Optogenetic activation of presynaptic inputs in lateral amygdala forms associative fear memory

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In Pavlovian fear conditioning, the lateral amygdala (LA) has been highlighted as a key brain site for association between sensory cues and aversive stimuli. However, learning-related changes are also found in upstream sensory regions such as thalamus and cortex. To isolate the essential neural circuit components for fear memory association, we tested whether direct activation of presynaptic sensory inputs in LA, without the participation of upstream activity, is sufficient to form fear memory in mice. Photic stimulation of axonal projections from the two main auditory brain regions, the medial geniculate nucleus of the thalamus and the secondary auditory cortex, was paired with aversive footshock. Twenty-four hours later, the same photostimulation induced robust conditioned freezing and this fear memory formation was disrupted when glutamatergic synaptic transmission was locally blocked in the LA. Therefore, our results prove for the first time that synapses between sensory input areas and the LA, previously implicated as a crucial brain site for fear memory formation, actually are sufficient to serve as a conditioned stimulus. Our results strongly support the idea that the LA may be sufficient to encode and store associations between neutral cue and aversive stimuli during natural fear conditioning as a critical part of a broad fear memory engram.

[Supplemental material is available for this article.]
associative memory. However, until now, this idea has never been directly tested.

We have performed such a test by using optogenetic photostimulation to specifically activate auditory axons and their presynaptic terminals in the mouse LA in a temporally and spatially restricted manner. We found that such selective activation of auditory synaptic inputs to the LA could induce robust freezing after pairing with an aversive stimulus. Therefore, our results directly demonstrate that the activity of auditory synaptic inputs to the LA is sufficient to serve as a CS in induction of learning-related changes and to support associative memory. Our findings indicate that associative learning occurs, and the memory for association between the CS and the US is formed, at this specific brain site during natural fear conditioning, thereby identifying the circuit components for a specific memory trace.

Results

AAV-mediated ChR2-Venus expression in auditory projections in the LA

To selectively evoke synaptic transmission between presynaptic auditory inputs and postsynaptic target neurons in the LA, we used ChR2-based photostimulation (Cardin et al. 2010; Johansen et al. 2012). ChR2 fused to the Venus fluorescent protein was expressed by an AAV2/1-hSyn-Chr2-Venus viral construct (AAV-Chr2). An AAV-EGFP construct was used to control for possible nonspecific effects related to viral infection or light exposure (Fig. 1A). These viruses were injected into the two major auditory brain regions that send direct projections to the LA: the MGm and the ventral part of the secondary auditory cortex (AuV) (Fig. 1B). Four weeks after virus injection, brains were sectioned and ChR2-Venus expression was observed under a fluorescence microscope. We detected ChR2 fluorescence on the membranes of neurons only in the targeted brain regions, MGm and the adjacent posterior intralaminar nucleus (PIN), as well as the AuV (Fig. 1C). Importantly, strong ChR2-Venus expression was also detected in the amygdala, indicating that ChR2 was efficiently expressed in the auditory projections and presynaptic terminals innervating the LA through the external and internal capsule (Fig. 1D).

To analyze the distribution of auditory projections within the amygdala, we quantified ChR2-Venus fluorescence intensity in various subdivisions of the amygdala: dorsal LA (LAd) and ventral LA (LAv), basal amygdala (BA) and the central nucleus of the amygdala (CE). The LA displayed substantial fluorescence, particularly in its dorsal division. A few axonal projections were also observed in the BA, while we barely detected fluorescence in the CE. One-way ANOVA revealed significant effect among subdivisions ($F_{(4, 45)} = 20.2$, $P < 0.0001$), and Bonferroni post hoc test confirmed statistical significance in LAd and LAv ($P < 0.0001$ and $P = 0.02132$, respectively), but not in BA and CE, in comparison with the adjacent piriform cortex that showed no ChR2-expression and thus was used to define the background fluorescence level (Fig. 1E).

Photostimulation of presynaptic auditory inputs in the LA

To characterize the functionality of ChR2 expressed in AuV and MGm neurons, we performed whole-cell patch-clamp recordings in brain slices prepared from mice in which AAV-ChR2 had been injected into the AuV and MGm. Brief photostimuli could reliably induce action potentials in both AuV and MGm neurons (Fig. 2A, top and middle). These responses were not blocked by a broad-spectrum glutamate receptor antagonist, kynurenic acid (2 mM), indicating that they were a direct result of photostimulation of these neurons rather than being an indirect consequence of activating excitatory synaptic input. Thus, both AuV and MGm neurons expressed ChR2 and could be photostimulated.

We next asked whether photostimulation of ChR2-expressing auditory afferent axons and terminals within the LA could evoke synaptic transmission. For this purpose, we measured excitatory postsynaptic currents (EPSCs) in LA neurons. Local conjoint photostimulation of AuV and MGm fibers within the LA induced robust EPSCs in 84% of our recordings from LA pyramidal neurons ($N = 50$). In control experiments, where only AuV or MGm were injected with AAV-ChR2, we found that a similar percentage of LA pyramidal neurons responded with EPSCs.

