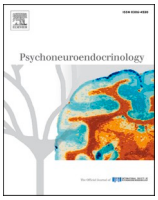
Psychoneuroendocrinology 143 (2022) 105859

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen



Social recognition in laboratory mice requires integration of behaviorally-induced somatosensory, auditory and olfactory cues

Shani Haskal de la Zerda, Shai Netser, Hen Magalnik, Mayan Briller, Dan Marzan, Sigal Glatt, Yasmin Abergel, Shlomo Wagner *

Sagol Department of Neurobiology, Integrated Brain and Behavior Research Center (IBBR), University of Haifa, Haifa, Israel

ARTICLE INFO

Keywords:
Olfactory signature
Sensory integration
Sex discrimination
Social behavior
Social recognition
Social whisking

ABSTRACT

In humans, discrimination between individuals, also termed social recognition, can rely on a single sensory modality, such as vision. By analogy, social recognition in rodents is thought to be based upon olfaction. Here, we hypothesized that social recognition in rodents relies upon integration of olfactory, auditory and somatosensory cues, hence requiring active behavior of social stimuli. Using distinct social recognition tests, we demonstrated that adult male mice do not exhibit recognition of familiar stimuli or learn the identity of novel stimuli that are inactive due to anesthesia. We further revealed that impairing the olfactory, somatosensory or auditory systems prevents behavioral recognition of familiar stimuli. Finally, we found that familiar and novel stimuli generate distinct movement patterns during social discrimination and that subjects react differentially to the movement of these stimuli. Thus, unlike what occurs in humans, social recognition in mice relies on integration of information from several sensory modalities.

1. Introduction

The ability to recognize or discriminate between individual conspecifics is crucial for the survival of members of gregarious species, as such ability guides appropriate interactions of these individuals with their social environment (Kendrick, 2006; Wiley, 2013). In the literature, social recognition is used as a generic term for both the ability of a subject to categorize conspecifics into different classes, such as sex, genetic relatedness and familiarity (henceforth termed social recognition), as well as for the ability to recall the learned idiosyncratic identity of a specific individual based on a previous encounter, also termed individual recognition (Choleris et al., 2009; Gheusi et al., 1994). In humans, social recognition can be based on cues detected by single sensory modalities. For example, humans can recognize a familiar person just by looking at their face (visual modality) or hearing their voice (auditory modality) (Anzellotti and Caramazza, 2017; Bruce and Young, 1986). Moreover, human social recognition can occur even without active engagement with a social partner, such as by looking at a sleeping individual. Such single-modality based social recognition appears to also hold true for other primates (Parr et al., 2000). The generality of this ability among mammals remains, however, unclear.

Mice and rats, the main mammalian laboratory models used in biomedical research (Russell, 2004), are social species known to exhibit social recognition (Brennan and Kendrick, 2006; Camats Perna and Engelmann, 2017; Kavaliers and Choleris, 2017; Yamazaki and Beauchamp, 2005). Specifically, during social interactions, these animals display higher investigative behavior towards novel conspecific individuals (henceforth termed social stimuli), as compared to those with whom they are familiar (Thor et al., 1982). Thus, in a social discrimination test, shorter times are dedicated by subjects for investigating a familiar stimulus, as compared to a novel one, reflecting recognition of the familiar stimulus (Engelmann et al., 1995). This type of social recognition, which is frequently used in the field of social neuroscience to assess typical social behavior (Kas et al., 2014), is widely assumed to be mediated by chemosensory cues released by the stimulus and received by the main and accessory olfactory systems of the subject (Brennan and Kendrick, 2006; Camats Perna and Engelmann, 2017; Yamazaki and Beauchamp, 2005). Therefore, in analogy to the human face, the identity of a social stimulus is thought to be represented by the passive signature of chemosensory cues (i.e. the olfactory signature), which distinguishes conspecifics (Camats Perna and Engelmann, 2017; Sanchez-Andrade and Kendrick, 2009). Still, despite reports that social recognition is impaired in anosmic animals (Dantzer et al., 1990; Matochik, 1988; Popik et al., 1991), the reliance of social recognition in

* Corresponding author.
E-mail address: shlomow@research.haifa.ac.il (S. Wagner).

https://doi.org/10.1016/j.psyneuen.2022.105859
Received 9 July 2021; Received in revised form 30 June 2022; Accepted 30 June 2022
Available online 5 July 2022

mice and rats solely on chemosensory cues has yet to be proven. Moreover, recent work showed that rat hippocampal CA1 pyramidal neurons respond to social cues, triggering the somatosensory and auditory systems in a social stimulus-specific manner (Rao et al., 2019) and that touch may by a crucial component of the social reward associated with social place preference (Kummer et al., 2011). Furthermore, a recent study showed that complex social stimuli allowing multimodal sensory stimulation attract more investigation by mouse subjects than social olfactory cues alone (Contestabile et al., 2021). These studies raise the possibility that mice and rats integrate multimodal information during social interactions which can serve as a more complex basis for social recognition than the olfactory signature alone (Rogers-Carter and Christianson, 2019).

In this study, we challenged the common assumption that social recognition in mice and rats is solely based upon chemosensory cues. We instead hypothesized that social recognition relies upon the integration of olfactory, auditory and somatosensory cues, hence requiring active behavior of social stimuli.

2. Materials and methods

2.1. Animals

Both mice and rats were commercially obtained (Envigo, Israel). Mice subjects were naïve C57BL/6 J adult (8–15 week-old) male mice, while stimuli mice were C57BL/6 J juvenile (21–30 day-old) male mice, naïve adult male and female C57BL/6 J mice, ICR (CD-1) male mice and Balb/C male mice (the latter two strain were used for social fear conditioning only). Mice were housed in groups of 2–5 per cage, at least one week prior to the test, on a 12 h light/12 h dark cycle, with lights being turn on at 7 p.m. each night. Rat subjects were adult (10–15 week-old) Sprague Dawley (SD) males, while stimuli were juvenile (21–30 day-old) SD male rats and adult SD male and female rats. Rats were kept in groups of 2–5 animals per cage, on a 12 h light/12 h dark cycle, with lights being turned on at 9 p.m. each night. All animals had ad libitum access to food (standard chow diet; Envigo RMS, Israel) and water. Behavioral experiments were performed during the dark phase, under dim red light. All experiments were performed according to the National Institutes of Health guide for the care and use of laboratory animals, and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Haifa.

2.2. Anesthesia

Mice were anesthetized with an intraperitoneal (i.p.) injection of a mixture of 100 mg/kg ketamine (100 mg/ml Clorketam, v′etoquinol) and 0.8 mg/kg medetomidine (1 mg/1 ml Domitor, Orion Pharma) in sterile saline (0.1 ml mix anesthesia/10 g BW). At the end of the session, mice were awakened with an injection of 0.1 ml/10 g BW atipamezole (4 mg/kg) in sterile saline (5 mg/ml Antisedan). Rats were first lightly anesthetized in a ventilated box with a few drops of 99.9% isoflurane, followed by subcutaneous injections of both 0.5 mg/kg medetomidine (0.05 ml/100 g BW) and 100 mg/kg ketamine (0.1 ml/100 g BW).

2.3. Experimental setups

2.3.1. Social discrimination

The experimental setups used for all types of social discrimination in both mice and rats have been previously described in detail (Netser et al., 2019). Briefly, each setup consisted of a Plexiglas arena placed in the middle of an acoustic chamber. Two Plexiglas triangular chambers were placed in two randomly selected opposing corners of the arena, in which an animal or an object (plastic toy) stimulus was placed. A metal mesh placed at the bottom of the triangular chamber allowed direct interaction with the stimulus through the mesh. A high-quality monochromatic camera (Flea3 USB3, Point Grey) equipped with a wide-angle lens was placed at the top of the acoustic chamber and connected to a computer, enabling a clear view and recording of subject behavior using commercial software (FlyCapture2, Flir).

2.3.2. Social fear conditioning

The setup used for social fear conditioning was a custom-made white Plexiglas arena similar in size to the main experimental setup (37 ×22×35 cm) but with a metal grid floor (H10–11 M, Coulbourn Instruments) connected to an electrical shock-delivering unit (precision regulated animal shocker H13–14, Coulbourn Instruments). The unit was modified to deliver a single pulse of 750 ms when manually triggered.

2.4. Behavioral paradigms

2.4.1. Familiarity discrimination test

The social discrimination paradigm consisted of 15-min habituation of subject mice to the arena containing two empty chambers. Simultaneously, stimuli mice were introduced into chambers outside the arena for acclimation. After habituation, the social discrimination test was conducted for 5 min when the subject was simultaneously introduced to social stimuli (one cage-mate [CM] and one novel stimuli) placed at opposite corners of the arena. When anesthetized stimuli were used, such individuals were anesthetized 15 min before the test and were kept anesthetized on a heating pad at 37 ℃ until the end of the experiment and between experiments. The stimuli received an additional dose of the anesthetic (33% of the initial dose) when signs of awakening appeared (i.e., whisker movement). At the end of an experiment, the stimuli were awakened by an injection of atipamezole and placed on the heating pad until fully awakened.

2.4.2. Sex discrimination test

Similar to the familiarity discrimination test (2.4.1) but using adult male and females social stimuli.

2.4.3. Social Preference/Social Novelty Preference paradigm

The SP/SNP paradigm was previously described in detail (Netser et al., 2019, 2017). Briefly, the paradigm involved a 15-min window of subject mice habituation to the arena presenting two empty chambers. Thereafter, social and object stimuli were randomly introduced to distinct corners of the arena, with the SP test being performed for 5 min. Upon termination of the SP test, the chambers housing the stimuli were removed from the arena, and the subject was left alone for 15 min. Then, the chambers were returned, this time to the other two corners of the arena, with one containing the same social stimulus used in the SP test (now as a familiar stimulus) and the other containing a novel one. At this point, the SNP test was performed for 5 min. Notably, the familiar stimulus was always placed in a different corner, relative to its position in the SP test. At the end of the SNP test, the subject was returned to its home cage, while stimuli were either left in the chambers for additional experiments or returned to their home cages.

Free interaction paradigm- a modification of the SP/SNP test described above, which contain a 5-min free interaction with juvenile stimulus without restriction instead of the SP test, after 15-min habituation to empty arena. 15 min after the free interaction, the subject perform the SNP test as described above, when the novel and familiar juvenile stimuli are confined to the triangular chambers.

2.4.4. Social fear conditioning paradigm

The SFC paradigm with mice consisted of 15-min habituation of the subjects to the arena presenting two empty chambers, followed by two consecutive SP tests with social stimuli of two distinct strains (C57BL/6 J and either ICR or Balb/C), separated by a 15-min interval. Thereafter, the subject was transferred to the social fear conditioning arena for 15-min habituation, followed by 5 min of the SFC procedure, in which the subject received a mild electrical foot shock (0.3–0.4 mA, 750 mSec)

each time it tried to interact with the stimulus chamber (ICR or Balb/C strains). In cases where ICR mice were used, five min after conditioning the subject was returned to the experiment arena for 15-min habituation and two more consecutive SP tests, performed as before conditioning. In the case where Balb/C mice were used, the animals also conducted a single SP test using either the C57BL/6 J or the Balb/C stimulus 24 h following the SFC session.

