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Conditioned odor aversion induces social anxiety towards females in wild-type and *TrpC2* knockout male mice

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Female-emitted pheromonal inputs possess an intrinsic rewarding value for conspecific males, promoting approach and investigation of the potential mating partner. In mice these inputs are detected mainly by the vomeronasal organ (VNO) and the main olfactory epithelium (MOE). We investigated the role of VNO-mediated inputs in experience-dependent plasticity of reproductive responses. We applied a sex-specific conditioned odor aversion (COA) paradigm on adult, wild-type (WT) male mice and on male mice impaired in VNO-mediated signal transduction (TrpC2$^{-/-}$). We found that WT males, which underwent COA to female-soiled bedding, lost their innate preference to female odors and presented lower motivation to approach a sexually receptive female. COA also abolished the testosterone surge normally seen following exposure to female odors. Moreover, the conditioned males displayed impairments in copulatory behaviors, which lasted for several weeks. Surprisingly, these males also exhibited phobic behaviors towards receptive females, including freezing and fleeing responses. In contrast, WT males which underwent COA specifically to male pheromones showed no change in olfactory preference and only a marginally significant elevation in intermale aggression. Finally, we show that TrpC2$^{-/-}$ males were able to acquire aversion to female-soiled bedding and presented similar behavioral alterations following COA in their responses to female cues. Our results demonstrate that the intrinsic rewarding value of female pheromones can be overridden through associative olfactory learning, which occurs independently of VNO inputs, probably through MOE signaling.

Keywords: Conditioned odor aversion (COA), experience-dependent plasticity, innate behavior, mice, pheromones, reproductive behavior, sexual preference, testosterone, *TrpC2*, vomeronasal organ

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Reproductive behaviors are considered to be stereotypic action patterns that ensure optimal execution and reproductive success with minimal energy investment (Alcock 2013). Correspondingly, those stereotypical actions are controlled by hard-wired brain circuits and determined prenatally under the control of sex steroids (Alexander *et al.* 2011; Choi *et al.* 2005). In rodents, reproductive behaviors are activated in response to external sensory stimuli, such as sex-specific pheromones, without the need for previous learning (Beny & Kimchi 2014; Halpern & Martinez-Marcos 2003). The innate nature of reproductive behaviors suggests that environmental conditions, experienced by the adult animal, will have a limited effect (if any) on the execution of those behaviors. Indeed, positive past experience enhances the initiation and expression of reproductive behaviors and of related physiological responses, serving the purpose of increasing reproductive success (Olsen 2011; Pfaus *et al.* 2001, 2012; Pitchers *et al.* 2013). However, the influence of negative experience on plasticity of innate reproductive behaviors and its limitations are less clear.

Pheromones, volatile and non-volatile compounds, are used for social communication in most mammals including rodents (Dulac & Torello 2003; Liberles 2014; Wyatt 2014; Zhang *et al.* 2013). Female pheromones elicit a set of copulatory behavioral and physiological responses in sexually naïve males, for example the rapid elevation of circulating luteinizing hormone (Coquelin *et al.* 1984; Gleason *et al.* 2009; Goldey & van Anders 2014) and testosterone levels (Amstislavskaya *et al.* 2013; Gleason *et al.* 2009; Swaney *et al.* 2012). Furthermore, female chemosensory signals are considered to be intrinsically rewarding and strongly attractive to males, eliciting anticipatory locomotion (Mendelson & Pfaus 1989; Trezza *et al.* 2011) and preference to approach and investigate female over male stimuli (Ago *et al.* 2015; Brown 1977). Accordingly, these chemosignals may serve as unconditioned natural reinforcers inducing associative conditioned learning (reviewed in Beny & Kimchi 2014; Griffiths & Brennan 2015; Pfaus *et al.* 2001).

Pheromones are detected by sensory neurons in the vomeronasal organ (VNO) and in the main olfactory epithelium (MOE), which in turn activate downstream brain structures to execute the appropriate behavioral response (Dulac & Wagner 2006; Keller *et al.* 2010; Mucignat-Caretta *et al.* 2012). The VNO of rodents covneys information regarding sex, age and social status and has a critical role in mediating innate reproductive behaviors and endocrine responses (Chamero *et al.* 2007; Dulac & Torello 2003; Isogai *et al.* 2011). Pheromone signal transduction through the VNO requires the TrpC2 ion channel, expressed predominantly in

sensory neurons of the VNO (Chamero *et al.* 2012; Omura & Mombaerts 2014; Yu 2015). Though TrpC2 mutant ($TrpC2^{-/-}$) males are able to successfully mate with females, these males are profoundly impaired in their ability to behaviorally distinguish between males and females, mounting both sexes with equal frequency. Moreover, they fail to initiate aggressive behavior towards an intruder male, unlike typical wild-type (WT) males (Chamero *et al.* 2007; Kimchi *et al.* 2007; Leypold *et al.* 2002; Stowers *et al.* 2002). This behavioral phenotype provides a unique tool to explore the role of the VNO in pheromone-mediated plasticity of innate reproductive circuits.

In this study, we designed a modified conditioned odor aversion (COA) procedure to test the effects of negative past experience associated with female pheromones, on a wide range of male-typical behavioral and physiological responses. As a source for pheromones we used fresh female-soiled bedding, a common stimulus employed in studies of pheromone-induced reproductive behaviors (Kimoto *et al.* 2005; Montani *et al.* 2013; Yokosuka *et al.* 1999). A similar procedure was used to examine whether it is possible to induce an aversion to male pheromones, and alter inter-male aggressive behavior. In addition, we employed the COA procedure on $TrpC2^{-/-}$ males to explore the role of VNO-mediated signals in sex-specific aversive olfactory learning.

Methods

Animals

Mutants of the *TrpC2* mouse line (B6;129S1-$Trpc2^{tm1Dlc}$/J, stock no: 021208; The Jackson Laboratory) were originally generated using 129/Sv (now termed 129S1; http://jaxmice.jax.org) embryonic stem cells, that have been backcrossed onto C57BL/6J strain background, as previously detailed (Stowers *et al.* 2002). Sexually naive adult (10–14 weeks-old) $TrpC2^{-/-}$ mice and their WT littermates were derived from an in house colony donated by Catherine Dulac (Harvard University) in 2008, and established from heterozygous breeding pairs. Data analysis of 1549 high-density single-nucleotide polymorphism (SNP) in $TrpC2^{-/-}$ mice (Chalfin *et al.* 2014) shows that ~61% of the SNPs originated from the genetic background of the 129S1 mouse strain while the remaining from the C57BL/6J strain (Table S1, Supporting Information). For the stimuli mice in the motivation assay (MA) and social interaction (SI) assay we used C57BL/6J inbred mice (Harlan laboratories). All mice were bred under standard pathogen-free laboratory conditions with food and water *ad libitum*. Two weeks before the initiation of behavioral assays, experiment mice were housed in single cages, and were transferred to a reversed 12/12 h light/dark cycle (lights off at 1000 h). All behavioral procedures were performed during the dark phase under dim red light, except for the MA and anxiety-related assays that were conducted under dim white light. The female-specific COA experiments comprised of 33 WT males for the Lithium Chloride (LiCl) group, 28 WT males for the saline group and 11 $TrpC2^{-/-}$ males for the LiCl and for the saline groups each. The male-specific COA experiment comprised of 11 WT males for the LiCl group and 11 WT males for the saline group each. The Institutional Animal Care and Use Committee of the Weizmann Institute of Science approved all experimental procedures.

