Extinction reverses olfactory fear-conditioned increases in neuron number and glomerular size

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Although much work has investigated the contribution of brain regions such as the amygdala, hippocampus, and prefrontal cortex to the processing of fear learning and memory, fewer studies have examined the role of sensory systems, in particular the olfactory system, in the detection and perception of cues involved in learning and memory. The primary sensory receptive field maps of the olfactory system are exquisitely organized and respond dynamically to cues in the environment, remaining plastic from development through adulthood. We have previously demonstrated that olfactory fear conditioning leads to increased odorant-specific receptor representation in the main olfactory epithelium and in glomeruli within the olfactory bulb. We now demonstrate that olfactory extinction training specific to the conditioned odor stimulus reverses the conditioning-associated freezing behavior and odor learning-induced structural changes in the olfactory epithelium and olfactory bulb in an odorant ligand-specific manner. These data suggest that learning-induced freezing behavior, structural alterations, and enhanced neural sensory representation can be reversed in adult mice following extinction training.

Increasing evidence suggests that the cellular, neuroanatomical, and receptive field organizations of vertebrate sensory systems are continually reshaped throughout adulthood by cues from the external environment. Activity-dependent changes are known to occur both during critical periods of development and also in the adult brain, allowing the animal to optimally perform behaviors based on the demands of the surrounding environment. Postmitotic organizational changes, along with activity-dependent plasticity, have been largely implicated in shaping sensory circuits from development through adulthood (1–4). In particular, the olfactory sensory system of adult mice exhibits functional and neuroanatomical learning-dependent changes following olfactory fear conditioning in adulthood (5–7). The M71-LacZ transgenic mouse line expresses LacZ under the M71 odorant receptor (OR) promoter (encoded by the olfactory receptor 151 gene, \textit{Olf151}) (8) in the M71 OR-expressing, acetophenone-responsive population of olfactory sensory neurons (OSNs). Using this line, we previously demonstrated an increased number of M71-expressing OSNs in the main olfactory epithelium (MOE) of adult mice following olfactory fear conditioning to acetophenone (5, 7), an odorant that activates the M71/M72 ORs (9, 10). This increase in receptor-specific OSNs within the MOE was directly correlated with an increase in the area of M71+ axons innervating the M71 glomeruli within the olfactory bulbs (OBs). Behaviorally, these olfactory fear-conditioned mice also exhibited enhanced fear-potentiated startle (FPS) and freezing specific to the conditioned odor stimulus. Notably such changes were never seen with equivalent odorant exposure alone but only when the odorant was paired with an aversive or appetitive cue (5, 7), suggesting the critical importance of behavioral learning facilitating these structural and functional alterations.

Reversing the behavioral and neuroanatomical effects of such emotional learning is important for our understanding of disorders such as posttraumatic stress disorder (PTSD), in which exposure-based psychotherapy is widely used for treatment. Notably, extinction training in rodent fear-conditioning models closely parallels many aspects of exposure-based psychotherapy in humans where exposure to nonreinforced presentations of the previously acquired conditioned stimulus (CS) reduces acquired fear responses such as freezing to the CS (11, 12). In the current study, we demonstrate that previously acquired structural changes within the primary olfactory system are reversed with olfactory fear extinction specific to the conditioned odorant cue.

Results

Behavioral Responses Following Olfactory Fear Acquisition and Extinction.

To investigate the effects of cue-specific olfactory fear extinction, 2–3-mo-old, odor-naive homozygous M71-LacZ transgenic adult mice were handled and left in their home cage (HC) or were conditioned to associate mild foot shocks (US, unconditioned stimulus) with acetophenone (CS) using a previously described fear-conditioning protocol (5, 7). Three weeks after the last olfactory fear-conditioning session, mice were handled only or were exposed to an olfactory extinction training protocol consisting of a total of 90 nonreinforced odor-alone presentations over 3 consecutive days, with 30 trials each day (Fig. 1 A and B). During the acquisition of olfactory fear conditioning, all groups acquired olfactory fear-like behaviors to the odor CS (as assessed by freezing behavior) at equivalent levels and rates (Fig. 1B). Mice undergoing olfactory fear extinction training exhibited decreased freezing to the odor CS (acetophenone) across extinction trials and sessions (Fig. 1C). One hour before sacrifice, all mice were exposed to five presentations of the odorant CS (acetophenone) to assess freezing behavior and long-term olfactory fear retention. Mice that had been fear-conditioned 3 or 6 wk before testing continued to

