and then "Analyze Data." Select the tracks you want to analyze.

17. Check all tracks by visualization of the data ("Experiment," then "Visualize Data"). Click into the first track picture to set the options ("Play," then "Options"). Set size to 10 sec or any other short period to detect and correct for "jumps" during the run and for deleting the first seconds when the mouse was placed in the arena. Further check the whole tracking to ensure that there are no "jumps;" if so delete those. Save the changes.

Setting the size to 10 sec is where the plus 5 to 10 sec under Recording Duration in step 4j comes from.

18. Collect the data:

a. Select all independent variables including mouse identification, cage number, sex, genotype, etc., by selecting "View," "Layout," "Independent Variables," and "Object/Actors."
b. Select the tab "Analysis," and define the following parameters:
   Input Filters: Minimum distance moved 1 cm
   Add parameters: In Zone (Total Duration [sec]) and Velocity (maximum). For "Parameter properties," tick the "use input filter" box for both parameters.

   Other parameters, such as "Distance moved (total)" and In Zone (Frequency, Total Duration [%], Latency to first occurrence), can be added for analysis.
c. Select the tabs "Data," "Nesting," and then "Zones." Choose Arena, Center, left chamber, right chamber, and if appropriate, left and right ring.
d. Click on "Calculate" in the "Analysis" tab.
e. Check if animals have a maximum velocity over 40, as in that case the chance of having missed a "jump" in the tracking is very likely! If so, go back to step 17 to check the visualization of the data and correct it.
f. Select the tab "File," "Export," and "Statistics" to export the data.

19. Analyze the total durations and the index (based on the durations) for group differences for both trials.

Habituation phase:

$$\text{Side preference index} = \frac{\text{time in right chamber}}{\text{time in right chamber} + \text{time in left chamber}} \times 100$$

Sociability phase:

$$\text{Sociability index} = \frac{\text{time in social chamber}}{\text{time in social chamber} + \text{time in non} - \text{social chamber}} \times 100$$

Data from left and right chamber/ring have to be restructured to "social" and "non-social" chamber/ring according to the side the stimulus animal was situated during the testing phase.

MEASURING SOCIAL INTERACTION IN THE THREE-CHAMBER SOCIABILITY CAGE BY A HUMAN OBSERVER

ALTERNATE PROTOCOL 1

Social behavior can also be monitored in the three-chamber sociability cage by measuring behavior with a handheld computer. In the social interaction phase, the durations spent in direct olfactory contact with the empty cylinder and the cylinder holding the stimulus animal are recorded.

The advantage of this protocol is that less preparation is needed, as setup of the video-based tracking is not required. Also, there is no major interruption between the habituation and the social interaction phase, as the animals do not need to be taken out of the arena, but can be confined to the central chamber. Also the trained observer tracks only the direct (olfactory) contact (interaction) of the experimental animal to the "social" or "non-social" cylinder, and not simply the close proximity to the objects. On the other hand, there is no video recording of behavior, making it impossible to re-analyze behavior at later time points. Also, other measures such as the distance travelled or analysis within different regions around the cylinders are not measured. This can be circumvented by including video recordings.

Materials

Experimental and stimulus mice (see Basic Protocol 2)
Disinfectant solution (e.g., Pursept-A Xpress)

Handheld computer (e.g., Psion Workabout) with event logging software (e.g., Noldus Pocket Observer)
Data sheet (see Basic Protocol 2)
Sound-attenuated testing room with adjustable light source
Lux meter
Sociability cage (see Basic Protocol 2)

Initial setup

1. House mice in groups of 4 to 5 separated by sex in individually ventilated cages in a temperature- and humidity-controlled environment (22°C to 24°C and 50% to 60%) on a 12/12 hr light/dark cycle for at least 2 weeks prior to and during the experiments. Do not handle mice other than described within this protocol for the experiment and for caretaking.

2. Prepare the Pocket Observer program settings for the social behavior test. Set the test duration for sociability at 10 min, and assign the following 3 keys:

   1 = neutral (i.e., experimental animal spends no time in olfactory investigation)
   2 = experimental animal shows olfactory investigation of stimulus animal (OVX)
   3 = experimental animal shows olfactory investigation of the empty cylinder.

If side preference during the habituation phase (10 min) is to be recorded, assign another 3 keys:

4 = center (i.e., experimental animal is in the central chamber)
5 = left (i.e., experimental animal is in the left chamber)
6 = right (i.e., experimental animal is in the right chamber).

3. Prepare a data sheet and assign stimulus animals (OVX females) to experimental animals, as well as the side/chamber into which the OVX females will be inserted. Make sure to alternate the position.

Testing day

4. Bring animals into the testing room at least 30 min prior to the start of the test to permit acclimatization.

   Test mice in the morning on a standard light/dark cycle, at least 1 hr after lights on.

   Avoid exposing mice to environmental stressors (such as loud noise) before and during the test. It may be reasonable to hang signs outside the room to inform others that testing is in progress inside the room.