2.5. Behavioral data analysis

Data analysis was performed using our custom-made TrackRodent software, as previously described (Netser et al., 2019, 2017).

2.6. Measuring movement of social stimulus using piezoelectric sensors

Setup - Movements of stimuli animals were measured using six piezoelectric ceramic discs (27 mm in diameter) connected in parallel, as previously described (Netser et al., 2020). Briefly, the discs were evenly distributed along the triangulated Plexiglass floor and adjusted using lamination foil. Signal from the piezo-discs were transferred to the analog input of a RHD2000 recording system (Intan Technologies) through a protective metal tube fixed to the inner wall of the triangulated chamber.

Analysis - All signals were analyzed using a custom-made MATLAB analysis program. Raw signals were recorded at 20 kHz. Signals were then down-sampled to 2000 Hz and band-pass filtered between 10 and 100 Hz using a Butterworth filter. Large movements were detected using a threshold of 10–30% of the maximum signal absolute value. For detecting a subject's tendency to investigate a stimulus animal after movement of the latter, we analyzed all periods meeting the criteria of no social investigation by the subject and no large movements by the stimulus for at least 4 s before a given movement by that animal. Varying this period between 2 and 8 s did not change the final results. For statistical analysis, the total investigation time within 4-sec window after the movement was considered for calculating the mean investigation time.

2.7. Modality impairment

2.7.1. Whisker tearing

Mice were lightly anesthetized (using 0.1 ml of the anesthesia mixture described above per mouse) and their whiskers were pulled off from both sides using tweezers and duct tape until all whiskers were completely removed.

2.7.2. Hearing loss

Mice (5 week-old) received a daily i.p. injection of 2 ml/kg BW gentamicin (50 mg/ml gentamicin sulphate, Biological Industries) for one week (Chen et al., 2012; Heydt et al., 2004). Tests were performed 1–2 weeks after the end of the injection period. Control mice received saline injections in the same manner.

2.7.3. Anosmia

Mice (8 week-old) received a single i.p. injection of 10 ml/kg BW methimazole (MMZ, Sigma-Aldrich) dissolved (10 mg/ml) in sterile double-distilled water (Blanco-Hernandez et al., 2012; Xie et al., 2011). Mice were subjected to all tests before receiving MMZ (control), and 1 week after MMZ treatment.

2.8. Tissue preparation and immunostaining

2.8.1. Fixating and sectioning

Mice were perfused following i.p. injection of a ketamine and medetomidine mix (overdose of a 0.8 ml anesthesia mixture per mouse). Twenty ml of saline were passed through the heart, followed by 20 ml of 4% paraformaldehyde (PFA). Both cochleae were removed and placed overnight in 4% PFA at 4 °C. For dissection of the nasal cavity, the mandibula was removed and the rest of the skull was placed overnight in 4% PFA. The following day, the cochleae and skulls were added to tubes containing 0.5 M EDTA, pH 8 (Sigma-Aldrich) for decalcification (~5 days for cochlea, ~7 days for skull). After the bones were decalcified, the organ of Corti was extracted from each cochlea and placed in Peel-A-Way embedding mold (Sigma-Aldrich) with 4% agar, positioned with apex facing upwards. The decalcified skulls were placed in the Peel-A-Way embedding mold with 4% agar, positioned with nostril facing up. Coronal sections (100 μm-thick) were sliced using a vibratome (Leica VT1200 S) under a magnifying binocular. For slicing the organ of Corti, the knife amplitude was 0.7 mm and the slicing speed was 0.02 mm/sec; for slicing the nasal cavity, the knife amplitude was 0.5 mm and the slicing speed was 0.01 mm/sec.

2.8.2. Immunostaining for olfactory marker protein (OMP)

Sections containing the vomeronasal organ (VNO), main olfactory epithelium (MOE) and the olfactory bulb were processed for immunostaining using the following protocol. After a 30 min incubation in 0.3% Triton X-100 in PBS (PBS-t), the sections were incubated in blocking mix containing 20% normal goat serum (NGS) in PBS-t for 2 h. Then, the sections were placed in primary antibody mix (2% NGS, mouse monoclonal αOMP antibodies (1:500; Santa Cruz) and PBS-t) overnight at 4^0 C. The next day, the sections were washed 3 times for 10 min each with PBS, and then incubated with Alexa 488-conjugated secondary antibodies in PBS (1:500; Abcam) for 2 h. The sections were subsequently washed 3 times with PBS, incubated for 3 min in DAPI solution (1:2000 DAPI (20 mg/ml); Sigma-Aldrich) and then washed again 3 times with PBS. The sections were placed on a slide (25 ×75×1.0 mm, superfrost plus, Fisherbrand) and once completely dry, were covered with mounting medium (Vectashield Hardset) and a coverslip (24 ×60 mm, Menzel-Glaser).

2.8.3. Phalloidin staining

Sections from the organ of Corti were incubated for 1 h at room temperature in a mix solution containing 10% NGS, phalloidin conjugated with Alexa 488 (1:2000, Abcam) and PBS. After incubation, the sections were washed 3 times with PBS for 10 min each and then counter-stained with DAPI in the same manner as described above.

2.8.4. Image analysis

All fluorescence images were acquired with a Nikon A1-Red Confocal Microscope using 10 × , 20x, and 40x objectives. To measure the mean fluorescence intensity (MFI), regions of interest (ROI) of the same size (230 μm x 166 μm for the organ of Corti; 379 μm x 133 μm for the MOE; 912 μm x 757 μm for the VNO) was reconstructed using ImageJ analysis software. Average mean gray values (mean±SEM, arbitrary units) were calculated from 3 random sections from each subject, subtracting background values for the same image and normalizing by dividing the values obtained by the average mean fluorescence intensity of the background. For quantification following gentamicin treatment, 3 mice served as controls and 5 mice were injected with gentamicin. For quantification of the MOE following MMZ treatment, 3 mice served as controls and 4 mice were injected with MMZ. For quantification of the VNO following MMZ treatment, 4 mice served as controls and 3 mice were injected with MMZ.

2.9. Statistical analysis

All frequentists statistical tests were performed using SPSS v21.0 (IBM) and the Bayesian matched statistics were performed using JASP v0.14.1. A Kolmogorov-Smirnov test was used to confirm the normal distribution of dependent variables. A one-tailed paired t-test was used to compare different conditions or stimuli for the same group, and a two-tailed independent t-test was used to compare a single parameter between two distinct groups. For comparing multiple groups and

parameters, a mixed analysis of variance (ANOVA) model was applied to the data. This model contained one random effect (ID), one within-effect (or two), one between-effect and the interaction between them (mixed three-way repeated ANOVA in the case of two within-repeated-factors and one between-factor). For comparison within a group using multiple parameters, a two-way (or three-way) repeated measures ANOVA model was applied to the data. This model contained one random effect (ID), two (or three) within-effects and the interactions between them. All ANOVA tests were followed, if the interaction was significant, by a post-hoc Student's t-test. Significance was set at 0.05. When the normal distribution of variables was rejected, an equivalent non-parametric test was performed on the variables. The Mann-Whitney U test was performed instead of the independent t-test for comparing two distinct groups, and the Kruskal-Wallis test was performed instead of one-way ANOVA to compare the effect of the independent variable on the dependent variable (with 3 levels). The Friedman test was performed instead of the ANOVA repeated measures test for comparing the effect of the repeated variable (each minute of the test) on the dependent variable (major movements). The Wilcoxon signed rank test was performed as a post-hoc test for the Friedman test, instead of the paired t-test for comparing two paired groups. All outliers were detected using SPSS and were excluded from the statistical analysis.

3. Results

3.1. Mice and rats do not exhibit behavioral discrimination between anesthetized novel and familiar social stimuli

To examine possible involvement of stimulus-related behavior in rodent social recognition, we employed our published behavioral system (Netser et al., 2017), which allows automated and precise detection of bouts of investigative behavior towards social and non-social stimuli (Fig. 1A). Using this system, we first analyzed the time dedicated by C57BL/6 J mice to investigate a CM and a novel social stimulus (Novel),

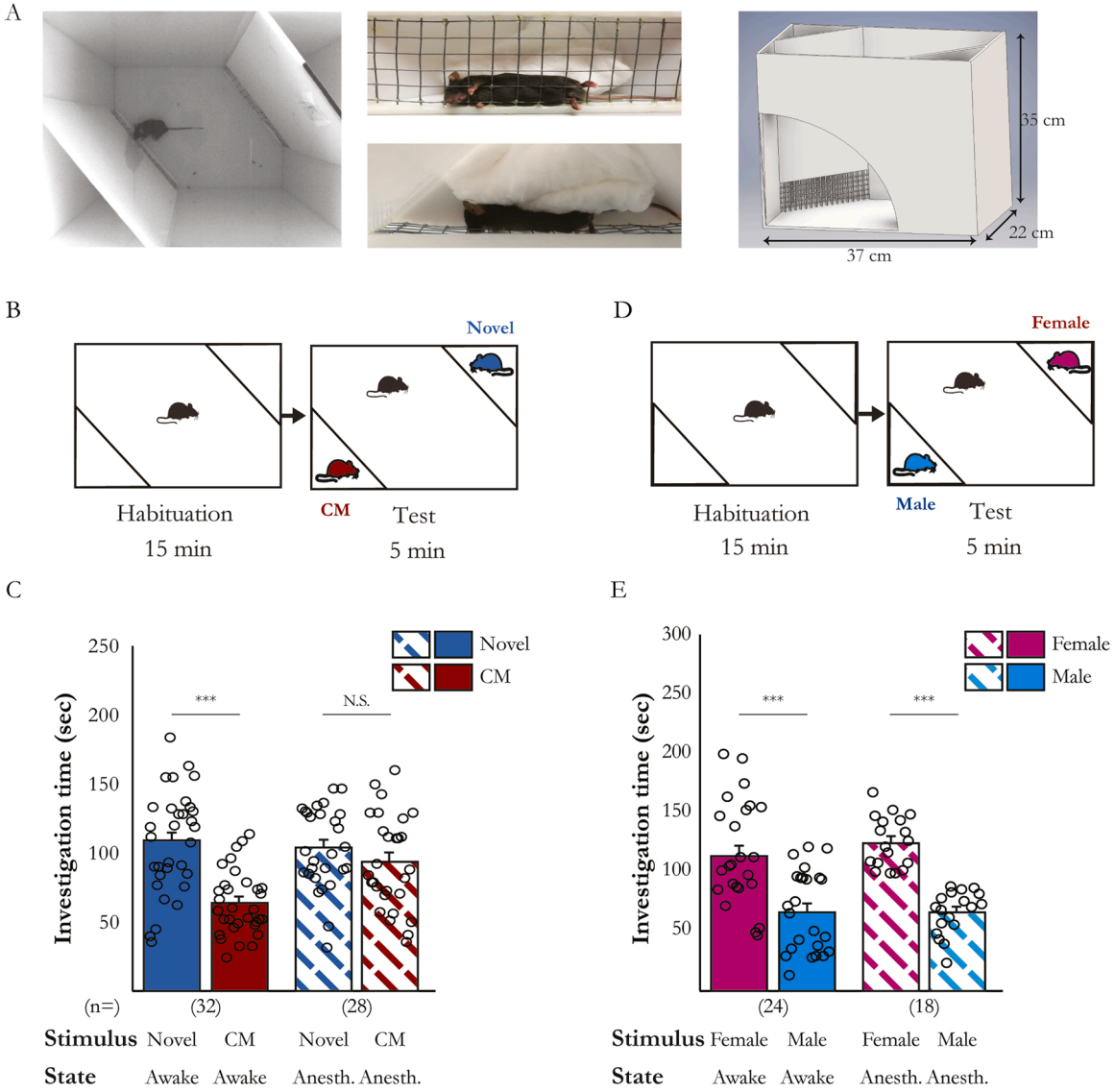


Fig. 1. Social but not sex recognition is impaired when social stimuli are anesthetized. (A)The behavioral arena, including triangular chambers for stimuli. The left panel shows a frame from the video recording of the experimental setup during an SP test. The middle panels provide a closer view on the position of anesthetized stimuli (front and upper views). The right panel shows a schematic description of the arena and chambers. (B)Schematic descriptions of the familiarity discrimination test. (C)Mean investigation time of awake (filled bars) and anesthetized (dashed bars) stimuli in the familiarity discrimination test. Number of tested subjects (n), stimulus type and state of stimulus are denoted below the bars. (D-E) Similarly to B-C, for sex discrimination. * **$p < 0.001$, post hoc 1-tail paired t-test following main effect in ANOVA.