Significance

Olfactory cues may be paired with traumatic experiences in humans (e.g., the smell of a physical abuser), and subsequent exposure to the environmental odor cue may serve as a reminder of the traumatic event and trigger posttraumatic stress disorder symptoms. Very few studies have investigated the mechanisms accompanying the processing of emotional learning at the level of specific sensory modalities. The present study demonstrates that extinction specific to the conditioned odor acetophenone (which activates the M71 receptor) reverses conditioning-associated increases in freezing behavior and M71-specific olfactory sensory neuron number and glomerular area in adult mice. These data highlight the potential to exploit sensory system plasticity as a means of ameliorating negative emotional memories that may be tied to peripheral sensory systems.


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Cue-Specific Olfactory Extinction 3 Wk Following Acquisition Reverses M71-Specific Neuroanatomical Enhancements. To investigate the neuroanatomical representation of neurons responsive to the conditioned odor, following sacrifice we used beta-galactosidase staining of the M71 OSNs in the MOE and the OB glomeruli for estimating OSN number counts in transgenic mouse lines in which OR genes are genetically tagged. The presented neuronal and glomerular data following olfactory fear acquisition and extinction suggest that (i) the effect of odor fear conditioning on olfactory sensory neuroanatomy is long lasting, as demonstrated by the increased numbers of OSNs and increased glomerular area specific to neurons responsive to the conditioned odor that is maintained at both 3 and 6 wk following training reduces the fear behavior to baseline when tested at the 6-wk time point.

**Fig. 1.** Olfactory fear extinction reverses conditioning-associated increases in freezing behavior. (A) Experimental time line of fear conditioning, extinction, and olfactory fear testing 1 h before sacrifice. (B) Mice acquire olfactory fear at similar rates across all groups (n = 9–12 per group) (extinction group has not yet undergone extinction training) [two-way repeated measures (RM) ANOVA, P = 0.4782, F(2,29) = 0.7569]. (C) Mice undergoing olfactory fear extinction exhibit decreased freezing to the odor CS across extinction sessions (n = 12). (D and E) Mice fear-conditioned 3 or 6 wk prior exhibit enhanced freezing to the odor CS compared with the HC and extinction groups (n = 9–12 per group) [D, two-way RM ANOVA, P < 0.0001, F(3,28) = 18.55; E, ANOVA, P < 0.0001, F(3,156) = 76.69]. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Fig. 2.** M71+ OSN numbers in the MOE are reversed with cue-specific olfactory extinction training. (A–C) Mice 6 wk post-fear conditioning (train to acetophenone 6 wk, gray bar) had a larger number of M71 OSNs in the MOE than HC (black bar) and extinction mice (train to acetophenone + extinction, white bar) [M71-LacZ, HC n = 16; train to acetophenone 6 wk, n = 21; train to acetophenone + extinction, n = 17; ANOVA, P < 0.0001, F(2, 51) = 15.72; HC versus train to acetophenone 6 wk, P < 0.001; train to acetophenone 6 wk versus train to acetophenone + extinction, P < 0.0001; HC versus train to acetophenone + extinction, P = n.s. (non-significant)]. (D) Mice 3 wk post-fear conditioning (train to acetophenone 3 wk, gray bar) and mice 6 wk post-fear conditioning (train to acetophenone 6 wk, gray bar) had an increased number of M71+ OSNs in the MOE than HC (black bar) and mice that were fear-conditioned to propanol, a non–M71-activating odorant (train to propanol 3 wk, gray bar with black spots) [M71-LacZ, HC n = 8; train to acetophenone 3 wk, n = 7; train to acetophenone 6 wk, n = 4; train to propanol 3 wk, n = 8; ANOVA, P < 0.0001, F(3, 23) = 18.38; HC versus train to acetophenone 3 wk, P < 0.0001; HC versus train to acetophenone 6 wk, P < 0.05; HC versus train to propanol 3 wk, P = n.s.; train to acetophenone 3 wk versus train to propanol 3 wk, P < 0.001; train to acetophenone 3 wk versus train to acetophenone 6 wk, P = n.s.]. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
following olfactory fear conditioning, and (ii) the effect of olfactory fear extinction on neuroanatomy is associated with decreased numbers of OSNs and decreased glomerular area relative to the trained groups; both of these effects are specific for the conditioned odor stimulus (acetophenone). Because the extinction group had undergone the same behavioral exposure as the 6 wk trained group before extinction, we conclude that the findings represent a dynamic reversal of the increased odorant-specific neuronal populations generated through olfactory fear conditioning. To ensure the cue specificity of our observed extinction effect, an additional control group was run with a separate set of animals; 3 wk following olfactory fear conditioning to acetophenone, this group received 3 consecutive days of propanol odor presentations (a non–M71-activating odorant) and was then killed 3 wk after the last exposure to propanol session (Fig. 4i). Propanol was used as a control odorant for several reasons: (i) Propanol has been shown to activate a region of the OBs distinct from those activated by acetophenone as assessed by glomerular activity patterns (gara.bio.uci.edu), (ii) previously published data have demonstrated that adult mice are able to discriminate between acetophenone and propanol at the level of fear behavior (5, 17), and (iii) adult mice fear-conditioned to propanol do not exhibit an enhanced M71-specific glomerular area compared with HC controls (5). Propanol is thus an ideal control odorant to test the cue specificity of our findings. Mice that were fear-conditioned 6 wk prior (with no olfactory extinction) and mice that were fear-conditioned to acetophenone and received non–cue-specific odor and context exposure (exposure to propanol) exhibited a significant increase in glomerular area at the level of the OB compared with HC control mice (Fig. 4B and Fig. S2). The observed increases in glomerular area were paralleled by increases in the number of M71 OSNs in the MOE compared with HC controls. These data demonstrate that it is the cue-specific extinction (and not merely the exposure to the context or a non-CS olfactory cue) that results in a reversal of conditioning-related glomerular size in the OB and OSN number in the MOE.