Conditioned odor aversion (COA) to female odors

The COA protocol included the following experimental stages (Fig. 1a): Male mice were tested for their baseline olfactory preference (OP) on days 1–2 of the experiment. Three conditioning sessions were performed on days 4–6 and a forth was performed on day 13 (see *Conditioning procedure*). On days 8–9 and 11, mice were tested in the OP and sexual MAs, respectively. On day 14, we conducted the SI assay with a receptive female intruder and this assay was repeated again on days 17, 20 and 31 with new unfamiliar females as an extinction procedure.

Conditioning (c) procedure

Animals were divided randomly into Lithium Chloride (LiCl, 0.2 M, 2% body weight, J.T. Baker) and saline-treated (0.9% NaCl) groups. One hour before each conditioning session, water and food were removed from the home cage. The saline and LiCl-treated groups were exposed to female-soiled bedding in their home cages for 12 min (Fig. 1c). Intraperitoneal (i.p.) injections of LiCl or saline were applied 5 min after the introduction of the bedding, and 7 min following the injections the bedding was removed from the subject's home cage. This conditioning pairing was conducted three times during three consecutive days (days 4–6). On day 7 mice were exposed in their home cage to clean bedding for 5 min in the same behavioral context as in the conditioning days, except no injections were applied.

On day 13, an additional conditioning session was conducted to the LiCl and saline groups, as described above, in order to strengthen the aversive learning.

Soiled bedding was taken from cages of unfamiliar sexually mature females housed in groups of four (4–6 month old, same strain as the tested mice). Females used as the source for the bedding were weekly exposed to soiled bedding of males to induce estrus. For bedding presentation, 30 ml of either female-soiled or clean bedding, were placed in an open polycarbonate cup (5 cm height, 7.5 cm diameter), which allowed physical access to the stimuli (i.e. exposure to both volatile and non-volatile pheromones). In a subset of experiments we induced aversion only to volatile compounds emanating from female-soiled bedding. This was achieved by placing the bedding in a closed polycarbonate cup with evenly distributed small holes (5 mm in diameter). Fresh soiled bedding was collected before the beginning of each experimental day.

Olfactory preference (OP) assay

Apparatus: The three-chamber apparatus consisted of two large side chambers and one narrow middle chamber as previously described (Karvat & Kimchi 2012; Zilkha *et al.* 2016a). *Procedure*: The OP assay was conducted before (days 1–2) and after (days 8–9) the conditioning phase. Prior to the first OP test only, a habituation day was conducted and the mice were placed in the three chamber apparatus for 15 min, while they were free to explore the entire apparatus, in order to rule out any side preferences. During the next 2 days, the mice were tested in the apparatus for their preference for female or male-soiled bedding vs. clean bedding (the order of the sex of the bedding stimuli was counter-balanced between the mice). Following a 10 min habituation period, 30 ml of clean bedding were placed in a polycarbonate cup (described above) and a similar amount of soiled bedding was placed in another, and each cup was stationed in a corner of one of the two main chambers (Fig. 1b). Mice were allowed to freely move between the chambers for 5 min. Experiments were recorded and analyzed for the time spent interacting with each of the cups using the EthoVision software (version 7.1.426 Noldus). An OP index was defined as the difference between the times spent sniffing the stimulus bedding and clean bedding divided by the total time spent sniffing either bedding, multiplied by 100. Then, the result for male bedding was subtracted from the result for female bedding, such that a positive value indicates that the preference is towards female over male bedding:

$$\frac{\text{Female-Clean}}{\text{Female}+\text{Clean}} \times 100 - \frac{\text{Male-Clean}}{\text{Male}+\text{Clean}} \times 100$$

Sexual motivation assay (MA)

Apparatus: Two red Plexiglass boxes, a start-box and a goal-box ($12.5 \times 8.5 \times 8\ cm^3$), were connected through a transparent narrow tunnel ($2.5 \times 2.5 \times 29\ cm^3$) that contained three thin openings on top, through which three barriers could be inserted (Fig. 1d). The barriers were Plexiglass rectangles of increasing heights, making passage