each located in triangular chambers at opposite corners of the experimental arena (familiarity discrimination test; Fig. 1B). The subject mice exhibited a clear preference for the novel social stimulus over the CM when these stimuli were awake, as reflected by the significantly longer time the subjects investigated the novel stimulus (t_{31} =5.622, $p < 0.001$, 1-tailed paired t-test; Fig. 1C, filled bars). We then exploited the unique design of our system to spread anesthetized social stimuli over the metal mesh of the triangular chamber (Fig. 1A, middle panel), such that the ventral side of the anesthetized animal, including facial and anogenital regions, were accessible for investigation by the subject. We reasoned that if social recognition is solely based upon chemosensory signatures passively transmitted by social stimuli, social recognition should also transpire even with anesthetized stimuli. However, if active behavior of the stimulus is also required for social recognition, then subjects would not be able to distinguish between anesthetized stimuli. We found that mice subjects did not exhibit any preference for the novel stimulus, relative to the CM, when both stimuli were anesthetized (t_{27} =0.969, p = 0.171, 1-tailed paired t-test; Fig. 1C, dashed bars). Notably, the total time of investigation did not decrease when the stimuli were anesthetized (awake: 173.04 ± 7.45; anesthesia: 197.78 ± 6.42; t_{58} =−2.479, p = 0.016, 2-tailed t-test), suggesting that the subjects were interested in the anesthetized animals at least as much as they were in awake stimuli. To examine whether novelty-preference was significantly reduced by anesthesia, we performed a 2 Group (awake vs anesthesia) x 2 Stimuli (novel vs CM) mixed-model ANOVA, that confirmed the presence of a significant Group x Stimuli effect ($F_{1,58}$ =7.406, p = 0.009). Similar results were found for Sprague-Dawley (SD) rats using the same paradigm (Group x Stimuli: $F_{1,29}$ =12.516, p = 0.001, mixed-model ANOVA; post hoc: awake: t_{15} =4.536, $p < 0.001$; anesthesia: t_{14} =−0.716, p = 0.243, 1-tailed paired t-test; Fig. S1). Thus, from a behavioral point of view, no social novelty preference was observed in test subjects when both stimuli are anesthetized. To ensure that these results indeed reflect direct investigation behavior rather than just location of the subject near the stimuli, we reanalyzed the video recordings using a head-position based algorithm. In accordance with our previous study (Netser et al., 2017), the head-position based algorithm results which were identical to those obtained using the body-mass center based algorithm (Group x Stimuli x Algorithm interaction: $F_{1,58}$ =1.557, p = 0.217 mixed three-way repeated ANOVA; Awake: Stimuli x Algorithm interaction: $F_{1,31}$ =0.021, p = 0.886, Anesthetized: Stimuli x Algorithm interaction: $F_{1,27}$ =1.74, p = 0.198, two-way repeated ANOVA; Fig. S2).

As a control test, we examined whether the preference of male mice to investigate a female more than a male conspecific (an innate preference, termed sex discrimination; Fig. 1D) also depends upon the behavior of the stimuli. We found that C57BL/6 J male mice preferred a female over a male even when both stimuli were anesthetized (awake: t_{23} =3.584, p = 0.001; anesthesia: t_{17} =7.880, $p < 0.001$, 1-tailed paired t-test), with no significant difference between the two conditions (Group x Stimuli interaction: $F_{1,40}$ =0.410, p = 0.513, mixed-model ANOVA; Fig. 1E). Thus, at least for mice, sex discrimination does not rely on the behavior of the stimuli but rather most likely on chemosensory cues per se.

To make sure that the mouse subjects did not exhibit social novelty preference towards anesthetized stimuli even temporarily, we have analyzed their behavioral dynamics as previously described by us (Netser et al., 2019). We found no significant difference in investigation time between the anesthetized stimuli at any time point of the session (1-min bins), while between awake stimuli we found a significant difference throughout most of the session (Dynamics: Anesthetized: Stimuli: $F_{1,27}$ =1.39, p = 0.249; Awake: Stimuli: $F_{1,31}$ =30.895, $p < 0.001$, two-way repeated ANOVA, with no significant interaction between Stimuli x Time due to constant preference towards the novel in the awake group and no preference in the anesthetized group; Fig. S3A-H). In contrast, the dynamics observed during the sex discrimination test did not change between awake and anesthetized stimuli (Dynamics: Anesthetized: Stimuli x Time interaction: $F_{4,68}$ =0.174, p = 0.951; Awake: Stimuli x Time interaction: $F_{4,92}$ =0.870, p = 0.480, two-way repeated ANOVA; Fig. S3I-P).

We then checked whether both stimuli need to be awake so as to allow discrimination between CM and novel stimuli. We found that anesthetizing the CM while keeping the novel stimulus awake caused a lack of familiarity discrimination (t(14) = 0.911, p = 0.189, paired t-test; Fig. S4A), whereas normal discrimination was observed in the opposite case (t(38) = 3.282, p = 0.001; Fig. S4B). These results are in accordance with our hypothesis that anesthetizing the CM caused a lack of behavioral recognition by the subject, which considered the CM to be a novel stimulus (although other explanations may be applied - see 3.3). Accordingly, when two CMs, one awake and one anesthetized, were used as stimuli, the subject discriminated between them and investigated the anesthetized CM for significantly more time, as if the anesthetized CM was considered as a novel stimulus (t(18) = 3.413, p = 0.002, paired t-test; Fig. S4C). Such discrimination between CMs was not observed if one of them was injected with saline rather than the anesthetic (t(18) = 0.844, p = 0.205, paired t-test; Fig. S4D), thereby ruling out the possibility that alarm pheromones released following the injection prevented CM recognition. It should be noted that a recent study (Contestabile et al., 2021) showed that mice did not discriminate between awake and anesthetized novel juveniles simultaneously presented to them, suggesting that the anesthesia itself does not draw higher investigation time.

Altogether, these results suggest that social recognition in both mice and rats relies on the behavior of the social stimuli.

3.2. *Active behavior of social stimuli is required for subjects to learn their identity*

Our results thus far can be explained by a requirement for active behavior of social stimuli for social recognition. An alternative explanation could be that the anesthesia modified the chemosensory signature of the CM, such that it could no longer be recognized by the subject. If this, however, was the case, then the subject should still be able to learn the identity of anesthetized novel stimuli and recognize them afterwards, while they are still anesthetized. Therefore, we used the social preference (SP)/social novelty preference (SNP) paradigm (Fig. 2 A) (Netser et al., 2017) to determine whether mice learn to identify an anesthetized social stimulus. As is apparent from Fig. 2B, when awake stimuli were used, subject mice exhibited a clear preference towards the novel social stimulus in both the SP (upper panel, t_{38} =−3.692, $p < 0.001$, 1-tailed paired t-test; Fig. 2B; Fig. S5A-C) and SNP (lower panel, t_{37} =5.137, $p < 0.001$, 1-tailed paired t-test; Fig. 2B; Fig. S5J-L) tests, suggesting proper social recognition learning. In contrast, when anesthetized stimuli were used for both tests (Fig. 2 C), the subjects did not exhibit discrimination between the novel and familiar social stimuli in the SNP test (lower panel, t_{14} =0.648, p = 0.264, 1-tailed paired t-test; Fig. 2 C; Fig. S5M-O), despite their normal investigation behavior towards the same anesthetized stimulus during the SP test (upper panel, t_{14} =−4.906, $p < 0.001$, 1-tailed paired t-test; Fig. 2 C; Fig. S5D-F). This lack of behavioral discrimination between novel and familiar stimuli during the SNP test was also observed if both social stimuli were anesthetized during the SP test and awake during the SNP test (SP - t_{17} =−6.1, $p < 0.001$, 1-tailed paired t-test; SNP - t_{17} =−0.577, p = 0.285, 1-tailed paired t-test; Fig. 2D; Fig. S5G-I, P-R). When comparing these three groups in both the SP and SNP tests using a mixed-model ANOVA analysis, we found a significant interaction between the group and the preference towards the novel stimulus in both tests, suggesting that even though the subjects tend to investigate the anesthetized novel stimulus significantly more than the awake novel stimulus during the SP test, they cannot differentiate between the same anesthetized stimulus and a novel one in the SNP test (SP: Group x Stimuli: $F_{2,69}$ =4.109, p = 0.021; SNP: Group x Stimuli: $F_{2,68}$ =4.022, p = 0.022, mixed-model ANOVA; Fig. 2B-D).