**Cue-Specific Olfactory Extinction Immediately Following Acquisition Blocks M71-Specific Neuroanatomical Enhancements.** We next wished to replicate the above observation as well as to determine whether the reversal of structure depends on the 6-wk time course from initial training. Thus, we performed an alternate time course of extinction training in which a separate group of odor and behavior-naïve mice received extinction training in the 3 d immediately following the 3 d of acquisition and were killed 3 wk following the last extinction session (Fig. 4C). Mice that were fear-conditioned to acetophenone but did not undergo olfactory extinction exhibited enhanced dorsal and medial glomerular area as well as increased M71+ OSN numbers compared with all control groups (Fig. 4 C and D and Fig. S3). Notably exposure to acetophenone only without prior conditioning (odorant presentations alone) did not produce an increased M71+ glomerular size or M71+ OSN number compared with HC controls, suggesting that it is the prior learned CS–US association that results in a corresponding enhanced neuroanatomical representation for the conditioned odor stimulus, rather than presentations of the odorant alone. Mice that received olfactory fear extinction immediately after conditioning had significantly decreased M71+ dorsal and medial glomerular areas as well as decreased M71+ OSN numbers compared with mice receiving only fear conditioning. These data demonstrate that the timing of cue-specific olfactory extinction immediately after acquisition also leads to a reversal in prior olfactory fear learning effects (as measured by the glomerular area in the OBs and OSN number in the MOE).

**Discussion**

These experiments demonstrate a reversal in neuroanatomical changes to OSN number and the glomerular area specific for the conditioned odor stimulus following an extinction protocol that occurs either 3 wk after or immediately following the fear learning event, demonstrating dynamic sensory epithelial plasticity with learning. Dynamic alterations in structural plasticity and neuroanatomical representation accompanying learning have been observed in other sensory systems; for example, studies in the primary auditory system have demonstrated that local tuning shifts produce a specific increase in the area of frequency representation within the tonotopic organization of primary auditory cortex (A1) (18, 19). A learning-dependent shift in primary representational area may be a common feature across primary sensory systems. Work in developmental rodent systems has also demonstrated behavioral and OB neural responses following an olfactory learning event during the preweaning period that is reversed with extinction training (20). However, the finding that at the level of the adult olfactory primary sensory system such changes are structurally reversible with a relatively small number of behavioral exposures is quite remarkable.