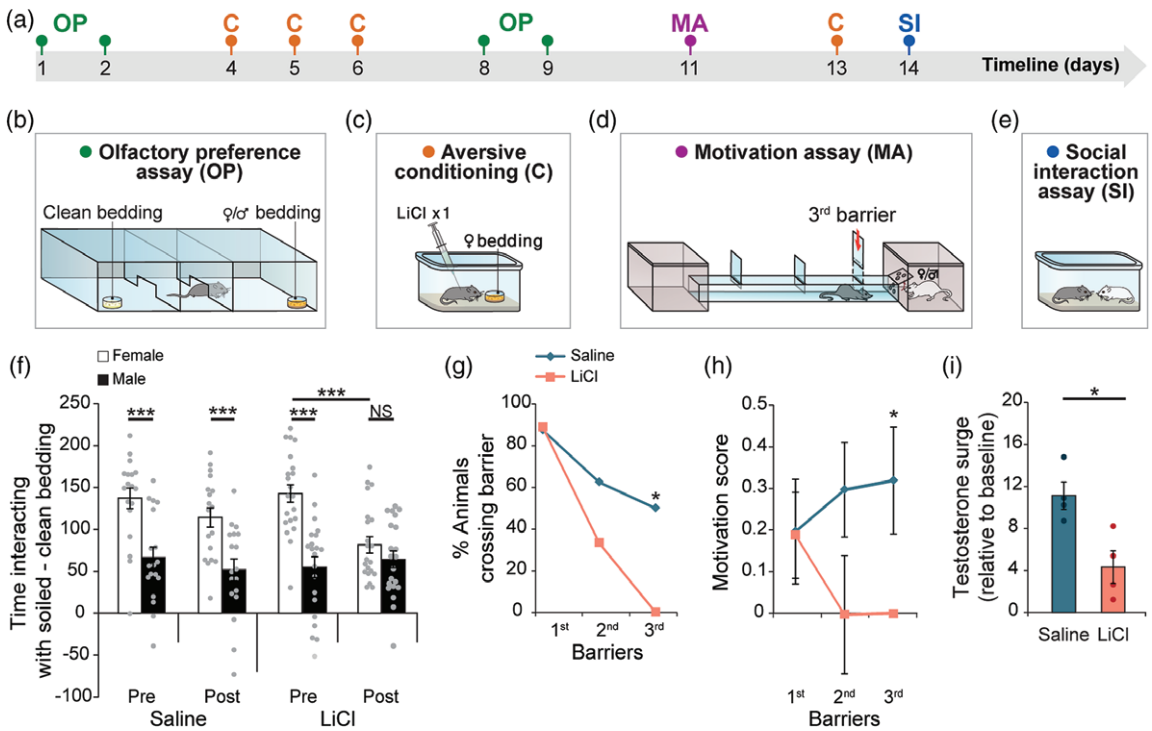

Figure 1: Female-specific COA alters innate precopulatory responses in WT males. (a) Timeline (days) of the conditioned odor aversion (COA) protocol. (b)–(e) Schematic illustrations of the different behavioral procedures. (f) Time (seconds) spent interacting with female and male bedding, subtracting the time (seconds) spent with the respective clean bedding, during the OP assay for the LiCl ($n = 21$) and saline ($n = 18$) groups. (g) Percentage of animals crossing the first, second and third barriers in the motivation assay ($n_{LiCl} = 9$, $n_{Saline} = 7$). (h) Motivation score to reach a female reinforcer for saline and LiCl-treated groups. (i) Testosterone surge for the saline- and LiCl-treated males ($n = 4$ per group) following exposure to female odors, relative to baseline levels of naïve animals. Values are displayed as mean ± SEM. $***P < 0.001$, $*P < 0.05$, NS: not significant.

underneath each successive barrier more difficult. The barriers differed in their distance from the bottom of the tunnel. The first easiest barrier allowed 15 mm of passage space, while the following barriers allowed 13, and then 11 mm. *Procedure*: A day before the test, mice were placed for 15 min of habituation in the apparatus and were allowed to freely move in the tunnel and between the boxes with no barriers. Then, they were tested for their motivation to overcome barriers to reach the goal-box that was either empty or contained a stimulus mouse, which was either a receptive female or a sexually mature male (8–10 weeks-old). A one-sided door ($4.5 \times 4.5\ cm^2$) separated the goal-box from the tunnel to prevent the stimulus mice from exiting the goal-box. In the first trial, the subject mouse had 10 min to cross the tunnel and reach the goal-box. If it succeeded, it was placed back in the start-box and the first barrier was placed for an additional 10 min, and so on until the third barrier was added. If the mouse failed to cross the tunnel after 10 min, the assay was stopped and no further trials were performed. The latency of the mouse to cross the tunnel and overcome 1, 2 or 3 barriers was measured using the Observer XT software (version 10.5.572 Noldus). The motivation score was defined as the difference in latency times between reaching an empty goal-box and reaching a stimulus goal-box, normalized to the total time of a single trial:

$$\frac{\text{Empty-Female}}{600}$$

The motivation score was calculated for each barrier, such that the motivation for the second barrier, for example, is calculated for the session where barriers 1 and 2 were present.

Social interaction (SI) assay

A sexually receptive and naïve 8 week-old female intruder was introduced into the home cage of the tested resident male for 15 min of SI, on days 14, 17, 20 and 31 of the experiment (Fig. 1e). For control experiments, the resident males were introduced with a sexually naïve 5 week-old intruder males swabbed with 140 µl urine collected from sexually mature and experienced male mice. Behavior was recorded using digital video cameras and was later scored by a single observer who was blind to the experimental conditions, using the Observer software. Behavioral parameters scored included olfactory investigation, sexual behavior parameters together with anxiety-related behaviors of avoidance and freezing, and aggressive behavior.

Anxiety-related assays

To examine anxiety-related behaviors we performed the open field assay and the elevated plus maze assay on a separate cohort of WT mice that underwent the conditioning phase followed by the MA. As described previously (Scott *et al.* 2015), the open field assay was performed in a white Plexiglas box ($50 \times 50 \times 40\ cm^3$) under dim white light (120 Lux). Each mouse was placed in the center of the apparatus and its movement patterns were recorded for 10 min, and analyzed using the EthoVision software. Total distance traveled and number of visits to the center of the apparatus were measured. For the elevated plus maze assay we used a polyvinyl chloride maze comprising a central part ($5 \times 5\ cm^2$), two opposing open arms ($30.5 \times 5\ cm^2$), and two opposing closed arms ($30.5 \times 5 \times 15\ cm^3$). The apparatus was raised to a height of 50 cm. The test was performed under dim white

light. Each mouse was placed in the center of the apparatus and its locomotion was recorded for 5 min. Time spent in the open arms and number of visits to the open arms were measured using EthoVision.

Testosterone and corticosterone quantification

Three days following the completion of the anxiety-related assays, male mice were exposed to female-soiled bedding in their home cages for 10 min. Immediately after the olfactory stimulation, the males were deeply anesthetized and blood samples were collected from the orbital sinus. The blood was centrifuged at 1000 *g* for 15 min and the supernatant containing the plasma was collected and stored at −80°C. Plasma testosterone and corticosterone levels were measured using commercial ELISA kits according to the manufacturer's protocol (Cayman, Ann Arbor, MI, USA; Figs. S3d and S4f). Additionally, plasma levels of naïve male mice were collected for quantification of baseline testosterone levels, using the same protocol.

COA to male odors

The COA protocol to male odors included the following stages, similarly to the COA to female odors: Male mice were tested for their baseline OP on days 1–2 of the experiment. Three conditioning sessions were performed on days 4–6, and an additional conditioning session was conducted on day 13. In the conditioning phase, the subject male was exposed to male bedding, followed by either saline or LiCl i.p. injection. Soiled bedding was taken from cages of unfamiliar sexually mature males housed in groups of 2 mice (4–6 month old, same strain as the tested mice). On days 8–9 and 14, mice were tested in the OP assay, and SI assay, respectively. The OP and SI assays were conducted exactly as described above. In the SI assay, the male subject was exposed to a male intruder and for control, to a receptive female intruder.

Statistical analysis

Time spent interacting with the stimulus beddings in the OP assay and the preference index was analyzed by repeated measures analysis of variance (ANOVA), followed by Fisher LSD post hoc analysis. Behaviors in the MA and SI assay as well as testosterone and corticosterone levels were analyzed using the Mann–Whitney *U*-test. When comparing between the percentages of animals preforming a specific behavior we used the Fisher exact test. Significance in the open field and elevated plus maze assays were all analyzed using the student's *t*-test. All statistical analyses were performed using the Statistica software (version 8.0 Statsoft, Tulsa, OK, USA). Significant results were considered for $P \leq 0.05$.