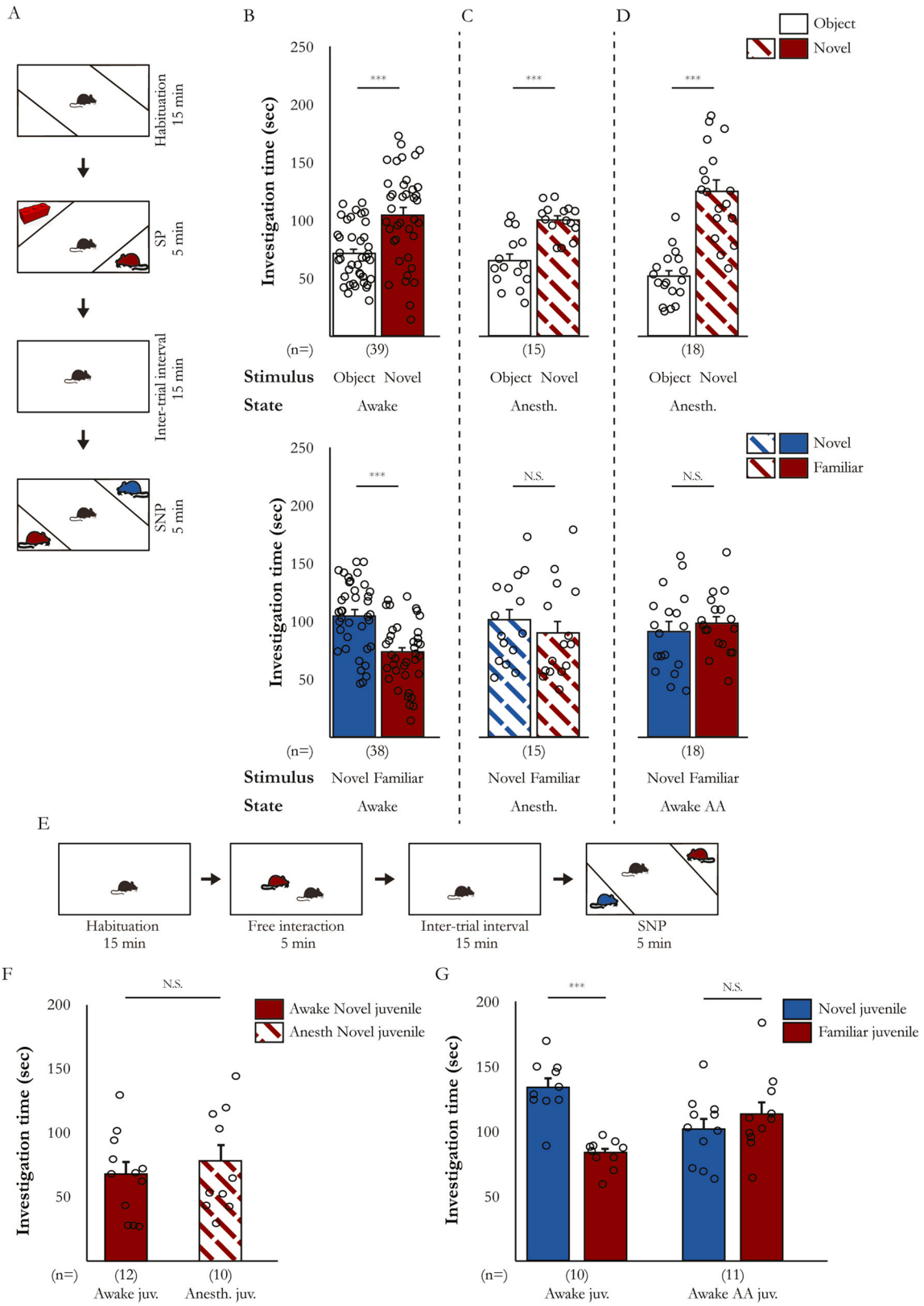


Fig. 2. Adult male mice do not learn to recognize a social stimulus while the stimulus is anesthetized. (A)Schematic description of the SP/SNP paradigm. (B)Mean investigation times during the SP (top) and SNP (bottom) tests, using awake stimuli in both tests. Number of tested subjects (n), stimulus type and state of stimulus are denoted below the bars.(C)As in B, using anesthetized stimuli in both tests.(D)As in B, with both social stimuli were anesthetized during the SP test and were awakened from anesthesia (Awake AA) during the SNP test. (E)Schematic description of the free interaction/SNP paradigm.(F)Mean interaction time of the subjects with a juvenile stimulus during free interaction session, using awake (filled bar) or anesthetized stimuli (dashed bar). (G)Mean investigation time of juvenile stimuli, either awake or awake after anesthesia (Awake AA), during the SNP test that followed free interaction with one of them. * **$p < 0.001$, post hoc 1-tailed paired t-test following main effect in ANOVA.

To make sure that the lack of discrimination between awake stimuli after anesthesia, as shown in Fig. 2D, is not due to traces of the anesthesia effect, we performed an experiment in which immediately after the SP test (conducted without anesthesia; t_{14} =4.322, $p < 0.001$), both familiar and novel stimuli were anesthetized for 5 min and then got awakened and conducted the SNP test 20 min later. In this case, the subjects did discriminate between the familiar and a novel stimulus during the SNP test (t_{14} =−3.620, $p = 0.001$; Fig. S6). This result exclude the possibility that stimulus animals did not fully recover following anesthesia.

To confirm that the restricted access to the anesthetized stimulus during the SP test was not the reason for the lack of social discrimination in the SNP test, we conducted similar experiment when the SP test was replaced by free interaction with a social stimulus (Fig. 2E). We found that following free interaction with an anesthetized juvenile, which did not attract less investigation time from the subject compared to an awake stimulus (t_{20} =0.649, $p = 0.524$, 2-tailed independent t-test; Fig. 2F), there was no significant preference towards the novel stimulus in the SNP test, even though both stimuli were awake (Group x Stimuli: $F_{1,19}$ =13.162, $p = 0.002$, mixed-model ANOVA, post hoc: awake: t_9 =6.479, $p < 0.001$; awake after anesthesia: t_{10} =−0.767, $p = 0.230$, 1-tailed paired t-test; Fig. 2G). This observation proves that the lack of discrimination between anesthetized stimuli is not due to a state of fear or surprise induced in the subject by the anesthetized conspecifics, as the lack of discrimination in this test is between awake stimuli.

Overall, these results suggest that it is unlikely that anesthesia modifies the olfactory signature of social stimuli. Instead, mice do not behaviorally exhibit recognition of an anesthetized novel social stimulus and thus further support a crucial role for the behavior of the stimulus for social recognition.

3.3. *Mice do not discriminate between anesthetized stimuli even following stimulus-specific social fear conditioning*

Thus far, we relied on the innate social novelty preference of the animals as driving their social recognition behavior. Yet, it may be possible that while subject mice indeed discern between novel and familiar anesthetized stimuli, they do not exhibit their innate novelty-seeking tendency towards these individuals. Therefore, we developed a behavioral paradigm that does not rely on the innate social novelty preference of mice but rather depends on their experience with a specific social stimulus. In this stimulus-specific social fear conditioning (SFC) paradigm (schematically described in Fig. 3A), we conducted two consecutive SP tests (separated by 15 min) with the subject before the SFC session (henceforth termed baseline tests). For each of these tests, we used a social stimulus from a specific mouse strain (C57BL/6 J and ICR; Fig. 3A upper panel) so as to enhance the ability of a subject to discriminate between these individuals which have distinct fur colors (black for C57BL/6 J and white for ICR). Twenty minutes after the second SP test, we conducted a 5-min SFC session using the same ICR stimulus used for the previous SP test, but in a different spatial context (Fig. 3A, middle panel). Twenty minutes later, we performed two SP tests (henceforth termed recall tests) with the same social stimuli as used before the SFC session (Fig. 3A, lower panel). As apparent in Fig. 3B for awake stimuli, during the baseline tests subject mice exhibited similar social preference to both stimuli over an empty chamber (C57: t_7 =8.220, $p < 0.001$; ICR: t_7 =5.104, $p = 0.001$, 1-tailed paired t-test; upper panel). However, during the recall tests subjects still showed clear social preference for the C57BL/6 J stimulus (t_7 =5.314, $p = 0.001$, 1-tailed paired t-test), but lost their preference towards the conditioned ICR mouse (t_7 =−0.448, $p = 0.334$, 1-tailed paired t-test). To examine whether social-preference was significantly reduced by the social fear conditioning, we performed a 2 Time points (before SFC vs. after SFC) x 2 Strain (C57 vs. ICR) x 2 Side (empty vs. social-stimulus) three-way repeated ANOVA, that confirmed the presence of a significant Time x Stimuli x Side effect ($F_{1,7}$ =19.309, $p = 0.003$). To rule out the possibilities that the order of SP tests, which was fixed in all cases (first with C57BL/6 J and then with ICR stimuli), or the specific combination of mouse strains affected the results, we conducted another experiment, this time using Balb/C instead of ICR mice. In this experiment, besides the abovementioned tests, the subjects were examined 24-h following the SFC session in a single recall SP test, either with the C57BL/6 J or with the Balb/C stimulus. As before, Subject mice showed a normal preference to both stimuli during the baseline tests (Time x Strain x Side: $F_{1,12}$ =14.907, $p = 0.002$, three-way repeated ANOVA; post hoc: C57: Before SFC: t_{12} =10.135, $p < 0.001$; Balb/C: Before SFC: t_{12} =2.971, $p = 0.006$, Fig. S7A), while in the recall test they exhibited social preference towards the C57BL/6 J stimulus and avoided the Balb/C stimulus (C57: After SFC: t_{12} =1.750, $p = 0.053$; Balb/C: After SFC: t_{12} =−3.622, $p = 0.002$, 1-tailed paired t-test; Fig. S7A). When examined with only one of the stimuli 24 h later, the animals showed the same results (analyzed separately for each of the groups) with avoidance behavior towards the fear-conditioned stimulus (Balb/C) exhibited only after the SFC (Stimuli x Time interaction: C57: $F_{1,5}$ =0.396, $p = 0.557$; Balb/C: $F_{1,6}$ =6.363, $p = 0.045$, two-way repeated ANOVA; post hoc: C57: Before SFC: t_5 =2.740, $p = 0.021$, 24 h after SFC: t_5 =5.609, $p = 0.001$; Balb/C: Before SFC: t_6 =2.173, $p = 0.036$, 24 h after SFC: t_6 =−1.796, $p = 0.061$, 1-tailed paired t-test; Fig. S7B-C). Thus, using this paradigm we could clearly discriminate between the two social stimuli on the basis of acquired social fear memory rather than any innate tendency.

We then considered whether subjects can discriminate between social stimuli anesthetized only during the recall tests. As apparent in Fig. 3 C (lower panel), subjects in this case showed similar social preference towards both anesthetized social stimuli, exactly as they did before the SFC towards the same stimuli while they were awake (C57: t_6 =4.152, $p = 0.003$; ICR: t_6 =7.145, $p < 0.001$, 1-tailed paired t-test; Fig. 3 C lower panel). Since this experiment is identical to the one shown in Fig. 3B, besides the recall tests, these results suggest that while the subjects did learn the identity of the fear-conditioned stimulus, they did not behaviorally recognize the anesthetized ICR stimulus after SFC as the fear-conditioned stimulus (Time x Strain x Side interaction: $F_{1,6}$ =0.903, $p = 0.379$, three-way repeated ANOVA). To rule out the possibility that the subjects do recognize to fear-conditioned stimulus but do not fear from it while it is anesthetized, since the fear conditioning was conducted with an awake stimulus, we performed the same experiment when both social stimuli were anesthetized throughout the course of the paradigm (Fig. 3D). In this case, subject mice exhibited a general social fear during the recall tests (Time x Strain x Side interaction: $F_{1,7}$ =6.326, $p = 0.04$, three-way repeated ANOVA) and still did not distinguish between the two stimuli (C57: t_7 =−0.870, $p = 0.265$; ICR: t_7 =−2.206, $p = 0.031$, 1-tailed paired t-test; Fig. 3D lower panel). Thus, in terms of behavior, it seems as if in this case they did not learn the specific identity of the anesthetized ICR stimulus during the SFC session, hence established a general social fear. This is in perfect accordance with the lack of behavioral discrimination between anesthetized social stimuli in the SP/SNP paradigm (Fig. 2 C). Thus, our results using both paradigms suggest that the stimulus behavior is needed for the subject to learn its identity, and that such learning is impaired when the stimulus is anesthetized. Altogether, these results further support our conclusion that social recognition between individuals of the same sex relies on their behavior and not only on chemosensory cues.