Learning-dependent alterations in olfactory plasticity were measured at 3 and 6 wk following olfactory fear conditioning, as alterations are not yet measureable at an earlier time point 3 d
following the last day of the olfactory fear-conditioning session (Fig. 3D and Fig. S4). Thus, the effect of olfactory fear extinction immediately after olfactory fear acquisition versus at 3 wk following acquisition may differ mechanistically based on the finding that at 3 wk following conditioning a neuroanatomical increase has been established, whereas it has not yet occurred in the 1–3 d following conditioning (Fig. 3D and Fig. S4). A more in-depth investigation into the time course following both the acquisition and extinction of olfactory fear could yield valuable insights into the mechanisms regulating our observed effects.

Further related to the observed time course of epithelial and glomerular structural changes after olfactory fear conditioning and extinction is their relation to changes in freezing behavior. As described above, structural changes have not yet emerged at 1–3 d after cue-specific olfactory fear conditioning or extinction, despite changes in freezing behavior. Furthermore, we demonstrate a decrease in freezing behavior between the 6 and 3 wk group (although we do not observe a decrease or reversal of structural changes in the OB or MOE at the 6-wk time point following olfactory fear conditioning to acetophenone). Thus, although it does not appear that the passage of time reverses our observed olfactory structural changes, the dissociation between our behavioral and structural effects at the 6-wk time point may reflect additional top–down processing mechanisms that are known to regulate behavioral responses such as freezing behavior. Together, these data suggest a potential role for higher processing regions, such as the amygdala or medial prefrontal cortex (mPFC), that may play important roles in the immediate behavioral readouts of a learning event, whereas changes in olfactory primary sensory representation may occur over a longer time period, thus influencing future sensitivity to the CS cue. Within our model, olfactory fear conditioning leading to an enhanced number of CS-responsive OSNs may serve to enhance the animals’ sensitivity to an important environmental cue, whereas following olfactory fear extinction, the accompanying decrease in CS-responsive OSNs may lead to a decrease in overall sensitivity to an extinguished odor. Complementary work in adult rodent systems has shown neurophysiological evidence of an in vivo odor-specific enhancement of the synaptic output of OSNs following an associative learning event (6). Thus, whereas regions such as the amygdala, hippocampus, or prefrontal cortex may allow for the inhibition of fear expression or the modulation of that inhibition (12, 21), the primary olfactory sensory system likely plays a major role in the CS sensitivity and responsiveness following a learning event and its extinction. A growing body of literature suggests that alterations in synaptic transmission within the basolateral amygdala (BLA) play an important role in the suppression of conditioned fear responses and extinction learning (22, 23); in fact, there is evidence of depotentiation of conditioning-related amygdala synaptic transmission that occurs following extinction training (24–27). These data are in line with our observation that extinction reverses aspects of conditioning-related effects, although the contribution of regions such as the amygdala in the regulation of primary sensory system structural changes remains an important area for future investigation.

Traditionally, extinction has been thought of not as an erasure or reversal of the initial fear memory but rather as leading to the formation of a new inhibitory memory, based on the behavioral properties of extinction such as spontaneous recovery (28). Interestingly, we do not observe a spontaneous recovery (the return of the conditioned fear response) of freezing behavior 3 wk following olfactory extinction (Fig. 1D and E), suggesting a robust and long-lasting effect of cue-specific extinction both at the level of freezing behavior and primary olfactory sensory system neuroanatomy. The spontaneous recovery of fear and threat responses depends on both the depth of extinction training as well as the length of time following olfactory extinction. Additional time points beyond the one included here (3 wk following extinction) could provide valuable insight into the long-lasting effects of olfactory fear extinction. Furthermore, although the behavioral effects of auditory cue-specific fear extinction have been thoroughly investigated, the long-lasting and robust reversal of freezing behavior following olfactory cue-specific extinction in adult animals is a previously unidentified finding within the field of fear extinction.