5. Habituate stimulus animals to the cylinders before the actual test phase for at least 10 min.

6. Using the light meter as a guide, adjust the light level to 200 lux (± 5 lux) at the center of the arena.

   It is essential that there is no or minimum noise and disturbance during the test.

   Keep handling to the absolute minimum necessary to transfer the mice from the cage to the testing apparatus.

7. Prepare your chair position so that you will be able to quietly observe the animal's behavior in the sociability cage.

   Place the cage low enough to be able to watch the experiment without leaning above the cage, or watch the live video on the computer screen instead. Looking into the cage from above will scare the animals and likely affect the experiment.

8. Ensure that the sociability cage is clean and dry before commencing the experiment.

Habituation phase

9. If side preference is to be measured, open the Pocket Observer program, and add specifics (mouse identification, sex, genotype/treatment/group) for the next mouse to test.

10. Place the experimental animal into the center of the sociability cage. Immediately start the Pocket Observer program, if applicable. Record the time spent in each chamber, whereby an entry into the chamber occurs when all paws are in a chamber.

11. After 10 min observation, confine the experimental animal to the center by closing the doors. If applicable save the collected data of the habituation phase, and open the next file for recording social interaction.

12. Insert both an empty cylinder and a cylinder with the stimulus animal inside to the left and right chamber, respectively, and according to the designated position on the data sheet.

Social interaction phase

13. Open the doors and immediately start the Pocket Observer program for measuring social interaction behavior.

14. After 10 min put the experimental animal back into its home cage.

    To avoid conflicts and stress, do not put already-tested mice back with their cage mates that are to be tested later. Put them in a fresh home cage, and take care to maintain the original housing groups.

15. Remove cylinders, fecal boli, and urine from the arena. Wipe the surface with disinfectant solution to remove any olfactory cues that may remain and to maintain hygiene standards and dry.

16. Test the next animal starting with step 9.

17. After having tested all animals, put them back into their home cages.

Data analysis

18. Analyze the total durations and the index (based on the durations) for group differences as in Basic Protocol 2, steps 15 through 19.

MEASURING SOCIAL INTERACTION IN THE THREE-CHAMBER SOCIABILITY TEST WITH A WORKSHOP APPARATUS AND JUVENILE VERSUS INANIMATE OBJECT AS STIMULI

ALTERNATE PROTOCOL 2

This version of the three-chamber sociability test is used to assess the likelihood that a mouse will spend time with a conspecific rather than with an inanimate object.

The recording of the test can be done with commercially available tracking software, such as ANY-maze (Stoelting Europe) or EthoVision (Noldus; see Basic Protocol 2). However, as active social interaction is difficult to track automatically and hard to distinguish from just spending time close to the social partner, it is recommended to score the social interaction behavior manually. This alternate protocol uses juvenile mice as social

partners, as they usually do not elicit aggressive behavior and can be used without any additional manipulations (e.g., ovariectomy). The use of a dummy mouse as inanimate object is meant to limit any bias that may arise if the object is generally less interesting to the mouse.

Materials

Experimental mice
Juvenile mice for social interaction

Test room equipped with adjustable lights for the test chamber
Three-chamber interaction apparatus
Sawdust bedding
Two cameras above the apparatus connected to a computer
Lux meter
Tracking software (e.g., ANY-maze or EthoVision) installed on the computer
Two round wire cages (e.g., Spectrum Diversified Designs, Galaxy Cup)
Objects to weigh down the wire cages
Dummy (toy) mice (non-social object)

Initial setup

1. House the test mice in a room adjacent to the test room for at least 1 week before testing.

   The choice of the test mice depends on the experimental design. These can, in principle, be of any strain (e.g., C57BL/6N) and both males and females. Tests should always be run within the same sex, though. The age of the mice should be older than 8 weeks. When testing older animals (e.g., older than 6 months) it should be taken into account that possible sensory/olfactory sensitivities can be diminished (see Basic Protocol 2). The animals can be bred in-house or ordered from a commercial supplier.

2. Place the three-chamber test apparatus in a test room with adjustable light.

   The three-chamber apparatus should contain a left and right chamber of 19 cm l × 25 cm w × 40 cm h and a center chamber of 12 cm l × 25 cm w × 40 cm h (total size 50 cm l × 25 cm w × 40 cm h) made of grey PVC. Dividing walls should have square openings on the floor (6 cm × 6 cm) allowing access to the side chambers. The openings should have sliding doors (opening upwards) to allow blocking access to the side chambers

   Ideally the apparatus is placed in a test chamber that is separated from the rest of the room (e.g., by a curtain). Within this test chamber the light should be adjustable and illuminate the apparatus evenly.

3. Fill the floor of the apparatus with sawdust bedding to a height of 1 cm.

   Bedding should ideally be the same bedding as used for housing of the experimental animals.

4. Ensure the doors connecting the middle with the two outer compartments of the apparatus are easily opened and closed.

5. Place two cameras on top of the apparatus, ensuring that the whole test arena is visible.

   Capturing the whole apparatus with one camera is also possible, but it has to be ensured that the two inner walls will not cause a blind angle where animals cannot be detected. Placing one camera directly over one of the two inner walls and the other camera over the third chamber will ensure that the full apparatus is visible. In animal tracking software, it is usually possible to merge two videos in one video file so that for the recording and scoring only one video file is obtained from two separate cameras.