3.4. *Familiarity discrimination depends upon somatosensory, auditory and chemosensory cues*

If stimuli behavior is indeed required for social recognition, one can ask what sensory modalities are recruited for detecting it? Since our behavioral experiments were performed under dim red light that is thought to be invisible to rats and mice (Gouras and Ekesten, 2004; Kojima et al., 2011) and since mice subjects did not discriminate between anesthetized C57BL/6 J and ICR stimuli following SFC, despite their markedly distinct (black vs. white) fur color, we reasoned that

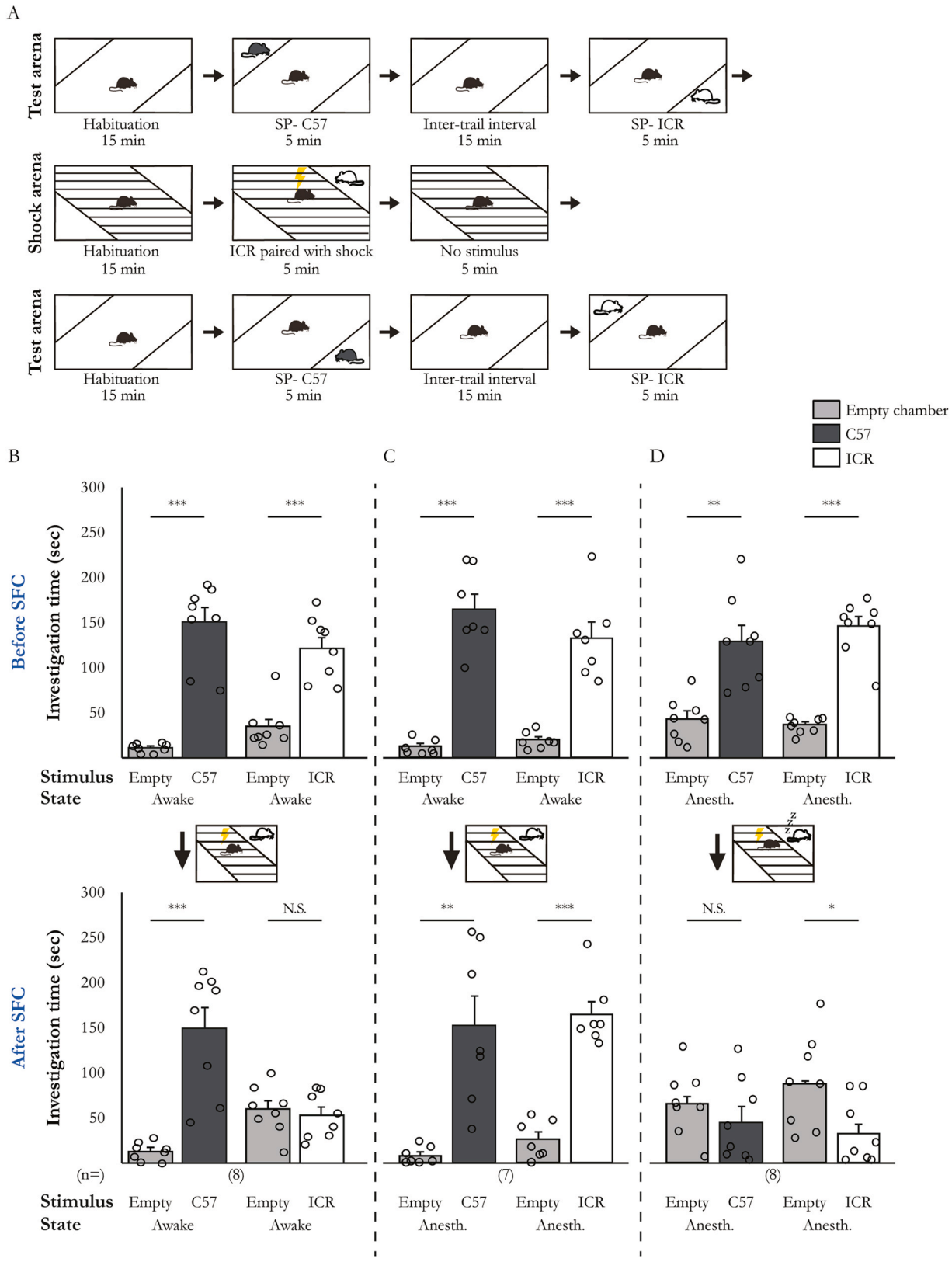


Fig. 3. Impaired discrimination between anesthetized social stimuli following social fear conditioning (SFC). (A)Schematic description of the SFC paradigm. Two SP tests (each with a distinct social stimulus) were conducted before (upper) and after (lower) the fear conditioning session (middle panel).(B)Mean investigation time of both stimuli during SP tests before (above) and after (below) SFC, using awake stimuli throughout the experiment. Stimulus type and state of stimulus are denoted below the bars. (C)Same as B, using stimuli that were anesthetized only after the SFC session. (D)Same as B, using stimuli that were anesthetized throughout the experiment. * **$p < 0.001$, * *$p < 0.01$, *$p < 0.05$, post hoc 1-tailed paired t-test following main effect in ANOVA.

vision most likely does not play a central role here. However, both somatosensory and auditory sensations can be used to detect touch, movement or vocalization of the stimuli (Rao et al., 2014). Therefore, we examined the effects of impairing somatosensory, auditory or olfactory sensations of the subjects on both familiarity discrimination and sex discrimination (Fig. 4A, B). We found that impairing somatosensation by removing the whiskers of subjects several days before the test abolished their familiarity discrimination (t_{14} =−0.160, p = 0.437, 1-tailed paired t-; Fig. 4 C, dashed bars; Fig. S8A-D), without affecting the total investigation time (whiskers: 178.42 ± 8.76, whiskerless: 164.34 ± 8.17; t_{14} =1.442, p = 0.170, 2-tailed paired t-test). In contrast, whiskerless mice showed normal sex discrimination (t_{18} =5.567, p < 0.001, 1-tailed paired t-test; Fig. 4D, dashed bars; Fig. S8M-P), although the total time was slightly reduced (whiskers - 181.38 ± 8.78, whiskerless - 123.68 ± 11.46; t_{41} =4.066, p < 0.001, 2-tailed t-test). To examine whether familiarity discrimination was significantly reduced by whiskers removal, we performed a 2 Time points (before whiskers removal vs. after whiskers removal) x 2 Stimuli (novel vs. CM) two-way repeated ANOVA, that confirmed the presence of a significant Time x Stimuli interaction effect ($F_{1,14}$ =16.367,

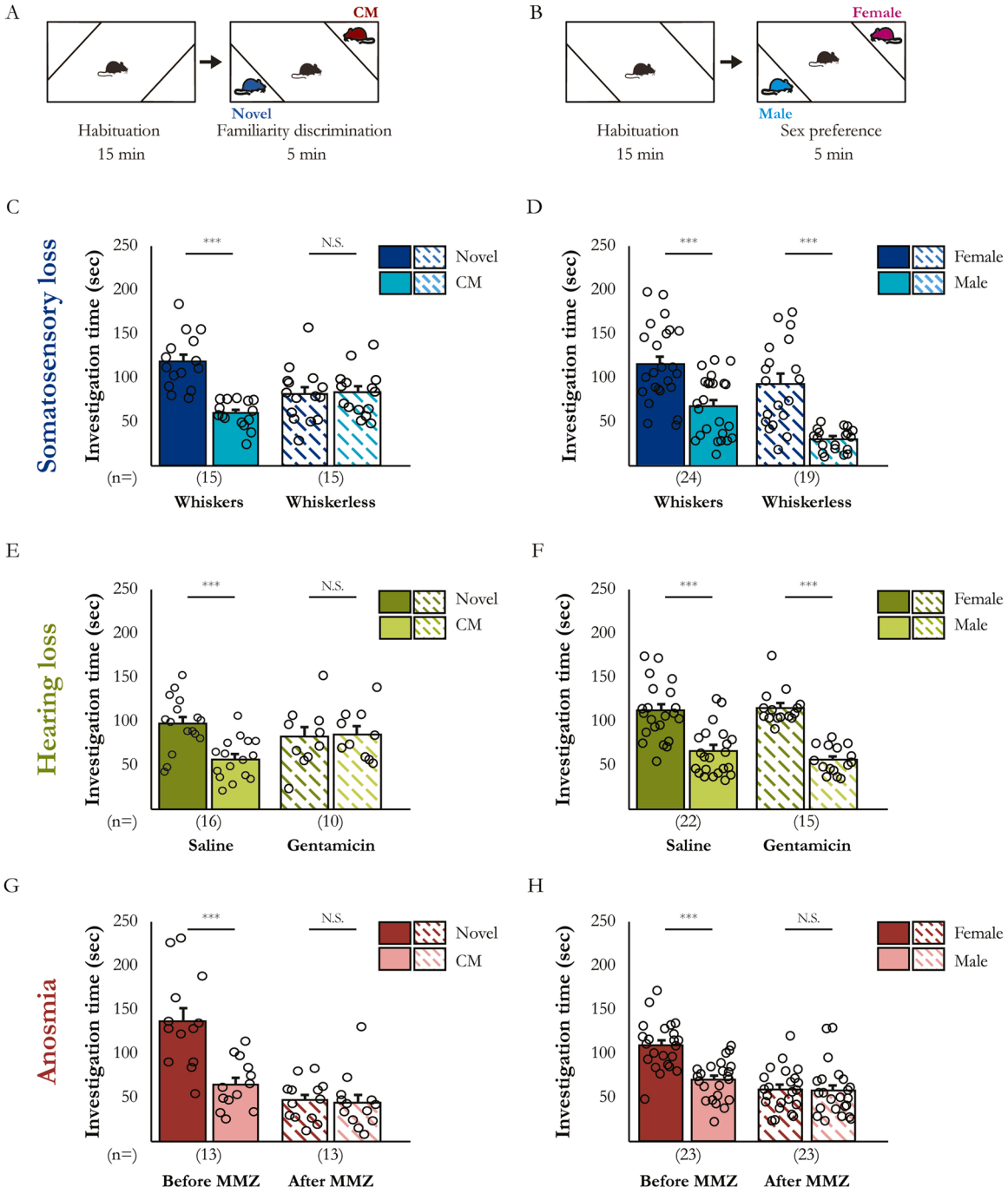


Fig. 4. Familiarity but not sex discrimination relies on the auditory and somatosensory systems. (A+B) Schematic descriptions of the familiarity (A) and sex (B) discrimination tests used. (C)Mean investigation time of the distinct stimuli by subjects with (filled bars) and without (dashed bars) whiskers, in the familiarity discrimination test. Number of tested subjects (n), and treatment are denoted below the bars.(D)As in C, for the sex discrimination test. (E-F) As in C-D, for subjects with an auditory system damaged by gentamicin injection. (G-H) As in C-D, for subjects with an olfactory system damaged by MMZ injection. * **p < 0.001, post hoc 1-tailed paired t-test following main effect in ANOVA.