Fig. 4. Exposure to the context and propanol (non-M71-activating odorant) does not result in reversal of M71+ glomerular size in the OB. (A) Experimental time line of fear conditioning, exposure to propanol, and sacrifice. The timing and extinction/exposure sessions were identical in timing and session duration as those used for the extinction to acetophenone group (except only propanol was presented). (B) Mice fear-conditioned to aceto 6 wk prior (train to aceto 6 wk, gray bar) and mice fear-conditioned to aceto that received exposure to the non-M71-activating odorant propanol in lieu of extinction (train to aceto + exposure to propanol 6 wk, checked bar) had increased M71+ glomerular area compared with HC (black bar) [M71-LacZ, HC, n = 52; train to aceto 6 wk, n = 34; train to aceto + exposure to propanol, n = 34; ANOVA, P < 0.0001, F(2, 117) = 20.92; HC versus train to aceto 6 wk, P < 0.0001; HC versus train to aceto + exposure to propanol, P < 0.0001; train to aceto 6 wk versus train to aceto + exposure to propanol, P = n.s.]. Olfactory fear extinction immediately following acquisition supports learning-related reversal of M71-specific neuroanatomical representation. (C) Alternate experimental time line of fear conditioning, extinction, and olfactory fear testing 1 h before sacrifice. M71+ glomerular area in the OB is reversed with cue-specific olfactory extinction training that occurs immediately after conditioning. One group remained in the HC and received handling on all behavioral session days (“Homecage”), a second group received odor (acetophenone) + shock pairings (“Train to aceto”), a third group received acetophenone odor-alone presentations (“Aceto exposure*”), and finally a fourth group received odor (acetophenone) + shock pairings followed by extinction sessions (“Train to aceto + extinction”) (n = 5–7 per group). (D) M71 glomerular area [M71-LacZ, HC, n = 13; train to aceto, n = 12; aceto exposure, n = 12; train to aceto + extinction, n = 10; ANOVA, P < 0.0001, F(3, 43) = 13.42; HC versus train to aceto, P < 0.0001; HC versus exposure to aceto, P = n.s.; HC versus train to aceto + extinction, P = n.s.; exposure to aceto versus train to aceto, P < 0.01; train to aceto versus trained to aceto + Extinction, P < 0.0001; exposure to aceto versus train to aceto + extinction, P = n.s.].
The mechanisms underlying these striking and robust structural changes accompanying the acquisition and extinction of olfactory fear learning remain to be elucidated. Mechanisms related to altered cell turnover of OSNs may play an important role. Previous work has shown that associative olfactory learning modulates the survival of newborn neurons and increases the survival of adult-born neurons in the OB (29–32). Although these studies investigated the contribution of olfactory neurogenesis to learning at the level of the OB, learning-dependent regulation of epithelial olfactory neurogenesis, in which OSNs are in direct contact with environmental cues, may play a similarly and perhaps even greater role in shaping sensory epithelial responses to learning. The functional significance of adult neurogenesis in the MOE, which occurs persistently throughout life (33), remains to be determined within this training context. M71-expressing neurons may have a selectively longer survival time following olfactory fear acquisition, perhaps through the learning-dependent release of neurotrophins such as brain-derived neurotrophic factor (BDNF) from the OBs. BDNF belongs to the neurotrophin family and has been shown to play an important role in learning and memory (34, 35). BDNF signaling through its primary receptor TrkB has also been shown to play a significant role in downstream effects underlying learning (36–40). Previous data from our laboratory have shown increased BDNF transcription and translation in the OB following olfactory fear conditioning (41). Furthermore, OSNs within the MOE express the BDNF receptor TrkB (42). BDNF-TrkB signaling may thus be well-suited to mediate the learning-dependent increases in M71+ OSN numbers observed following olfactory fear conditioning. Conversely, cue-specific olfactory fear extinction may revert these neurotrophic effects, thus inducing the selective M71-specific cell death.

Epigenetic regulation of M71 receptor expression within the MOE may also play a role in the dynamic increase following training and subsequent reversal of M71-specific OSN number following extinction. Epigenetic processes such as histone acetylation and DNA methylation have been shown to play important roles in the mechanisms underlying fear acquisition and extinction across brain regions that include the hippocampus and the amygdala (43–46). Furthermore, epigenetic mechanisms are known to regulate the choice and maintenance underlying the (singularity of) OR expression (47, 48). Cellular mechanisms underlying odor preference also include dynamic regulation of cell death and apoptotic mechanisms, based on evidence that behavioral extinction paradigms can induce the death of newborn bulbary cells previously selected to survive (32). Processes regulating cell survival may modulate memory strength based on the biological relevance of particular odor associations in the environment. In fact, recent work has shown that a histone H2B variant, H2be, expressed exclusively by OSNs, displays activity-dependent expression, which contributes to the transcriptional activity and life span of the OSN (49). Furthermore, histone variant exchange has been recently shown to play an important role in fear conditioning in the hippocampus and the cortex (50).