Results

Female-specific COA impairs innate precopulatory responses in WT males

In the following set of experiments, the conditioned males had full physical access to female-soiled bedding during the aversive conditioning session. Differences in the time spent investigating female- and male-soiled bedding before and after the conditioning phase were evaluated for the saline- and LiCl-treated males. Repeated measures ANOVA showed a significant interaction between 'group' × 'phase' × 'stimuli' ($F_{1,37} = 8.81$, $P < 0.01$). WT males, which underwent female-specific COA (LiCl group) lost their preference for female odors and spent similar amounts of time interacting with female- and male-soiled bedding (Fig. 1f), stemming from a significant decrease in time spent interacting with female bedding ($P < 0.001$, Fig. 1f). In the OP index for female- vs. male-soiled bedding, a significant interaction between 'group' and 'phase' was found ($F_{1,37)} = 5.1$, $P < 0.05$, Fig. S1a). Post-hoc analysis showed a significant decrease in the OP index following the LiCl conditioning ($P < 0.001$), but not following saline conditioning (Fig. S1a, Video S1). In the MA, nearly 60% of males from the saline group crossed the third barrier in order to reach a goal-box containing an unfamiliar sexually receptive female, whereas none of the males from the LiCl group crossed the third barrier ($P < 0.05$, Fig. 1g, Video S2). In accordance, LiCl WT males received a significantly lower motivation score to cross the third barrier and reach the unfamiliar female ($Z = 2.5$, $P < 0.05$; Fig. 1h), whereas the motivation score to reach a male was similar in the LiCl and saline groups ($Z = 1.16$, $P = 0.38$; Fig. S2a). LiCl WT males also displayed a significantly lower female-induced testosterone surge compared to the saline WT males ($Z = 2.3$, $P < 0.05$; Fig. 1i and Fig. S3a,c).

Female-specific COA induces long-lasting deficits in the sexual behaviors of WT males

In order to examine whether COA will affect consummatory (copulatory) behaviors, we tested the sexual behavior of the males towards an unfamiliar sexually receptive female in a 15 min SI assay. We found that the LiCl group spent significantly less time engaging in sexual behavior ($Z = 2.36$, $P < 0.05$; Fig. 2a) and performed significantly less pelvic thrusts ($Z = 2.86$, $P < 0.01$; Fig. 2b) compared to the saline-treated males. No difference was detected between the groups in the duration of aggressive behavior towards a male intruder ($Z = -0.03$, $P = 0.97$; Fig. S2b).

We next evaluated the long-term effects of aversive olfactory conditioning on consummatory behavior towards unfamiliar females at days 17, 20 and 31 from the beginning of the experiment. Surprisingly, LiCl males kept presenting significantly shorter durations of sexual behavior (repeated measures ANOVA; 'group' effect: $F_{1,13} = 7.16$, $P < 0.05$, Fig. 2c) and lower number of pelvic thrust events ('group' effect: $F_{1,13} = 6.32$, $P < 0.05$, Fig. 2d) even almost a month following the first conditioning session.

Female-specific COA induces social anxiety responses

During the SI assay we measured the distance of the subject male from the intruder female. The average distance from the receptive female was significantly larger in the LiCl males compared to saline males ($t_{20} = -2.21$, $P < 0.05$; Fig. 3a). Unexpectedly, a fleeing behavior was observed towards the female among 75% of LiCl males, compared to only 30% of males from the saline group ($P = 0.08$, Fig. 3b, Video S3). LiCl WT males exhibited high levels of anxiety manifested in a significant increase in female-directed avoidance events ($Z = -1.95$, $P = 0.05$; Fig. 3c), and a significantly greater duration of freezing behaviors ($Z = -2.04$, $P < 0.05$; Fig. 3d). In contrast, both LiCl and saline groups presented no significant differences in anxiety-related behaviors towards male intruders ($Z_{\text{Freezing}} = -1.25$, $P = 0.2$; $Z_{\text{Avoidance}} = 0.7$, $P = 0.47$; Fig. S2c,d). Finally, we measured blood levels of corticosterone following exposure to female odors. No difference was found in corticosterone levels between saline and LiCl groups

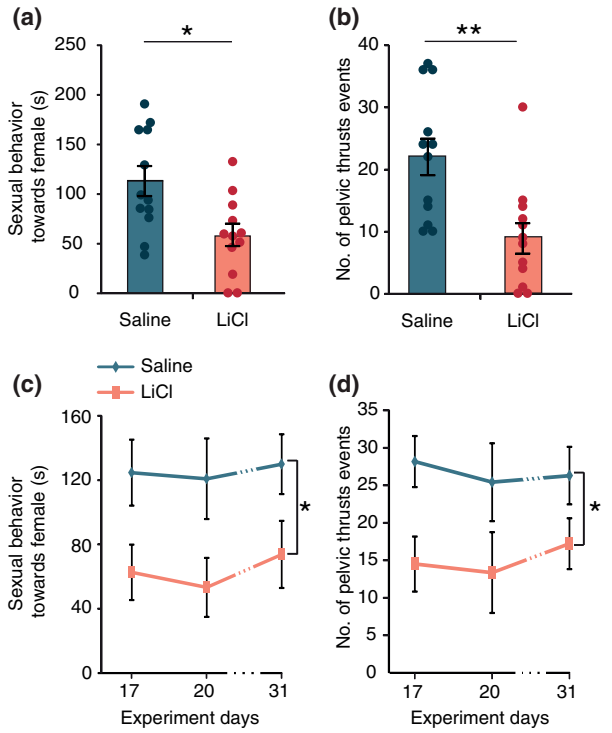

Figure 2: Female-specific COA induces long-lasting deficits in sexual behaviors of WT males. (a) Sexual behavior duration and (b) pelvic thrusts towards a female intruder in the SI assay ($n = 12$ per group). (c) Sexual behavior duration and (d) pelvic thrusts towards females in the SI assay conducted at days 17, 20 and 31 of the experiment. Values are displayed as mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

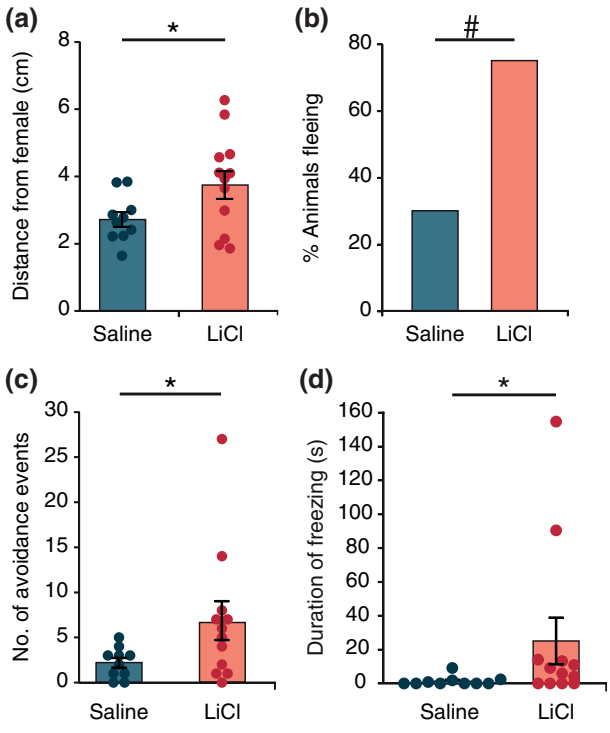

Figure 3: Female-specific COA induces social anxiety-like behaviors. (a) Distance from the female intruder in the SI assay ($n_{LiCl} = 12$, $n_{Saline} = 10$). (b) Percentage of animals fleeing from the female intruder. (c) Female-directed avoidance events and (d) freezing duration for saline and LiCl-treated males. Values are displayed as mean ± SEM. $^{*}P < 0.05$, $\#P = 0.08$.