6. Adjust the lighting of the apparatus to 10 to 20 lux.

   In this protocol dim illumination is used to reduce anxiety in the mice and thereby enhance explorative behavior. It is recommended to use dim light conditions especially in mouse strains that are known to display high anxiety–like behavior in novel environments.

7. Program the animal tracking software, and make sure animal tracking and video recording works properly.

8. Habituate the juvenile mice to the wire cages.

   Stimulus mice should be juveniles, between 4 and 6 weeks of age, to avoid aggressive encounters with the test mouse. The preferred strain to use is the relatively calm BALB/c strain, but the test also works with animals from the same strain as the test animals (e.g., C57BL/6N). Juveniles should not be re-used for more than four test animals.

   Habituation should be done at least twice for 30 min each on the 2 days before testing. The mice are placed under the wire cages, each in a separate chamber and left there undisturbed for 30 min.

9. Habituate the test animals to the apparatus 2 days and 1 day before testing. Close the doors separating the apparatus chambers, and place the test animals in the middle chamber. Allow the animals to explore this chamber for 10 min.

   It is possible to record the locomotor behavior of the animals during the two habituation trials to exclude differences in general locomotion.

Testing day

10. On the test day, prepare the animal-tracking software and re-measure the light conditions (adjust when necessary).

11. In each of the outer chambers, place a wire cage with either a dummy mouse or a live juvenile mouse underneath, according to the assigned side on the data sheet.

    Keeping stranger animals in wire cages serves the purpose of preventing any type of direct physical contact (allowing nose contact but prevent fighting), as well as ensuring that all social approach is initiated only by the test animal.

    Alternate the chamber with the juvenile mouse in a semi-random fashion between test animals to avoid a side bias. Weigh down the wire cages by placing a same-sized object on top (e.g., a metal cylinder, square, or filled water bottle) to make sure (1) that the juvenile cannot move the wire cage and (2) that the experimental animal cannot climb on the wire cage.

    Many people use general inanimate objects as counterparts for the live mice, but we prefer to use inanimate dummy mice to avoid a preference based on non-social aspects of live mice, such as shape. Dummy mice can be obtained in pet stores as toys for cats.

12. Start the video tracking. Immediately introduce a test animal to the middle chamber of the apparatus for 5 min, but do not allow access to outer chambers.

13. Open both chamber doors, and allow the test animal to explore the full apparatus for 10 min.

14. At the end of the test, remove the test animal from the arena, and place it back in the home cage.

    To avoid conflicts and stress do not put already-tested mice back with their cage mates that are to be tested later. Put them in a fresh home cage, and take care to keep the original housing groups.

15. Shuffle the bedding of the test arena around a bit, and remove feces where possible.

    It is recommended to renew the sawdust bedding after four animals.

16. Repeat steps 11 to 15 for each test animal.

Data analysis

17. Calculate the percent of time spent in social interaction as follows:

$$\text{Percent time spent in social interaction} = \frac{\text{time spent in social interaction}}{\text{total time spent interacting with dummy or live mouse}} \times 100$$

Data analysis should be based both on the automatic video tracking—rendering time spent in each compartment, distance travelled, and time spent in the immediate vicinity of the wire cage—and also on manual rescoring of actual social interaction behavior.

SUPPORT PROTOCOL 1

MEASURING SOCIAL MEMORY IN THE THREE-CHAMBER SOCIABILITY CAGE

Social memory assesses a different feature of social behavior, namely how well social interaction partners are identified and remembered. Under normal conditions a mouse would spend more time investigating a novel social partner compared to a previously encountered conspecific (Kohl et al., 2015; Richter, Wolf, & Engelmann, 2005). As the test setup is very similar to the sociability test described above, both tests can be easily combined, but can also be run separately.

The social memory test can be run with any of the above-described sociability protocols. In this Support Protocol we describe the approach used for the three-chamber interaction apparatus from Alternate Protocol 2. Applying the social memory test to the Basic Protocol 2 and Alternate Protocol 1 is similar. Here, an unknown OVX female and the already-known OVX female (from the sociability phase) are introduced after a certain retention time, and testing is carried out analogous to the described protocols above.

Additional Materials *(also see Alternate Protocol 2)*

Mice not previously encountered

1. Perform steps 1 through 15 as described for the three-chamber sociability test in Alternate Protocol 2.

   Make sure to have a sufficient number of juvenile mice for the social interaction, as two different mice are needed for a single test run.

   If you are not interested in sociability and only in social memory, it is also possible to use two live mice in the acquisition trial instead of a dummy mouse and one live mouse.

2. Place a previously encountered mouse under the wire cage in one of the outer chambers and a never-encountered mouse under the wire cage in the other outer chamber.

   Alternate the location of the known and unknown social partners in a semi-random fashion to avoid a side bias.

3. After an inter-trial interval of 10 min, start video recording again, and re-introduce the test mouse in the middle chamber of the apparatus with access to the outer chambers blocked for 5 min.