Overall our results demonstrate that extinction training specific to a conditioned odor reverses the conditioning-associated increases in freezing behavior and structure (summary schematic in Fig. S5). Mice undergoing olfactory fear extinction 3 wk following acquisition exhibited decreased freezing to the odor CS across extinction sessions. The observed reduction in within-session freezing during extinction is maintained 3 wk following the extinction session, suggesting a long-term retention of extinction learning. Furthermore, the changes in freezing behavior accompanying the acquisition and extinction of olfactory fear conditioning are paralleled by neuroanatomical changes in the neuronal populations (M71+) responsive to the cue CS (acetophenone). Mice fear-conditioned 3 or 6 wk prior had a larger number of M71+ OSNs in the MOE compared with controls and mice that had undergone extinction, suggesting that cue-specific olfactory extinction reverses the primary olfactory sensory neuroanatomical changes that accompany the initial learning event. Our laboratory has previously found that the effects of olfactory fear acquisition (increased numbers of M71 receptor-expressing OSNs, increased M71-specific glomerular area, and enhanced behavioral sensitivity to acetophenone) are transmitted across generations (7). Although the present study investigates exclusively the effect of cue-specific olfactory extinction in an adult F0 generation, the possibility that the effects of extinction may be transmitted to subsequent generations is extremely intriguing and is an important direction for future investigations. Also of note, recent observations of plasticity within the human olfactory system suggest the intriguing possibility that the phenomena observed here may occur in humans as well (51–53). Overall the data described in the present study support the dynamic alterations of olfactory sensory representation with learning and shed light on how a sensory system responds to a therapeutic intervention such as extinction learning following fear conditioning.

Materials and Methods

Animals. Adult M71-RES-tauLaCZ transgenic mice (8) were maintained in a mixed 129/Sv × C57BL/6 background (Jackson Laboratories) and were used in all behavioral and neuroanatomical experiments. All mice were 2–3 mo old at the time of olfactory fear conditioning. For each training time course, behavioral groups were formed with mice from at least four litters, controlling for sex and age, such that each group was age-matched and had equivalent numbers of males and females. All mice were experiment- and odor-naive at the start of the experiment. Mice were housed in a temperature-controlled vivarium on a 12 h light/dark cycle in standard group cages (≤4 mice per cage) and were given ad libitum access to food and water. All experiments were performed during the light cycle and were approved by the Emory University Institutional Review Board following the National Institutes of Health Internal Animal Care and Use Committee standards.

Olfactory Fear Conditioning, Extinction, and Testing. Fear training, testing, and extinction were conducted using startle response systems (SR-LAB, San Diego Instruments) that had been modified to deliver discrete odor stimuli as previously described (5, 7, 17). Please refer to SI Materials and Methods for the specific conditioning and extinction protocols as well as the training time course details used throughout the paper.

Freezing Behavior Data Analysis. Within-session freezing during conditioning, extinction, and testing was determined as described in Jones et al. (17). Freezing behavior data acquisition and analysis are described briefly in SI Materials and Methods.

Beta-Galactosidase Staining of the MOE OSNs and OB Glomeruli. Following sacrifice, MOE and OBs of M71-LaCZ mice were processed for beta-galactosidase staining as previously described (5, 7). Please refer to SI Materials and Methods for a detailed description of beta-gal staining.

Quantification of M71-Positive OSNs in the MOE. Following staining, the lateral whole-mount MOE was imaged using a microscope-mounted digital camera, and beta-gal-stained blue OSNs were counted manually by experimenters blind to the experimental groups. Please refer to SI Materials and Methods for a detailed description of M71+ OSN quantitation in the MOE.

Measurement of Glomerular Area in the OB. Beta-gal–stained glomeruli were imaged using a microscope-mounted digital camera to capture high-resolution images of dorsal and medial glomeruli at 40× magnification. Please refer to SI Materials and Methods for a detailed description of glomerular area measurement in the OB.

Statistics. Freezing was analyzed by two-way ANOVA (Fig. 1 B and D) or one-way ANOVA (Fig. 1E). The glomerular area was analyzed by one-way ANOVA (Figs. 3C and 4 and Figs. S1, S2 C and D, and S3 C and D) or Student’s t test (Fig. 3D and Figs. S3B and S4). OSN number was analyzed by one-way ANOVA (Fig. 2F and Figs. S2 A and B and S3B). All OSN counts and glomerular area data were covaried by sex, with no effect of sex observed. Glomerular size to OSN number correlation was analyzed by linear regression. All ANOVA main effects or interactions were followed by Tukey post hoc tests, unless otherwise noted.
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