($Z = 0.87$, $P = 0.37$; Fig. S4e). In addition, no difference was found between the groups in anxiety-related behaviors measured by the elevated plus maze and open field assays (Fig. S4a–d).

Volatile female pheromones are sufficient to induce conditioned aversion

A separate cohort of WT males underwent a slightly modified COA limiting the exposure only to the volatile odors emanating from female-soiled bedding. LiCl-treated males showed a significant reduction in OP towards females following the aversive conditioning (repeated measures ANOVA; interaction effect: $F_{1,20} = 5.53$, $P < 0.05$; Fig. S5a). Moreover, aversive conditioning using volatile female odors was sufficient to cause a decrease in sexual behavior ($Z = 2.31$, $P < 0.05$, Fig. S5b) and an increase in avoidance from an unfamiliar receptive female ($Z = -3.11$, $P < 0.01$; Fig. S5c) in LiCl males compared to the males from the saline group.

Male-specific COA does not alter OP

Next, we examined whether we can alter innate behaviors towards male conspecifics by inducing a specific aversion to male-soiled bedding. Repeated measures ANOVA of time interacting with female- and male-soiled bedding revealed a significant effect for the stimulus ($F_{1,15} = 49.63$, $P < 0.001$, Fig. 4a). A marginally significant decrease in time spent interacting with male bedding after the conditioning phase was detected in the LiCl group ($P = 0.06$). The COA did not alter OP towards female odors as no significant main effects of 'phase' or 'group' were found when analyzing the OP index ('phase': $F_{1,15} = 0.95$, $P = 0.34$; 'group': $F_{1,15} = 0.34$, $P = 0.56$, Fig. S1b). In the SI assay, 100% of the LiCl males executed aggressive attacks towards a male intruder, compared to 60% of males from the saline group ($P = 0.08$, Fig. 4b). However, no difference was found between the groups in either aggressive score ($Z = -1.25$, $P = 0.21$, Fig. 4c), or in anxiety-related behaviors such as freezing or avoiding the male intruder ($Z_{Freezing} = 0$, $P = 1$; $Z_{Avoidance} = -0.48$, $P = 0.63$; Fig. 4d,e). As for the behavior towards receptive females, we found no difference between the groups in any of the sexual behavioral parameters or anxiety-related behaviors examined (Fig. S6).

Female-specific COA impairs precopulatory and copulatory behaviors in TrpC2$^{-/-}$ males

We employed a female-specific COA protocol on adult, sexually naïve, TrpC2$^{-/-}$ male mice, using the same methodologies applied on the WT males (Fig. 1a–e).

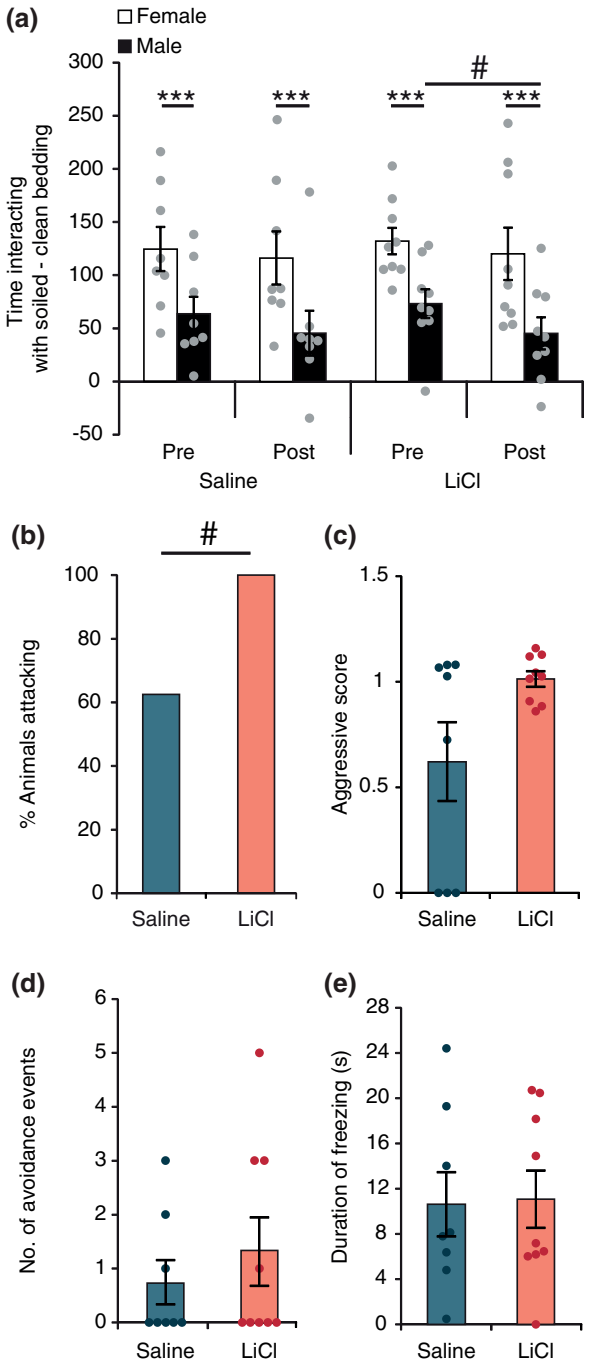

Figure 4: Male-specific COA does not alter inter-male aggressive behaviors. (a) Time (seconds) spent interacting with female and male bedding, subtracting the time (seconds) spent with clean bedding in the OP assay for the LiCl ($n=9$) and saline ($n=8$) groups. (b) Percentage of animals presenting aggressive behavior towards a male intruder in the SI assay. (c) Aggression score calculated from aggressive duration and latency to attack. (d) Number of avoidance events and (e) duration of freezing among the saline- and LiCl-treated males. Values are displayed as mean ± SEM. ***$P<0.001$, #$P=0.06$ (a), #$P=0.08$ (b).