   Depending on the strain of the mice and the overall conditions, the length of the inter-trial interval can be varied.

4. Open the doors to both chambers, and allow the test animal to explore the full apparatus for 10 min.

5. At the end of the test, remove the test animal from the arena, and place it back in the home cage.

   To avoid conflicts and stress do not put already-tested mice back with their cage mates that are to be tested later. Put them in a fresh home cage, and take care to maintain the original housing groups.

6. Shuffle the bedding of the test arena around a bit, and remove feces where possible.

7. Repeat steps 1 to 6 for each test animal.

8. Calculate the following:

$$\text{Percent time spent in social interaction} = \frac{\text{time spent in social interaction}}{\text{total time spent interacting with ~~dummy or live mouse~~}} \times 100$$

   Data analysis should be based both on the automatic video tracking—rendering time spent in each compartment, distance travelled, and time spent in the direct vicinity of the wire cage—and also on manual rescoring of actual social interaction behavior.

COMMENTARY

Background Information

As outlined by Insel and Winslow (1998), the study of complex social behaviors provides an important basic science for psychiatry because the detailed understanding of the role of neurotransmitters, neuropeptides, neural circuits, and genetic pathways involved in biologically relevant social behaviors is necessary for understanding their role in psychopathology.

Parental care is a collection of the strongest natural behaviors and is important to ensure the survival of the offspring (Krasnegor & Bridges, 1990; Numan & Insel, 2003). A particularly robust maternal behavior is elicited by the emission of USVs from pups, which are isolated from the nest and unable to maintain their body temperature (Noirot, 1966; Sewell, 1970). These USVs trigger the mother to search for the pup and retrieve it back into the nest. The exhibition of pup-retrieving behavior is restricted to dams after parturition or virgin females and males who interact with newborn pups for several days (experienced virgins and sires). Naïve virgins will usually ignore unfamiliar pups while males often show aggression towards unfamiliar pups (Cohen et al., 2011; Dulac, O'Connell, & Wu, 2014). Hence, pup retrieval behavior can either be induced by internal mechanisms (e.g., hormonal changes) or by social learning of the caring behavior demonstrated by the mother.

Since pup retrieval can be quantified simply by counting the number of retrieved pups and measuring the response time of the adult animal, it is particularly simple and robust to probe parental responsiveness in mothers, sires, experienced virgins, naïve virgins, and naïve males for various scientific questions. Due to the causal link between the behavior and a sensory stimulus, it is widely used in behavioral neuroscience to investigate the impact of maternity on the processing of sensory information or multisensory integration (Cohen et al., 2011; Liu & Schreiner, 2007). Moreover, mutant studies have highlighted the importance of particular signaling pathways for the development of maternal behavior (Sheleg et al., 2017), while pharmacological research has studied the effect of antipsychotic drugs on pup retrieval (Silva, Bernardi, & Felicio, 2001).

Social memory (i.e., the ability to recognize a familiar conspecific) is the foundation of all mammalian social relationships (Bielsky & Young, 2004; Ferguson, Young, & Insel, 2002), and the first step in this behavior is social approach (also called affiliation), which is the most frequently investigated form of social interactions. While the first test protocols developed to measure social approach and recognition behavior frequently, but not exclusively, brought the stimulus (animal) together with the tested animal in a single-chamber arena (File & Seth, 2003; Gheusi, Bluthe, Goodall, & Dantzer, 1994; Winslow, 2003), the three-chamber arena version described here was developed explicitly to allow for a better characterization of mouse models for autism (Moy et al., 2004) and for automation (Nadler et al., 2004). Thus, the three-chamber arena makes it easier than a single-chamber test arena to dissociate the choice of entry either

into the chamber containing the familiar stimulus, the chamber containing the novel stimulus, or the empty middle chamber, and it simplifies the automated measurements of these choices (Nadler et al., 2004).

Critical Parameters

Pup retrieval

In principle, every mouse strain should be suitable for pup retrieval, but there might be differences in the responsiveness and strength of the caring behavior between strains. We recommend using the CBA/CaJ strain, which is known for its breeding and maternal qualities as well as a comparably late hearing loss onset (Zheng et al., 1999).

The pups used for the assay should be between the age of P3 and P7 to make sure they are not too weak for the behavioral test but still young enough to be immobile to not cluster together on their own during the assay. Moreover, do not choose pups lying outside the nest, since the mother sometimes isolates dying or unhealthy pups and would most probably not retrieve the pup in the behavioral test. It is also very helpful to co-house a naïve virgin together with the pregnant animal before parturition. This ensures alloparental care of the pups during the behavioral test of the mothers and provides the additional possibility to extend the behavioral test on experienced virgins. If you use the same pups for repeated behavioral tests, make sure to let the pups rest for at least 20 min between retrieval sessions to reduce stress and hypothermia.

Sociability

Behavioral parameters vary according to age, sex, and strain. This has to be taken into account when designing the experiment. Also the experimenter's performance, including level of experience and training, especially when measuring behavior by a handheld computer, can have an effect on the results. As such, it is highly recommended to train with a small cohort of non-experimental animals when doing it for the first time. This improves the organization of the workflow, workspace, and confidence in the whole procedure.