$p = 0.001$). For the sex discrimination test we did not find a significant interaction between intact whiskers and the preference of the female stimulus ($F_{1,41} = 0.679$, $p = 0.415$, mixed-model ANOVA).

To assess the effect of hearing loss, we examined the behavior of animals centrally injected with gentamicin, an antibiotic that kills cochlear hair cells (Chen et al., 2012; Heydt et al., 2004) (Fig. S9), and compared this behavior to that of saline-injected animals. Like whiskerless animals, mice with hearing loss exhibited a lack of familiarity discrimination (Group x Stimuli: $F_{1,24} = 4.789$, $p = 0.039$ mixed-model ANOVA; *Post hoc*: Saline - $t_{15} = 3.777$, $p = 0.001$; Gentamicin - $t_9 = -0.159$, $p = 0.438$, 1-tailed paired t-test; Fig. 4E; Fig. S8E-H). In contrast, hearing loss did not affect sex discrimination behavior (Group x Stimuli: $F_{1,35} = 0.802$, $p = 0.377$, mixed-model ANOVA; *Post hoc*: Saline - $t_{21} = 4.195$, $p < 0.001$; Gentamicin - $t_{14} = 7.874$, $p < 0.001$, 1-tailed paired t-test; Fig. 4 F; Fig. S8Q-T). No change in total investigation time was observed in any of the tests (familiarity discrimination test: Saline: 154.81 ± 8.46, Gentamicin: 168 ± 8.19; $t_{24} = -1.052$, $p = 0.302$; sex discrimination test: Saline: 178.18 ± 6.61, Gentamicin: 172.38 ± 5.46; $t_{35} = 0.631$, $p = 0.532$, 2-tailed t-test).

To examine the effects of anosmia, we compared the behavior of animals before and after a single injection of Methimazole (MMZ), a drug used for treating hyperthyroidism and which is known to kill olfactory sensory neurons (Blanco-Hernandez et al., 2012; Crisafulli et al., 2018) (Fig. S10). As apparent in Fig. 4G-H, MMZ-induced anosmia abolished not only familiarity discrimination (Time x Stimuli: $F_{1,12} = 8.163$, $p = 0.014$, two-way repeated ANOVA; *Post hoc*: before MMZ – $t_{12} = 3.790$, $p = 0.001$, 1-tailed paired t-test; after MMZ – $t_{12} = 0.311$, $p = 0.381$, 1-tailed paired t-test; Fig. 4 G; Fig. S8I-L) but also sex discrimination (Time x Stimuli: $F_{1,22} = 10.485$, $p = 0.004$, two-way repeated ANOVA; *Post hoc*: before MMZ – $t_{22} = 5.306$, $p < 0.001$; after MMZ – $t_{22} = 0.249$, $p = 0.403$, 1-tailed paired t-test; Fig. 4H; Fig. S8U-X). Moreover, anosmia also caused a significant reduction in total investigation time in both tests (familiarity discrimination test: before MMZ: 201.89 ± 14.32, after MMZ: 91.66 ± 12.87; $t_{12} = 7.222$, $p < 0.001$; sex discrimination test: before MMZ: 180 ± 7.26, after MMZ: 116.79 ± 7.86; $t_{22} = 6.832$, $p < 0.001$, 2-tailed paired t-test), suggesting a reduction in general motivation for social interaction. Thus, sex discrimination relies on olfaction but not on hearing or whisker-dependent somatosensation, while familiarity discrimination seems to require all three modalities.

3.5. *Movement of stimuli can produce somatosensory and auditory cues required for social recognition*

The results presented so far suggest that both the auditory and somatosensory modalities are involved in social discrimination. We, therefore, looked for stimulus behavior-generated cues that may be detected by both modalities. A primary candidate is the movement of the stimulus, as movement produces both somatosensory and auditory cues. To examine if there are any differences in the movements generated by a CM and a novel social stimulus during the familiarity discrimination test, we used our recently described movement monitoring system (Netser et al., 2020) comprising an array of piezo-electric sensors placed at the floor of the triangular chambers containing the social stimuli. We then recorded the electrical signals generated by the sensors, which reflect the movement of each stimulus, during familiarity discrimination tests performed by 35 male C57BL/6 J subjects. The raw piezo signal trace recorded along the time course of each session was normalized to the peak signal separately for each chamber so as to correct for differences in mass and strength among the various stimuli. We subsequently quantified the number of major movements, defined by peaks that crossed a threshold ranging between 10% and 30% of the maximal signal. We found that at all threshold levels, novel stimuli generated significantly higher numbers of major movements, especially during the first 2–3 min of the test (threshold: 10%: $\chi^2(9) = 58.353$, $p < 0.001$; 20%: $\chi^2(9) = 44.257$, $p < 0.001$; 30%: $\chi^2(9) = 27.26$, $p < 0.001$, Friedman test; Fig. 5A-C). Thus, during the early phase of the social discrimination test, novel stimuli seemed to be more active than were CMs. To examine if the behavior of the subject was affected by the movement of the stimuli, we measured the time spent by the subject investigating each of the stimuli following a major movement made by that individual (using a threshold level of 25%), as compared to when no major movement occurred. We found that at resting conditions (i.e., after a 4-s break in the investigation behavior), subject mice showed a reduction in their likelihood to restart investigating the CM following it making a major movement (Fig. 5D, left, red line), as compared to periods when no major movement was observed (green line). The differences between periods of movement or no movement of the CM were found to be statistically significant ($Z = -3.513$, $p < 0.001$, Wilcoxon signed rank test; Fig. 5D-E, left). No such tendency was found towards the novel social stimulus ($Z = -0.915$, $p = 0.180$, Wilcoxon signed rank test; Fig. 5D-E, right). As a control, we made the same calculations for periods that followed a 4 s window of stimulus investigation and found no effect of major movements in this case (CM: $Z = -0.231$, $p = 0.485$; Novel: $Z = -0.556$, $p = 0.289$, Wilcoxon signed rank test; Fig. 5F-G). To confirm that these differences were not caused by a preference of the subjects for investigating a novel stimulus, we analyzed the results separately for subjects who preferred the novel stimulus (Fig. 5H-J) and those who preferred the CM (Fig. 5K-M). We found no significant differences between the groups (Group x Stimuli x movements interaction: $F_{1,29} = 1.166$, $p = 0.289$, mixed three-way repeated ANOVA; Fig. 5 J+M).

Overall, these results reveal that familiar and novel stimuli exhibit different movement patterns in the familiarity discrimination test, and that subjects react to these patterns in a stimulus-dependent manner. The observation that novel stimuli produce more major movements as compared to CM regardless of the subjects' preference (Fig. 5H-M) suggests that this difference between novel and familiar stimuli is not driven by the preference of the subjects, thus it may subserve discrimination between the stimuli.

Altogether, the results of this study suggest that subject mice integrate chemosensory and behavior-generated cues, acquired via multiple sensory modalities, including the olfactory, auditory and somatosensory modalities, to discriminate between same-sex stimuli.

4. Discussion

In this study, we explored the role of stimulus behavior in social recognition of male C57BL/6 J mice. Male mice were examined, as they show robust preference in both familiarity- and sex-discrimination tests, while in our hands female C57BL/6 J subjects does not show behavioral preference in the sex-discrimination test (unpublished results). We hypothesized that social recognition in these animals does not depend solely on chemosensory signature passively emitted by the stimulus (i.e., the signaler) and detected by the subject (i.e., the receiver), in analogy to face recognition by humans. Instead, social recognition involves both activity of the stimulus and integration of information arriving via several sensory modalities by the subject. To test this hypothesis, we analyzed sex and familiarity discrimination by subject mice when challenged with sets of awake and anesthetized stimuli.

4.1. *Working with anesthetized stimuli*

The number of behavioral studies exploring social recognition that used anesthetized stimuli is surprisingly small. Anesthetized same-sex conspecifics were found to elicit defensive responses and ultrasonic vocalizations in rats (Blanchard et al., 1993). Latané and Glass (Latane and Glass, 1968) reported a reduction in the level of contacts made by rats with anesthetized stimuli as compared to freely moving stimuli and concluded that movement is important for the attractiveness of social stimuli. It should be noted that we did not observe any general difference in the investigation time between anesthetized and awake stimuli,