We found that TrpC2$^{-/-}$ males develop a female-specific aversion, manifested in a significant interaction effect of 'group' × 'phase' × 'stimuli' ($F_{1,20}=7.8$, $P<0.05$, Fig. 5a). Post hoc analysis revealed a significant decrease in the time LiCl TrpC2$^{-/-}$ mice spent investigating female-soiled bedding ($P<0.001$), while the time spent investigating male bedding remained the same (Fig. 5a). Since prior to the conditioning procedure the TrpC2$^{-/-}$ males had no preference to female or male odors, the aversive conditioning actually resulted in an OP towards males, with the TrpC2$^{-/-}$ males spending more time investigating the male- over female-soiled bedding ($P<0.001$, Fig. 5a). Overall, statistical analysis of the OP index shows an interaction effect of 'group' × 'phase' ($F_{1,20}=6.96$, $P<0.05$; Fig. S1c). Following the conditioning procedure the sexual preference was changed in favor of male odors in LiCl males ($P<0.01$), while among the saline males, no change in the preference index was detected throughout the experiment (Fig. S1c). In the MA, LiCl TrpC2$^{-/-}$ males received significant lower motivation scores to cross the third barrier and approach a receptive female ($Z=1.92$, $P=0.05$; Fig. 5b), however, there was no difference in the motivation score to reach a male conspecific between the groups (Fig. S7a). Unlike WT males, the LiCl and saline TrpC2$^{-/-}$ males showed no significant difference in testosterone levels following exposure to a female ($Z=-0.86$, $P=0.38$; Fig. 5c, Fig. S3b,c).

Following the aversive conditioning to female odors we tested TrpC2$^{-/-}$ males in the SI assay. It was previously reported that TrpC2$^{-/-}$ males mount both females and males indiscriminately (Leypold *et al.* 2002; Stowers *et al.* 2002). When presented with a receptive female, LiCl TrpC2$^{-/-}$ males spent significantly less time engaging in sexual behavior than saline-treated TrpC2$^{-/-}$ males ($Z=2.04$, $P<0.05$; Fig. 5d). However, no difference was detected in the duration of sexual behavior towards male intruders between the saline and LiCl group ($Z=0.15$, $P=0.87$; Fig. S7b). Moreover, LiCl-conditioned males exhibited more female-directed avoidance and freezing responses ($Z_{Avoidance}=-2.98$, $P_{Avoidance}<0.01$; $Z_{Freezing}=-2.45$, $P_{Freezing}<0.05$; Fig. 5e,f), but there were no differences in their freezing or avoidance responses towards a male conspecific (Fig. S7c,d). These data suggest that TrpC2$^{-/-}$ males were able to distinguish between male and female odors, acquiring a specific aversion to female signals, which caused a robust alteration in their behavior exclusively towards females.

Discussion

Whenever sex-specific pheromones are detected through the VNO and/or the MOE of the male mouse, they convey crucial information regarding the identity of the animal, and generate an appropriate innate reproductive response. Whether theses innate responses are sensitive to environmental changes and to what extent is less clear. The present study aimed to explore experience-dependent plasticity in innate reproductive behaviors triggered as a response to sex-specific pheromones and the involvement of the VNO in such plasticity.

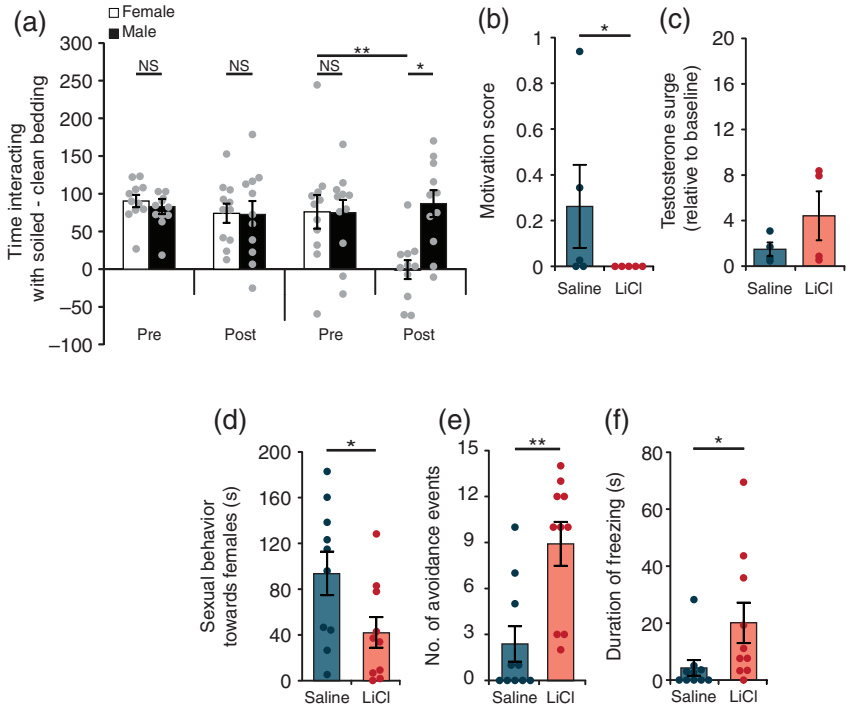

Figure 5: Female-specific COA induces alterations in precopulatory and copulatory behaviors in TrpC2$^{-/-}$ males. (a) Time (seconds) spent interacting with female- and male-soiled bedding, subtracting the time (seconds) spent with clean bedding in the OP assay ($n = 11$ for saline and LiCl groups). (b) Motivation score to cross the third barrier and reach a female reinforcer ($n = 5$ for saline and LiCl groups). (c) Testosterone surge of saline- and LiCl-treated males following exposure to female odors, relative to baseline levels of naïve animals ($n = 4$ per group). (d) Sexual behavior duration, (e) no. of avoidance events, and (f) freezing durations measured for saline and LiCl-treated groups in the SI assay ($n = 10$ per group). Values are displayed as mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$, NS: not significant.

Olfactory aversive conditioning using LiCl-induced malaise associated with female-soiled bedding, either with or without full physical access to the bedding, led to the development of aversive responses in WT adult males. LiCl-treated WT males lost their innate odor preference for females, and exhibited less motivation to invest effort in order to reach a female reinforcer. Additionally, the LiCl conditioning led to the abolition of the typical testosterone surge following exposure to female odors (Gleason *et al.* 2009; Shulman & Spritzer 2014).

The literature regarding the effect of aversive conditioning on reproductive behavior of male rodents is inconclusive and limited mainly to rats. Some early reports demonstrated that pairing LiCl injection with copulation (in rats) or vaginal secretions (in hamsters) reduces precopulatory behaviors (Agmo 2002; Johnston & Zahorik 1975; Zahorik & Johnston 1976). However, similar experiments on male mice showed that odor aversion to female urine caused only minor and transient changes in precopulatory behaviors such as olfactory preference and ultrasonic vocalizations elicited by female urine (Kay & Nyby 1992). We found that a negative experience associated with female pheromones altered precopulatory behavioral responses to the extent of eliminating sexual preference towards female odors and reducing sexual motivation. Moreover, it altered physiological responses induced by female pheromones in male mice.