When comparing two groups (e.g., mutants and wild-types) it is always important that the groups are tested concurrently to control for circadian rhythms and any other potentially confounding effects of the testing day (e.g., the experimenter's experience and training). Males and females should not be tested concurrently as the presence of the other sex may influence behavior. For this reason we test males and females on subsequent days, at the same time of day whenever possible.

Any exceptional stress to the animals (e.g., cage change, noise, increased handling prior to testing) should be avoided.

Troubleshooting

Pup retrieval

One particular problem that sometimes occurs while testing naïve animals is attacks by the adult animals towards the pups. However, this is more common for naïve males. Terminate the experiment immediately if you observe an adult animal attacking pups.

Ideally, a clean and neutral cage is used for the behavioral test to ensure comparability between different animal groups. However, sometimes mothers, sires, or experienced virgins do not retrieve pups in unfamiliar cages. In that case, the behavioral assay could also be performed in the respective home-cage of the tested mouse.

Sociability

The cylinders should always be sealed with a lid, as mice can climb the bars. Usually, it is the experimental animal that climbs the bars and not the stimulus animal (OVX 129SV females) in our experience. Nonetheless it is necessary to record on the data sheet if an animal has climbed, as it might influence results. Generally the mice seldom climb the bars, and even less frequently it is required to induce them to descend by presenting a hand above the cylinder and, if necessary, guiding the animal downwards.

If juvenile animals are showing aggressive behavior (e.g., tail rattling, a fast shaking movement of the tail), they should be replaced by younger animals. If an animal is not detected by the video tracking system, adjust the tracking settings and ensure that there are no blind angles due to camera settings.

Make sure you do not get any reflections from the walls or the floor, because these could be detected by the video tracking system and lead to false tracking of the mouse. It might be possible to avoid this by narrowing the borders of the arena and zones, so that reflections are outside the relevant area.

It is advisable to test in a separate cohort of mice if there is a general side preference to one of the outer chambers. This could be the case if illumination is not equal, if one chamber is closer to the experimenter compared to the other, or if there are any external sensory cues that interfere with the test.