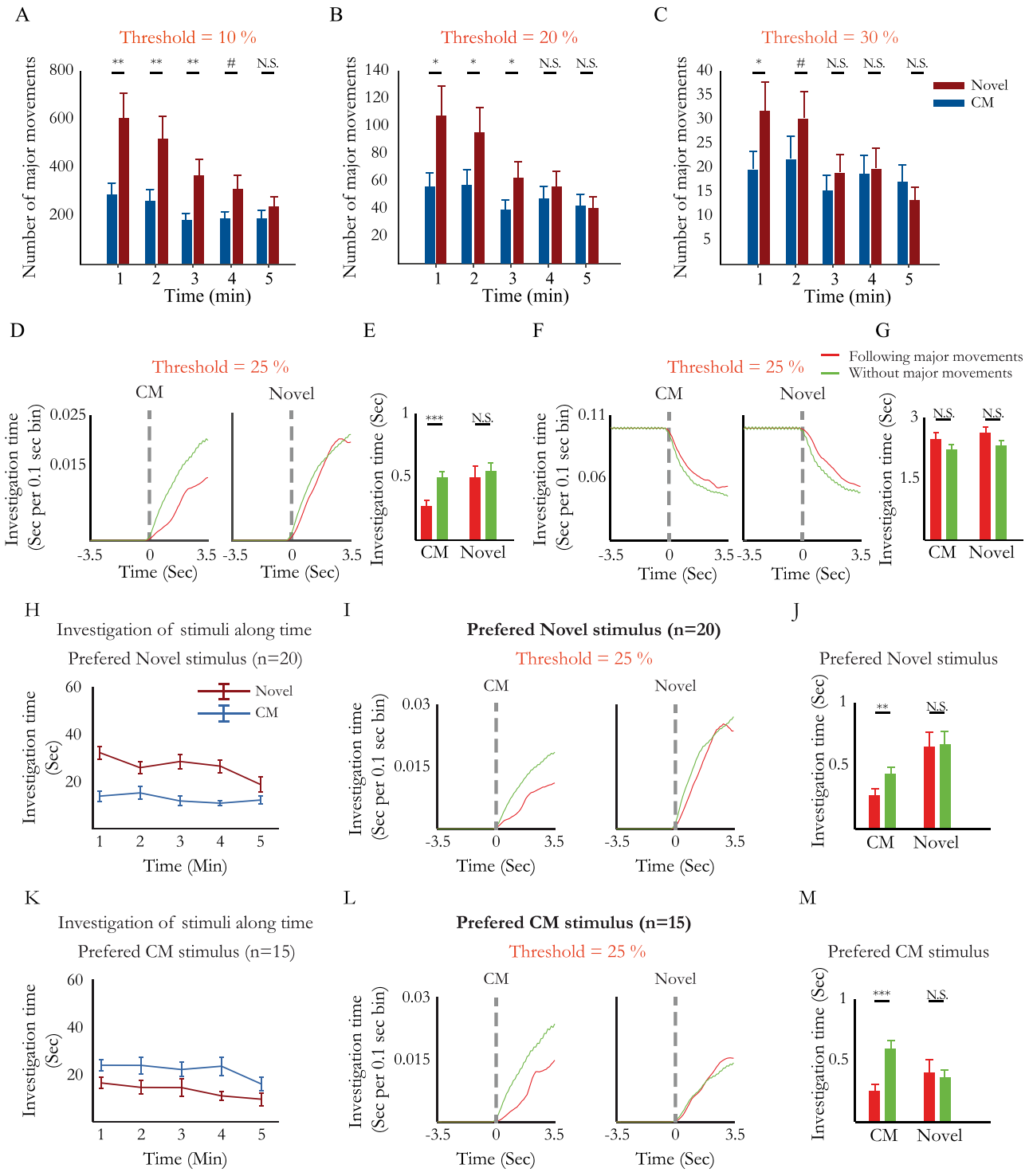


Fig. 5. Differential movement by social stimuli during familiarity discrimination draws distinct subject responses. (A) Mean number of major movements made by each of the stimuli, using a threshold of 10% of the maximal signal for defining a major movement (min1: $Z = -2.842$, $p = 0.002$; min2: $Z = -2.424$, $p = 0.008$; min3: $Z = -2.703$, $p = 0.003$; min4: $Z = -1.458$, $p = 0.072$; min5: $Z = -1.097$, $p = 0.136$, 1-tailed Wilcoxon signed rank test). (B)Same as A, using a threshold of 20% (min1: $Z = -2.138$, $p = 0.016$; min2: $Z = -1.941$, $p = 0.026$; min3: $Z = -1.778$, $p = 0.037$; min4: $Z = -0.475$, $p = 0.317$; min5: $Z = -0.205$, $p = 0.418$, 1-tailed Wilcoxon signed rank test).(C)Same as A, using a threshold of 30% (min1: $Z = -1.760$, $p = 0.039$; min2: $Z = -1.582$, $p = 0.056$; min3: $Z = -0.915$, $p = 0.18$; min4: $Z = -0.299$, $p = 0.382$; min5: $Z = -0.655$, $p = 0.256$, 1-tailed Wilcoxon signed rank test).(D)Mean investigation time of the distinct stimuli, after 4-sec period with no investigation by the subject, with (red) and without (green) major movement made by the CM (left) or novel social stimulus (right). Time 0 marks the beginning of movement. (E)Statistical analysis of the results shown in D, summed across the 3.5-sec period after time 0.(F)Same as D, after 4-sec period of stimulus investigation.(G) Same as E, for the results shown in F.(H) Mean investigation time of subjects along the time course of the familiarity discrimination test, for the group of mice that preferred novel stimulus over their CM, using 1 min bins. (I)Same as D, for subjects that preferred the novel social stimulus over the CM. (J) Same as E, for the results shown in I. (K)Same as H, for the group of mice that preferred their CM over the novel stimulus. (L)Same as D, for subject that preferred the CM. (M)Same as E, for the results shown in O. * **$p < 0.001$, * *$p < 0.01$, *$p < 0.05$, #$p < 0.1$, Wilcoxon signed rank test.

suggesting no reduction in attraction of social stimuli due to anesthesia. Similar results were recently reported by Contestabile et al. (2021). Notably, a recent study using a novel methodology for assessing social investigation over a 100 min period found that adult male mice investigated anesthetized CMs more than they did novel conspecifics, with each being encountered separately (Ito et al., 2019). However, this difference was observed only after 10 min of exposure to the stimulus; no difference was found during the first 10 min, in accordance with our results. Thus, it may be possible mice do recognize familiar conspecifics even without active behavior exhibited by them, if given enough time. Future experiments using longer exposure times may examine this possibility.

One concern regarding our use of anesthetized animals is the possibility that the anesthesia procedure we employed changed the odor of the social stimulus, thus interfering with subject recognition of animals previously encountered prior to the anesthesia. Multiple observations from our experiments, however, argue against this possibility. First, subjects did not differentiate saline-injected from non-injected CMs, suggesting no interference by injection-induced release of alarm pheromones. Second, the anesthesia had no effect on the ability of the subjects to discriminate between male and female stimuli, suggesting that such treatment did not mask chemosensory cues emitted by these individuals. Third, subjects did not learn to recognize an anesthetized stimulus even if this individual was still anesthetized during the discrimination test, thus ruling out the possibility that anesthesia induced a novel chemosensory signature the social stimulus. Finally, the full agreement of the results of sensory impairment experiments, all conducted with awake stimuli, with the results of social discrimination experiments conducted with anesthetized stimuli, suggests that all these results reflect the same dependence of familiarity discrimination on cues generated by the behavior of social stimuli.

4.2. Social recognition by chemosensory cues

A large body of evidence suggests that chemosensory cues can alone mediate several types of social recognition, such as mate and kin recognition (Hurst, 2009; Hurst and Beynon, 2010; Kavaliers and Choleris, 2017; Yamazaki and Beauchamp, 2005). In the case of familiarity recognition, the picture is less clear. Several studies have shown that anosmic rats and mice lose their ability to discriminate between familiar and novel conspecifics (Dantzer et al., 1990; Matochik, 1988; Popik et al., 1991). These results, which are in agreement with the data presented here, does not rule out the involvement of other sensory modalities in familiarity discrimination. Multiple other studies have used operant conditioning to demonstrate that rats and mice can learn to discriminate between odors of social stimuli even if the animals involved are almost genetically identical (Gheusi et al., 1994; Hopp et al., 1985; Kwak et al., 2009). Operant conditioning is, however, well known for revealing astonishing capabilities of discrimination between almost identical complex sets of signals. For example, mice can be trained to discriminate between sensory stimuli on the basis of activity of a single cortical neuron (Houweling and Brecht, 2008). Such skills may define the limits of learning capabilities, yet are not necessarily employed in natural conditions. Other studies involving more ethologically relevant habituation-based learning showed that small rodents can discriminate between chemosensory cues derived from individual conspecifics based on either the highly polymorphic major histocompatibility complex (MHC) or a combinatorial repertoire of major urine proteins (MUPs) (Hurst et al., 2001; Roberts et al., 2018; Yamazaki and Beauchamp, 2005). However, these studies largely focused on body odors, such as urine, instead of the social stimulus itself. As the mechanisms employed for recognition may differ in the context of encountering an individual conspecific than when encountering odors derived from the same individual, it is hard to draw conclusions regarding the sensory modalities involved in recognition of the actual conspecifics from these experiments (Wiley, 2013).

Notably, a recent elegant study by Contestabile et al. (2021) explored the type of sensory stimuli underlying approach behavior towards novel social stimuli. In accordance with our results, they found that while olfactory stimuli may be the most important cues in attracting mouse subject to a conspecific, complex social stimuli allowing multimodal sensory (auditory and tactile) stimulation are significantly more attractive than social stimuli allowing olfactory cues alone. It should be noted, however, that unlike our study, this study did not explore the aspect of social novelty preference, which may be driven by stimuli which are distinct from those driving social preference.

4.3. Using stimulus-specific social fear conditioning for social discrimination

In the present study, we employed a novel paradigm of stimulus-specific social fear conditioning (SFC) in order to rule out the possibility that by anesthetizing the stimuli we interfered with the innate tendency of rodents to interact more intensively with novel individuals. The ability of SFC to induce general avoidance of social stimuli, thus to overcome the innate attractiveness of social interactions in small rodents was previously demonstrated by several laboratories (Toth et al., 2012; Xu et al., 2019). Here we presented a protocol that enables induction of avoidance behavior towards a specific individual, allowing other conspecifics to retain their attractiveness. We used this protocol to show that C57BL/6 J mice did not discriminate between fear-conditioned and neutral social stimuli if these animals were anesthetized either during the conditioning session or during the discrimination test, despite the unpleasant experience associated with the conditioned stimulus. Notably, a previous study employed a similar approach involving aversive conditioning to female odors showed that *TrpC2* knockout mice could be conditioned to avoid female stimuli, despite their lack of innate discrimination between male and female stimuli (Beny and Kimchi, 2016). Therefore, our SFC results support the conclusion that mice do not behaviorally discriminate between anesthetized stimuli due to recognition failure, rather than because of motivation problems.

4.4. Sensory modalities that mediate familiarity recognition

To examine which sensory modalities are involved in detection of behaviorally induced social cues that contribute to familiarity recognition, we used established methods to induce impairments in either the olfactory, somatosensory or auditory modalities of subject mice. We found that impairing any of these modalities abolished social discrimination between a novel social stimulus and a CM. The fact that only olfactory impairment interfered with sex recognition suggests that our treatments were specific to the targeted modalities and did not induce a general behavioral state leading to a complete lack of discrimination between stimuli. One limitation of our approach is that the administration of MMZ or gentamicin could also affect other brain systems. Thus, we cannot rule out the possibility that these treatments cause behavioral changes in ways which are unrelated to sensory impairment.

Multiple seminal studies from the Brecht laboratory showed that social interactions of rats involve intensive bouts of facial touch, which are temporally coordinated with ultrasonic vocalizations emitted by the subjects (Rao et al., 2014). They also demonstrated that such facial contacts trigger social-specific responses of single units in the somatosensory cortex (Lenschow and Brecht, 2015), while also modulating neuronal responses to social vocalizations in the auditory cortex (Rao et al., 2014). These studies suggest the existence of a substrate for the integration of sensory cues emitted by rats during close social interaction by the somatosensory, auditory and other cortices (Ebbesen et al., 2019). However, C57BL/6 J mice do not normally emit ultrasonic vocalizations during male-male interactions (Portfors, 2007). Thus, we hypothesized that at least in mice, movements by social stimuli can supply cues that are detected by both the somatosensory and auditory modalities, both during close contact and remote interactions between

the animals. To test this possibility, we applied a novel experimental system based on piezoelectric sensors, which produces electrical signals that are proportional to the movement of the social stimulus within its chamber. Using this system, we found that novel stimuli are more active in the chamber during the discrimination test than CMs. We also found that subject mice responded to major movements of the stimulus in a stimulus-dependent manner. While these results do not prove that the differential movement of the stimuli is the basis for their recognition by the subject, they do show that such behavior can serve as a source of behavior-dependent social cues, which contribute to familiarity recognition in mice.

4.5. Conclusions

Overall, our results suggest that in rats and mice some aspects of social recognition, such as familiarity recognition, require integration of information from several sensory modalities, including the somatosensory and auditory modalities.

Ethics approval

All experiments were performed according to the National Institutes of Health guide for the care and use of laboratory animals, and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Haifa (Ethical approvals # 367/15, 448/16, 490/17, 503/17, 528/17, 608/19, 701/20).