Notably, we showed that aversive conditioning to female pheromones was sufficient to induce deficits in copulatory behaviors towards an unfamiliar sexually receptive female (that was not the donor of the odor stimulus used in the conditioning phase). Moreover, the effect of the aversive conditioning on this consummatory sexual behavior was stable for almost a month following the first conditioning day and resistant to extinction.

Female pheromones are highly rewarding for males, eliciting preferential approach and precopulatory behaviors, which terminate in the consummatory sexual act (Ago *et al.* 2015; Malkesman *et al.* 2010; Veening & Coolen 2014). These behaviors do not require any learning, but might be positively reinforced following sexual experience (Ago *et al.* 2015; Been *et al.* 2013; Pitchers *et al.* 2010). We argue that by associating a highly rewarding stimulus, as female pheromones, with a stimulus possessing an opposite rewarding value, i.e. LiCl-induced malaise, we caused the devaluation of the salience of female signals. The alteration in rewarding value severely impaired innate behavioral and physiological reactions typically seen towards females.

Further supporting our hypothesis is the observed effect of COA on testosterone levels following exposure to female pheromones. Testosterone plays an essential role in mediating the rewarding properties of female cues, as was shown in adult male hamsters where development of conditioned place preference to vaginal secretions was prevented following castration (Harding & McGinnis 2004) and restored following testosterone treatment (Bell & Sisk 2013). Thus, the testosterone surge induced by female pheromones seems to serve as a neuroendocrine response mediating the intrinsic rewarding value of females, which is required for stimulating male precopulatory behaviors (Bell *et al.* 2013; Petrulis 2013). In our study, LiCl-conditioned WT males lacked female-induced testosterone surge, indicating a reduction in the intrinsic rewarding value of female chemosignals following COA. This reduction in rewarding value manifested, in turn, in sexual behavioral deficits.

Remarkably, the aversive conditioning also triggered strong phobic and anxiety-related behavioral responses towards conspecific females, including freezing, avoidance and escape behaviors (Video S3). To our knowledge, such strong repulsive behavioral responses were never observed in reaction to conspecific females in aversion studies (Johnston *et al.* 1978; Peters 1983), and are typically emerging in the presence of predator-related signals (Brechbuhl *et al.* 2013; Papes *et al.* 2010) or strong aversive physical external stimuli (Jaisinghani & Rosenkranz 2015). Importantly, no differences were found between the saline and LiCl group in plasma corticosterone levels following exposure to female bedding, or in general anxiety assays. This suggests that the anxiety-like responses directed towards female conspecifics were not the result of the generally higher anxiety levels of LiCl-treated mice and may be classified as anxiety-related responses specifically triggered by social interactions. Human studies report that there is no difference in baseline cortisol levels between patients with social anxiety disorder and healthy controls (Condren *et al.* 2002; Martin *et al.* 2010). Moreover, similarly to our corticosterone results, some studies found no difference in cortisol following exposure to a social stimulus (Martel *et al.* 1999; Uhde *et al.* 1994). However, in our case this could be a result of a ceiling affect as the female stimulus might induce a maximal corticosterone elevation in saline-treated males, such that further increases cannot be seen (Kavaliers *et al.* 2001; Thornton *et al.* 2014).

In most cases, once a sexually mature WT male is introduced to a male intruder it will initiate an instinctive aggressive attack towards the intruder (Mackintosh 1970). This innate behavior is mainly mediated by pheromone signals (Chamero *et al.* 2007; Montani *et al.* 2013) and is thought to be reinforcing, as males perform operant responses to gain opportunity for an aggressive attack (Fish *et al.* 2005; May & Kennedy 2009). Thus, male pheromones might also possess rewarding properties. We explored whether we can change the rewarding properties of male odors by inducing a male-specific COA, and thus affect the innate aggressive behavior typically triggered by those odors. Our results show that aversive conditioning associated with male odors led to marginally significant effects in the behavior towards male stimuli, manifested in decreased time spent investigating conspecific male bedding, and increased inter-male aggression. On the other hand, male-specific aversion did not alter sexual preference or any other behavior towards females.

We demonstrated that the same aversive conditioning protocol of female or male odors led to different effects on the corresponding innate behavior. While female-specific COA resulted in the generalization of the aversion to females and the sexual act, male-specific COA had only minor effects on the behavioral repertoire. As the COA to male-soiled bedding was performed using the same protocol of COA to female-soiled bedding we suggest that the differential response observed is related solely to the nature of the conditioned stimulus (female vs. male bedding). We propose that this difference might emerge from the distinct motivational saliences assigned to female and male pheromones by male mice. According to the prediction error learning theory, an error is generated when an outcome is different from its prediction (either better than expected or worse than predicted). This leads to an updated prediction, which drives learning and behavioral change. However, when an outcome matches its predication, no error is generated and the behavior remains the same (Hollerman & Schultz 1998; Pignatelli & Bonci 2015; Schultz 2015). In our case, an unpleasant physiological response in males to the presentation of female odors is extremely unexpected thereby triggering learning. On the other hand, the association between male odors and an unpleasant outcome might be more expected, thus it is reasonable that only minor (if any) associative learning will occur, as previously demonstrated (Waelti *et al.* 2001). This may indicate that female and male odors fall at different places of the 'saliency spectrum', which ranges from aversion to reward (Pignatelli & Bonci 2015). Additionally, previous studies have demonstrated that factors such as odor type might also affect the magnitude of aversive learning as some odorants cannot be conditioned to induce aversion (Holder & Garcia 1987; Panhuber 1982). These characteristics might also explain the differential intensities of the COA between female and male odors.

Next, we successfully induced COA paradigm in TrpC2$^{-/-}$ males, which are deficient in VNO pheromone-mediated signal transduction. TrpC2$^{-/-}$ males managed to acquire a specific aversion to female signals, altering precopulatory and copulatory behaviors towards females, whereas behaviors towards male stimuli were not changed.

Previous studies show that TrpC2$^{-/-}$ males do not exhibit the innate mating preference towards females typically presented by WT males (Chalfin *et al.* 2014; Kimchi *et al.* 2007; Leypold *et al.* 2002; Stowers *et al.* 2002). This behavioral phenotype was explained by the inability of TrpC2$^{-/-}$ males to detect VNO-mediated signals, which is considered essential for the discrimination between sex-specific pheromones (Ibarra-Soria *et al.* 2014; Isogai *et al.* 2011; Stowers *et al.* 2002). Our results demonstrate for the first time that TrpC2$^{-/-}$ males can acquire sex-specific COA, similar to the abilities of WT males, and thus support previous studies reporting that sexually naïve males with surgically ablated VNOs can discriminate between female and male conspecifics' urine (Keller *et al.* 2006; Liu *et al.* 2010; Pankevich *et al.* 2004).