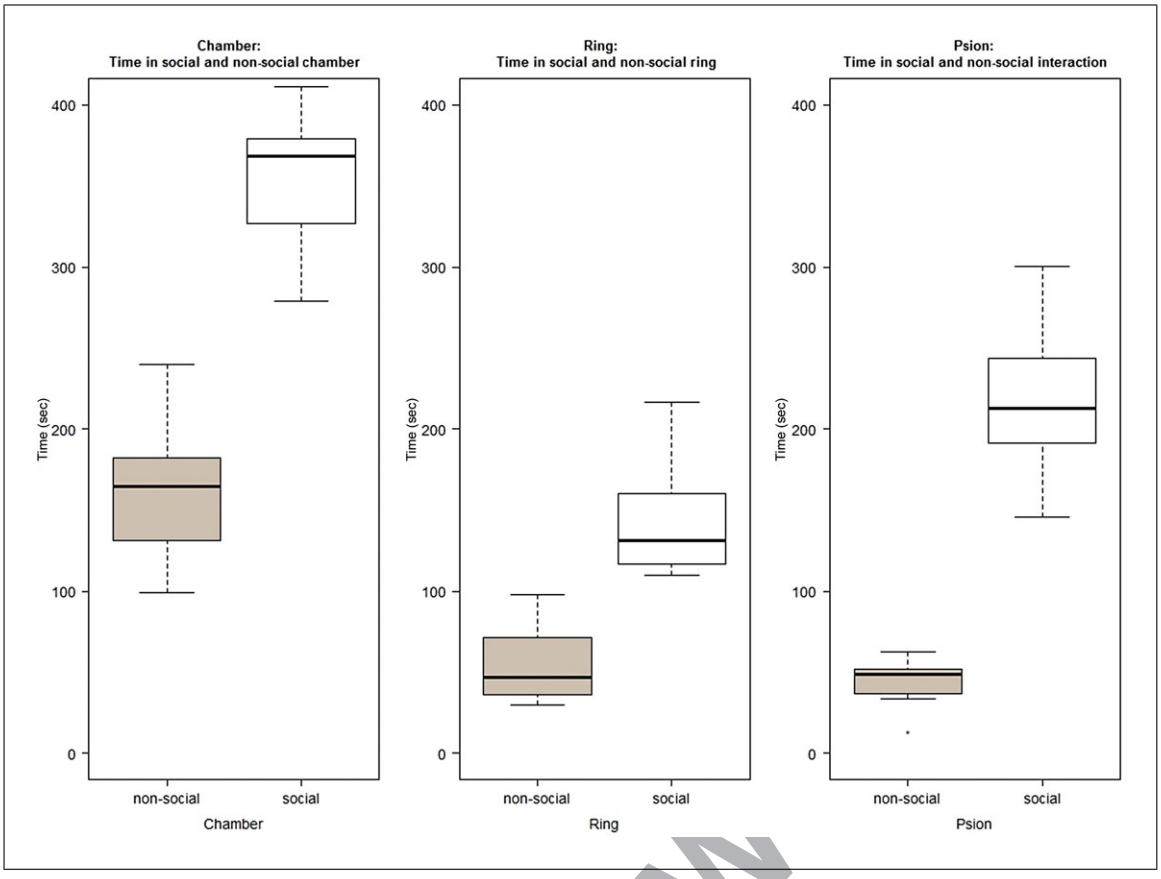


Figure 3 Example data of time spent on different sides obtained by 12 male C57BL/6N mice tested as in Basic Protocol 2. The graphs show the time the experimental animals spent within the sociability phase at the "social" or "non-social" side. Different data extraction methods are shown: "chamber" data collected with EthoVision of the whole "social" or "non-social" chamber, "ring" data collected by EthoVision when the center of the mouse's body is within a 3-cm broad ring around the cylinders, and "psion" data collected by a handheld computer scoring contact with either "social" or "non-social." For all extractions methods the difference between respective "social" and "non-social" are significantly different, as tested by paired *t*-tests (chamber: $t = 9.021$, df $= 11$, $p < .001$; ring: $t = 10.472$, df $= 11$, $p < .001$; psion: $t = 12.962$, df $= 11$, $p < .001$).

If the experimental animals do not spend longer times in the chamber with the stimulus animal, check the habituation phase for side preferences. If there is a clear preference for one side, make sure to eliminate any possible causes that could exert such an influence (see above). If the obtained results of the (control) group do not match the anticipated results, a possible explanation might lie in alterations of olfactory sensitivity. Especially if animals are older, olfactory insensitivity might occur, and this should be evaluated (e.g., see Basic Protocol 5 of Hölter et al., 2015).

Anticipated Results

Pup retrieval

From previous studies employing the pup retrieval assay including mothers, experienced virgins, and naïve virgins, it would be expected that 90% to 100 % of the mothers and experienced virgins retrieve all pups while naïve virgins do not retrieve pups (Cohen et al., 2011; Marlin, Mitre, D'Amour, Chao, & Froemke, 2015). The response latency for pup retrieval is expected to be a bit longer for experienced virgins.

Sociability

Intact social behavior is represented by a significantly longer investigation time of the stimulus animal compare to the "non-social" object (cylinder or dummy mouse) in the sociability phase (Fig. 3). A sociability index (Fig. 4) can be calculated as time spent with stimulus animal ("social") divided by sum of the time spent with object ("non-social") plus stimulus animal ("social") × 100.

Social memory

Intact social memory is indicated by a preference for the chamber with the previously unknown juvenile (or unknown OVX female) and by higher interaction times.