CRediT author contribution statement

Shani Haskal de la Zerda: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Visualization. **Shai Netser:** Conceptualization, Formal analysis, Methodology, Project administration, Software, Validation. **Hen Magalnik:** Investigation, **Mayan Briller:** Investigation. **Dan Marzan:** Investigation, Methodology. **Sigal Glatt:** Investigation, Methodology. **Yasmin Abergel:** Investigation, Methodology, **Shlomo Wagner:**Conceptualization, Funding acquisition, Project administration, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the Human Frontier Science Program (HFSP grant RGP0019/2015), the Israel Science Foundation (ISF grants #1350/12, 1361/17), a binational joint program of the Ministry of Europe and Foreign Affairs (MEAE) and the Ministry of Higher Education, Research and Innovation (MESRI) of France and the Ministry of Science and Technology of Israel (grant #3-16545) and the Ministry of Science and Technology of Israel (Grant #3-12068).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2022.105859.

References

Anzellotti, S., Caramazza, A., 2017. Multimodal representations of person identity individuated with fMRI. Cortex 89, 85–97.
Beny, Y., Kimchi, T., 2016. Conditioned odor aversion induces social anxiety towards females in wild-type and TrpC2 knockout male mice. Genes Brain Behav. 15, 722–732.
Blanchard, R.J., Yudko, E.B., Blanchard, D.C., Taukulis, H.K., 1993. High-frequency (35–70 kHz) ultrasonic vocalizations in rats confronted with anesthetized conspecifics: effects of gepirone, ethanol, and diazepam. Pharm. Biochem Behav. 44, 313–319.
Blanco-Hernandez, E., Valle-Leija, P., Zomosa-Signoret, V., Drucker-Colin, R., Vidaltamayo, R., 2012. Odor memory stability after reinnervation of the olfactory bulb. PLoS One 7, e46338.
Brennan, P.A., Kendrick, K.M., 2006. Mammalian social odours: attraction and individual recognition. Philos. Trans. R. Soc. Lond. B Biol. Sci. 361, 2061–2078.
Bruce, V., Young, A., 1986. Understanding face recognition. Br. J. Psychol. 77 (Pt 3), 305–327.
Camats Perna, J., Engelmann, M., 2017. Recognizing others: rodent's social memories. Curr. Top. Behav. Neurosci. 30, 25–45.
Chen, L., Xiong, S., Liu, Y., Shang, X., 2012. Effect of different gentamicin dose on the plasticity of the ribbon synapses in cochlear inner hair cells of C57BL/6J mice. Mol. Neurobiol. 46, 487–494.
Choleris, E., Clipperton-Allen, A.E., Phan, A., Kavaliers, M., 2009. Neuroendocrinology of social information processing in rats and mice. Front Neuroendocr. 30, 442–459.
Contestabile, A., Casarotto, G., Girard, B., Tzanoulinou, S., Bellone, C., 2021. Deconstructing the contribution of sensory cues in social approach. Eur. J. Neurosci. 53, 3199–3211.
Crisafulli, U., Xavier, A.M., Dos Santos, F.B., Cambiaghi, T.D., Chang, S.Y., Porcionatto, M., Castilho, B.A., Malnic, B., Glezer, I., 2018. Topical dexamethasone administration impairs protein synthesis and neuronal regeneration in the olfactory epithelium. Front Mol. Neurosci. 11, 50.
Dantzer, R., Tazi, A., Bluthe, R.M., 1990. Cerebral lateralization of olfactory-mediated affective processes in rats. Behav. Brain Res 40, 53–60.
Ebbesen, C.L., Bobrov, E., Rao, R.P., Brecht, M., 2019. Highly structured, partner-sex- and subject-sex-dependent cortical responses during social facial touch. Nat. Commun. 10, 4634.
Engelmann, M., Wotjak, C.T., Landgraf, R., 1995. Social discrimination procedure: an alternative method to investigate juvenile recognition abilities in rats. Physiol. Behav. 58, 315–321.
Gheusi, G., Bluthe, R.M., Goodall, G., Dantzer, R., 1994. Social and individual recognition in rodents: Methodological aspects and neurobiological bases. Behav. Process. 33, 59–87.
Gouras, P., Ekesten, B., 2004. Why do mice have ultra-violet vision? Exp. Eye Res 79, 887–892.
Heydt, J.L., Cunningham, L.L., Rubel, E.W., Coltrera, M.D., 2004. Round window gentamicin application: an inner ear hair cell damage protocol for the mouse. Hear Res 192, 65–74.
Hopp, S.L., Owren, M.J., Marion, J.R., 1985. Olfactory discrimination of individual littermates in rats (Rattus norvegicus). J. Comp. Psychol. 99, 248–251.
Houweling, A.R., Brecht, M., 2008. Behavioural report of single neuron stimulation in somatosensory cortex. Nature 451, 65–68.
Hurst, J.L., 2009. Female recognition and assessment of males through scent. Behav. Brain Res 200, 295–303.
Hurst, J.L., Beynon, R.J., 2010. Making progress in genetic kin recognition among vertebrates. J. Biol. 9, 13.
Hurst, J.L., Payne, C.E., Nevison, C.M., Marie, A.D., Humphries, R.E., Robertson, D.H., Cavaggioni, A., Beynon, R.J., 2001. Individual recognition in mice mediated by major urinary proteins. Nature 414, 631–634.
Ito, W., Huang, H., Brayman, V., Morozov, A., 2019. Impaired social contacts with familiar anesthetized conspecific in CA3-restricted brain-derived neurotrophic factor knockout mice. Genes Brain Behav. 18, e12513.
Kas, M.J., Glennon, J.C., Buitelaar, J., Ey, E., Biemans, B., Crawley, J., Ring, R.H., Lajonchere, C., Esclassan, F., Talpos, J., Noldus, L.P., Burbach, J.P., Steckler, T., 2014. Assessing behavioural and cognitive domains of autism spectrum disorders in rodents: current status and future perspectives. Psychopharmacol. (Berl.) 231, 1125–1146.
Kavaliers, M., Choleris, E., 2017. Social cognition and the neurobiology of rodent mate choice. Integr. Comp. Biol. 57, 846–856.
Kendrick, K.M., 2006. Introduction. The neurobiology of social recognition, attraction and bonding. Philos. Trans. R. Soc. Lond. B Biol. Sci. 361, 2057–2059.
Kojima, D., Mori, S., Torii, M., Wada, A., Morishita, R., Fukada, Y., 2011. UV-sensitive photoreceptor protein OPN5 in humans and mice. PLoS One 6, e26388.
Kummer, K., Klement, S., Eggart, V., Mayr, M.J., Saria, A., Zernig, G., 2011. Conditioned place preference for social interaction in rats: contribution of sensory components. Front Behav. Neurosci. 5, 80.
Kwak, J., Opiekun, M.C., Matsumura, K., Preti, G., Yamazaki, K., Beauchamp, G.K., 2009. Major histocompatibility complex-regulated odortypes: peptide-free urinary volatile signals. Physiol. Behav. 96, 184–188.
Latane, B., Glass, D.C., 1968. Social and nonsocial attraction in rats. J. Pers. Soc. Psychol. 9, 142–146.
Lenschow, C., Brecht, M., 2015. Barrel cortex membrane potential dynamics in social touch. Neuron 85, 718–725.
Matochik, J.A., 1988. Role of the main olfactory system in recognition between individual spiny mice. Physiol. Behav. 42, 217–222.
Netser, S., Haskal, S., Magalnik, H., Wagner, S., 2017. A novel system for tracking social preference dynamics in mice reveals sex- and strain-specific characteristics. Mol. Autism 8, 53.
Netser, S., Haskal, S., Magalnik, H., Bizer, A., Wagner, S., 2019. A system for tracking the dynamics of social preference behavior in small rodents. J. Vis. Exp. 153.
Netser, S., Meyer, A., Magalnik, H., Zylbertal, A., de la Zerda, S.H., Briller, M., Bizer, A., Grinevich, V., Wagner, S., 2020. Distinct dynamics of social motivation drive

differential social behavior in laboratory rat and mouse strains. Nat. Commun. 11, 5908.
Parr, L.A., Winslow, J.T., Hopkins, W.D., de Waal, F.B.M., 2000. Recognizing facial cues: individual discrimination by chimpanzees (Pan troglodytes) and rhesus monkeys (Macaca mulatta). J. Comp. Psychol. 114, 47–60.
Popik, P., Vetulani, J., Bisaga, A., van Ree, J.M., 1991. Recognition cue in the rat's social memory paradigm. J. Basic Clin. Physiol. Pharm. 2, 315–327.
Portfors, C.V., 2007. Types and functions of ultrasonic vocalizations in laboratory rats and mice. J. Am. Assoc. Lab Anim. Sci. 46, 28–34.
Rao, R.P., Mielke, F., Bobrov, E., Brecht, M., 2014. Vocalization-whisking coordination and multisensory integration of social signals in rat auditory cortex. Elife 3, e03185.
Rao, R.P., von Heimendahl, M., Bahr, V., Brecht, M., 2019. Neuronal responses to conspecifics in the ventral CA1. Cell Rep. 27 (3460–3472), e3463.
Roberts, S.A., Prescott, M.C., Davidson, A.J., McLean, L., Beynon, R.J., Hurst, J.L., 2018. Individual odour signatures that mice learn are shaped by involatile major urinary proteins (MUPs). BMC Biol. 16, 48.
Rogers-Carter, M.M., Christianson, J.P., 2019. An insular view of the social decision-making network. Neurosci. Biobehav Rev. 103, 119–132.
Russell, W.M.S., 2004. The use of non-human animals in research: a guide for scientists. Atla-Alter. Lab Anim. 32, 119–120.
Sanchez-Andrade, G., Kendrick, K.M., 2009. The main olfactory system and social learning in mammals. Behav. Brain Res. 200, 323–335.
Thor, D.H., Wainwright, K.L., Holloway, W.R., 1982. Persistence of attention to a novel conspecific: some developmental variables in laboratory rats. Dev. Psychobiol. 15, 1–8.
Toth, I., Neumann, I.D., Slattery, D.A., 2012. Social fear conditioning: a novel and specific animal model to study social anxiety disorder. Neuropsychopharmacology 37, 1433–1443.
Wiley, R.H., 2013. Specificity and multiplicity in the recognition of individuals: implications for the evolution of social behaviour. Biol. Rev. Camb. Philos. Soc. 88, 179–195.
Xie, F., Zhou, X., Genter, M.B., Behr, M., Gu, J., Ding, X., 2011. The tissue-specific toxicity of methimazole in the mouse olfactory mucosa is partly mediated through target-tissue metabolic activation by CYP2A5. Drug Metab. Dispos. 39, 947–951.
Xu, H., Liu, L., Tian, Y., Wang, J., Li, J., Zheng, J., Zhao, H., He, M., Xu, T.L., Duan, S., Xu, H., 2019. A disinhibitory microcircuit mediates conditioned social fear in the prefrontal cortex. Neuron 102 (668–682), e665.
Yamazaki, K., Beauchamp, G.K., 2005. Chemosensory recognition of olfactory individuality. Chem. Senses 30 (Suppl 1), i142–i143.