Sexual odor preference and female-induced testosterone surge are a result of assigning highly rewarding properties to female signals (Ago *et al.* 2015; Malkesman *et al.* 2010). The results obtained from TrpC2$^{-/-}$ males suggest that signaling through the VNO is not necessary for sexual discrimination but for sexual preference and motivation. Thus, they suggest that VNO inputs might be crucial for appropriate neuronal representation of the reward features of female vs. male signals.

Furthermore, since plasticity in reproductive behaviors can be readily induced in the absence of VNO-mediated inputs, we propose that female odors detected by the MOE mediate this aversive learning process. Given that the MOE is specialized in detecting volatile odors (Liberles 2014; Liberles & Buck 2006; Zhang *et al.* 2013), this conclusion resonates with our results showing that aversion towards female-emitted volatile odors was sufficient to alter male-specific precopulatory and copulatory behaviors. Collectively, as studies have shown, chemosignals detected by both the MOE and VNO inputs play an important role in assigning the reward

value of female-derived signals (Korzan *et al.* 2013; Stowers & Kuo 2015). Our results further suggest that the VNO mediates the innate value of female pheromones, while the MOE mediates their acquired value. Nevertheless, although TrpC2 is predominantly expressed in the VNO, recently it was shown to be expressed in some small subpopulations of neurons in the MOE (Omura & Mombaerts 2014). In addition, studies have demonstrated the activation of VNO neurons in cultured TrpC2$^{-/-}$ slices (Kim *et al.* 2011; Zhang *et al.* 2010). Thus, we propose that aversive olfactory conditioning occurs independently of TrpC2 signal transduction in the male mouse olfactory system. Further research is required to determine whether similar behavioral plasticity can be induced in females (Zilkha *et al.* 2016b).

To conclude, our findings demonstrate that transient aversive conditioning to female odors can overwrite the innate positive biological message of female stimulus with an acquired one, with only few conditioning trials of a short exposure to the conditioned stimulus. Furthermore, they prove that neural circuits underlying innate male-typical sexual behaviors are flexible and can be easily reshaped to mediate a more adaptive behavior that can even exceed the natural boundaries of response to sex-specific stimulus. Finally, our findings suggest that behavioral plasticity can occur in the absence of a fully functioning VNO in male mice.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: Olfactory preference indexes. (a) Olfactory preference index of WT males following aversion to female odors. (b) Olfactory preference index of WT males following aversion to male odors. (c) Olfactory preference index of TrpC2$^{-/-}$ males following aversion to female odors. Dashed line represents no preference for either female or male bedding. For index calculation see methods. Values are displayed as mean ± SEM. $***P < 0.001$, $**P < 0.01$.

Figure S2: Female-specific COA does not affect male-directed behaviors in WT males. (a) Motivation scores for LiCl ($n = 9$) and saline ($n = 8$) groups to cross three barriers and reach a male reinforcer. (b) Aggressive behavior duration, (c) freezing durations and (d) avoidance events towards a male intruder in the social interaction assay ($n_{LiCl} = 12$, $n_{Saline} = 9$). Values are displayed as mean ± SEM.

Figure S3: Concentrations of plasma testosterone levels. (a) Testosterone levels (ng/ml) of WT saline- and LiCl-treated males ($n = 4$ per group) following exposure to female odors. (b) Testosterone levels (ng/ml) of TrpC2$^{-/-}$ saline- and LiCl-treated males ($n = 4$ per group) following exposure to female odors. (c) Baseline testosterone levels (ng/ml) of WT ($n = 6$) and TrpC2$^{-/-}$ ($n = 5$) naïve male mice. Values are displayed as mean ± SEM. (d) Standard curve of ELISA plate including trendline and linear equation. $*P < 0.05$.

Figure S4: Female-specific COA does not affect corticosterone levels or anxiety-related behaviors. (a) Time spent in open arms and (b) number of visits to the open arms in the elevated plus maze. (c) Total distance traveled and (d) number of visits to the center zone in the open field assay ($n_{LiCl} = 8$, $n_{Saline} = 5$). (e) Plasma corticosterone levels (ng/ml) of saline and LiCl-treated groups ($n_{LiCl} = 12$, $n_{Saline} = 8$) following exposure to female odors. Values are displayed as mean ± SEM. (f) Standard curve including trendline and linear equation for the corticosterone ELISA assay.

Figure S5: Volatile female pheromones are sufficient to induce conditioned aversion. (a) Sexual preference index of LiCl ($n = 12$) and saline ($n = 10$) treated males, before and after the aversive conditioning. (b) Number of sexual behavior events and (c) avoidance events towards the female intruder in the LiCl ($n = 11$) and saline ($n = 9$) groups. Values are displayed as mean ± SEM. $**P < 0.01$, $*P < 0.05$.

Figure S6: Male-specific COA has no effect on female-directed behaviors in WT males. (a) Sexual behavior duration, (b) latency to mount a female and (c) number of avoidance events measured in the social interaction assay with a receptive female intruder for saline- and LiCl-treated males ($n_{LiCl} = 9$, $n_{Saline} = 9$). Values are displayed as mean ± SEM.

Figure S7: TrpC2$^{-/-}$ males do not generalize the aversion to male stimuli. (a) Motivation scores for LiCl and saline males (n = 5 per group) to cross three barriers and reach a male reinforcer. (b) Sexual behavior duration, (c) freezing duration and (d) avoidance events towards a male intruder in the social interaction assay (n = 10 per group). Values are displayed as mean ± SEM.

Table S1: Data of high-density single-nucleotide polymorphism (SNP) array (Chalfin *et al.* 2014). Genotyping data of the entire chromosome 7, with 2611 SNP markers for C57BL/6 J and 129S1 inbred mouse strains and the TrpC2$^{-/-}$ mouse strain.

Video S1: WT males prefer to interact with female odors in the olfactory preference assay. The video shows a WT male exploring the 3-chamber apparatus and the cups containing clean and female-soiled bedding. The final screenshot presents the track of the mouse for the entire assay (extracted from the EthoVision software).

Video S2: Motivation of typical males to reach a receptive female. The video displays a WT male investing effort to cross three barriers in order to reach a goal box containing a receptive female.

Video S3: LiCl conditioning alters the expected response of males towards a female intruder, from sexual approach to avoidance. In this video, WT males following either saline or LiCl conditioning were introduced with a female intruder in their home cage. In the first part of the movie a saline male performs olfactory investigation of the female followed by consummatory sexual behavior. In contrast, the second part presents a LiCl male avoiding the female and exhibiting short freezing responses.