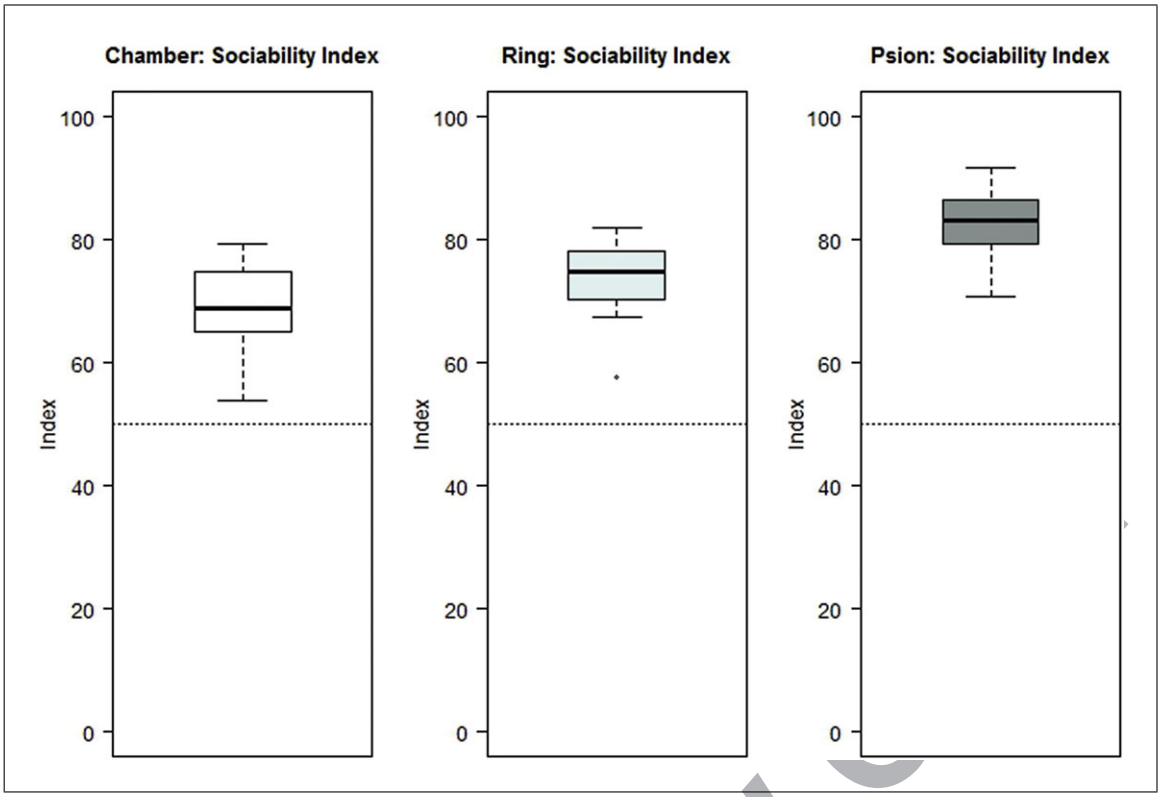


Figure 4 Example data of the sociability index obtained by 12 male C57BL/6N mice tested as in Basic Protocol 2. The graphs show the sociability index. Different data extraction methods are shown: "chamber" data collected with EthoVision of the whole "social" or "non-social" chamber, "ring" data collected by EthoVision when the center of the mouse's body is within a 3-cm broad ring around the cylinders, and "psion" data collected by a handheld computer scoring contact with either "social" or "non-social." At 50% (dotted line) the animals would spend the same amount of time at the "social" and "non-social" side. Values above 50% depict a preference for "social." With a one sample *t*-test, the significant difference to 50% is analyzed. All extraction methods show a significant difference (chamber: $t = 8.9342$, df $= 11$, $p < .001$; ring: $t = 12.487$, df $= 11$, $p < .001$, psion: $t = 19.511$, df $= 11$, $p < .001$).

Time Considerations

Pup retrieval (Basic Protocol 1)

The time needed to perform the experiments depends mainly on the number of pups available for the behavioral test. With 5 pups, testing 1 animal takes about 40 min including the 20 to 30 min habituation time and another 5 to 10 min for the actual test. During the resting period of the pups, the next adult animal can be placed into the test cage for habituation. The test can then start ~60 min after the beginning of the first habituation. In this manner, 3 to 4 animals can be tested within 2 hr. If 10 pups are available, the behavioral assays can be interleaved so that almost double the number of animals can be tested in the same time.

Three-chamber sociability cage (Basic Protocol 2 and Alternate Protocol 1)

Using one sociability cage with video tracking (and scoring by a handheld computer), 1 person can test 8 mice in 4 hr using this protocol. These calculations include testing and cleaning time; additional time has to be considered for preparation before testing and for data analysis as well as quality control.

Three-chamber sociability test, social memory test (Alternate Protocol 2 and Support Protocol 1)

In our experience it is possible for one person to run up to four tests in parallel with slight time delay. This means that on the testing day (the most time consuming day) it would be possible to test up to 32 mice for the three-chamber sociability test, or 16 mice when the test is combined with the social memory test.

Video

The video discussed in this article can only be accessed from the online version of this article.

Acknowledgements

We thank the team of animal care takers of the German Mouse Clinic at Helmholtz

Zentrum München for sustained excellent cooperation. This work has been funded in part by the German Federal Ministry of Education and Research (Infrafrontier grant 01KX1012) and the German Research Foundation (DFG) Collaborative Research Centre 870 (SFB 870).

Literature Cited

Berton, O., McClung, C. A., Dileone, R. J., Krishnan, V., Renthal, W., Russo, S. J., ... Nestler, E. J. (2006). Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science*, *311*, 864–868. doi: 10.1126/science.1120972

Bielsky, I. F., & Young, L. J. (2004). Oxytocin, vasopressin, and social recognition in mammals. *Peptides*, *25*, 1565–1574. doi: 10.1016/j.peptides.2004.05.019

Brooks, L. R., Le, C. D., Chung, W. C., & Tsai, P. S. (2012). Maternal behavior in transgenic mice with reduced fibroblast growth factor receptor function in gonadotropin-releasing hormone neurons. *Behavioral and Brain Functions: BBF*, *8*, 47. doi: 10.1186/1744-9081-8-47

Cohen, L., Rothschild, G., & Mizrahi, A. (2011). Multisensory integration of natural odors and sounds in the auditory cortex. *Neuron*, *72*, 357–369. doi: 10.1016/j.neuron.2011.08.019

Dulac, C., O'Connell, L. A., & Wu, Z. (2014). Neural control of maternal and paternal behaviors. *Science*, *345*, 765–770. doi: 10.1126/science.1253291

Ferguson, J. N., Young, L. J., & Insel, T. R. (2002). The neuroendocrine basis of social recognition. *Frontiers in Neuroendocrinology*, *23*, 200–224. doi: 10.1006/frne.2002.0229

File, S. E., & Seth, P. (2003). A review of 25 years of the social interaction test. *European Journal of Pharmacology*, *463*, 35–53. doi: 10.1016/S0014-2999(03)01273-1

Gheusi, G., Bluthe, R. M., Goodall, G., & Dantzer, R. (1994). Social and individual recognition in rodents: Methodological aspects and neurobiological bases. *Behavioural Processes*, *33*, 59–87. doi: 10.1016/0376-6357(94)90060-4

Golden, S. A., Covington, H. E., 3rd, Berton, O., & Russo, S. J. (2011). A standardized protocol for repeated social defeat stress in mice. *Nature Protocols*, *6*, 1183–1191. doi: 10.1038/nprot.2011.361

Hartmann, J., Wagner, K. V., Dedic, N., Marinescu, D., Scharf, S. H., Wang, X. D., ... Schmidt, M. V. (2012). Fkbp52 heterozygosity alters behavioral, endocrine and neurogenetic parameters under basal and chronic stress conditions in mice. *Psychoneuroendocrinology*, *37*, 2009–2021. doi: 10.1016/j.psyneuen.2012.04.017

Hölter, S. M., Garrett, L., Einicke, J., Sperling, B., Dirscherl, P., Zimprich, A., ... Wurst, W. (2015). Assessing cognition in mice. *Current Protocols in Mouse Biology*, *5*, 331–358. doi: 10.1002/9780470942390.mo150068

Insel, T. R., & Winslow, J. T. (1998). Serotonin and neuropeptides in affiliative behaviors. *Biological Psychiatry*, *44*, 207–219. doi: 10.1016/S0006-3223(98)00094-8

Kohl, C., Wang, X. D., Grosse, J., Fournier, C., Harbich, D., Westerholz, S., ... Schmidt, M. V. (2015). Hippocampal neuroligin-2 links early-life stress with impaired social recognition and increased aggression in adult mice. *Psychoneuroendocrinology*, *55*, 128–143. doi: 10.1016/j.psyneuen.2015.02.016

Krasnegor, N. A., & Bridges, R. S. (1990). *Mammalian parenting: Biochemical, neurobiological and behavioural determinants*. Oxford, United Kingdom: Oxford University Press.

Liu, R. C., & Schreiner, C. E. (2007). Auditory cortical detection and discrimination correlates with communicative significance. *PLoS Biology*, *5*, e173. doi: 10.1371/journal.pbio.0050173

Marlin, B. J., Mitre, M., D'Amour, J. A., Chao, M. V., & Froemke, R. C. (2015). Oxytocin enables maternal behaviour by balancing cortical inhibition. *Nature*, *520*, 499–504. doi: 10.1038/nature14402

Masana, M., Su, Y. A., Liebl, C., Wang, X. D., Jansen, L., Westerholz, S., ... Muller, M. B. (2014). The stress-inducible actin-interacting protein DRR1 shapes social behavior. *Psychoneuroendocrinology*, *48*, 98–110. doi: 10.1016/j.psyneuen.2014.06.009

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., ... Crawley, J. N. (2004). Sociability and preference for social novelty in five inbred strains: An approach to assess autistic-like behavior in mice. *Genes, Brain, and Behavior*, *3*, 287–302. doi: 10.1111/j.1601-1848.2004.00076.x

Nadler, J. J., Moy, S. S., Dold, G., Trang, D., Simmons, N., Perez, A., ... Crawley, J. N. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes, Brain, and Behavior*, *3*, 303–314. doi: 10.1111/j.1601-183X.2004.00071.x

Noirot, E. (1966). Ultra-sounds in young rodents. I. Changes with age in albino mice. *Animal Behaviour*, *14*, 459–462. doi: 10.1016/S0003-3472(66)80045-3

Noirot, E. (1972). Ultrasounds and maternal behavior in small rodents. *Developmental Psychobiology*, *5*, 371–387. doi: 10.1002/dev.420050410

Numan, M., & Insel, T. R. (2003). *The neurobiology of parental behavior* (Vol. *1*). New York: Springer.

Richter, K., Wolf, G., & Engelmann, M. (2005). Social recognition memory requires two stages of protein synthesis in mice. *Learning & Memory*, *12*, 407–413. doi: 10.1101/lm.97505

Sewell, G. D. (1970). Ultrasonic communication in rodents. *Nature*, *227*, 410. doi: 10.1038/227410a0

Sheleg, M., Yu, Q., Go, C., Wagner, G. C., Kusnecov, A. W., & Zhou, R. (2017). Decreased maternal behavior and anxiety in ephrin-A5-/-

mice. *Genes, Brain, and Behavior*, *16*, 271–284. doi: 10.1111/gbb.12319

Silva, M. R., Bernardi, M. M., & Felicio, L. F. (2001). Effects of dopamine receptor antagonists on ongoing maternal behavior in rats. *Pharmacology, Biochemistry, and Behavior*, *68*, 461–468. doi: 10.1016/S0091-3057(01)00471-3

Umemura, S., Imai, S., Mimura, A., Fujiwara, M., & Ebihara, S. (2015). Impaired maternal behavior in Usp46 mutant mice: A model for transgenerational transmission of maternal care. *PLoS One*, *10*, e0136016. doi: 10.1371/journal.pone.0136016

Winslow, J. T. (2003). Mouse social recognition and preference. *Current Protocols in Neuroscience*, *22*, 8.16.1-8.16.16. doi: 10.1002/0471142301.ns0816s22

Zheng, Q. Y., Johnson, K. R., & Erway, L. C. (1999). Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hearing Research*, *130*, 94–107. doi: 10.1016/S0378-5955(99)00003-9