Figure 1. ChR2-expression in the auditory pathways projecting from auditory thalamus and cortex to the LA. (A) AAV constructs expressing ChR2-Venus or control EGFP. (B) AAV viruses were injected into ipsilateral MGm and AuV, which directly project to the LA. (C) ChR2-Venus expression was observed in the auditory thalamus (right) and cortex (left) with reciprocal projections from each other. Single injections of AAV-EGFP into either AuV or MGm specifically yielded infected neurons without reciprocal axonal projection (top insets). ChR2-Venus expression was detected on the membranes of infected neurons (bottom insets). (D) Amygdala showed ChR2-Venus expression representing axonal projections and terminals from auditory afferents. (E) Fluorescence in amygdala subdivisions was analyzed in the dorsal and ventral LA, BA, and CE ($n = 10$). Data are expressed as mean ± SEM. (***$P < 0.001$, *$P < 0.05$). (Au1) Primary auditory cortex, (AuV) ventral part of secondary auditory cortex, (MGm/PIN) medial division of medial geniculate/posterior intralaminar nucleus, (LAd) dorsal part of lateral amygdala, (LAv) ventral part of lateral amygdala, (BA) basal amygdala, (CE) central amygdala, and Base is background fluorescence measured in piriform cortex.
auditory inputs to the LA with no relevant activation of upstream MGm or AuV. Such photostimuli were therefore used as a CS in the behavioral experiments described in the following section.

**Fear conditioning with the optogenetic CS**

We next asked whether selective photostimulation of auditory presynaptic inputs in the LA can serve as a CS to form fear memories. For this purpose, virus-injected mice with an implanted guide cannula were subjected to fear conditioning. These mice received five pairings of photostimuli applied to the LA (Fig. 3A); also see Materials and Methods for details) along with a footshock that served as the US. To determine whether this optical conditioning could form long-term fear memory, we examined retention of responses to the same photostimuli by putting the mice in a novel chamber 24 h after fear conditioning (Fig. 3A). Freezing behavior elicited by the photostimuli was then used as an index of fear memory. Mice injected with AAV-ChR2 exhibited robust freezing in response to photostimulation during such testing, indicating that activation of auditory presynaptic input in the LA could act as a CS for fear conditioning and could form long-term fear memories (Fig. 3B; see also Supplemental movie). There was significant interaction between the group and the CS presentation ($F_{(3,20)} = 6.51, P = 0.00167, \text{two-way repeated-measures ANOVA}$); only the group expressing ChR2 showed significant induction of freezing compared with pre-CS levels ($P < 0.0001$, Bonferroni post hoc test), whereas control mice injected with AAV-EGFP did not.

As another control, ChR2-expressing mice were given the CS alone during training, without the US. These mice did not show any noticeable behavioral responses to the light during either training or retention periods (Fig. 3B). This result is important because it demonstrates that simply activating auditory inputs to the LA is insufficient to elicit unconditioned freezing behavior. Moreover, ChR2-expressing mice trained with an unpaired protocol, in which photostimuli and electrical footshocks were explicitly unpaired, failed to show significant freezing in response to subsequent presentation of photostimuli (Fig. 3B). This suggests that information about the contingency of the CS and the US can be represented at LA synapses without requiring processes in any other brain areas.

Because pairing of the CS and the US did not show significant freezing compared with the baseline in a few ChR2-expressing mice, we further analyzed ChR2-Venus expression in these mice. For this analysis, mice were reclassified into two groups: those exhibiting >30% freezing (Success) and those with <30% freezing (Fail) (Fig. 3C). We found that in the Fail group all mice showed low ChR2-Venus fluorescence ($<7$ arbitrary units, a.u.), whereas in the Success group all showed higher fluorescence (10 a.u. or greater). There was no significant correlation between freezing percentage and fluorescence intensity within either the Success or Fail groups (Fig. 3D), suggesting that the amount of auditory inputs activated affected the threshold level for a CS to form fear memory rather than the strength of the memory.

**Optogenetic fear memory is mediated by glutamatergic synaptic transmission in LA**

To establish that fear conditioning was exclusively mediated by synaptic transmission between the presynaptic auditory inputs and postsynaptic LA neurons, we locally blocked glutamatergic synaptic transmission in the LA in anesthetized mice by injecting microinfusion of kynurenic acid (2 mM). Ten minutes after administration of kynurenic acid, the peak amplitude of EPSCs evoked in the LA by photostimulation of auditory inputs was decreased to 15% of their control values measured prior to drug administration.
This is consistent with our in vitro slice results (Fig. 2A) and confirms that EFPs evoked in the LA in response to photostimulation are mediated by glutamatergic excitatory synapses (Sah et al. 2003). When the same animal was retested 20 h later, the amplitudes of light-evoked EFP had recovered up to \( \approx 80\% \) of the values measured prior to drug treatment, indicating that the effect of kynurenic acid was reversible.

These results allowed us to next use kynurenic acid to determine the role of excitatory synaptic transmission in the behavioral response to photostimulation. For this purpose, either kynurenic acid or control vehicle solution was locally infused into the LA immediately prior to training and mice were then fear conditioned with the optogenetic CS as described earlier. When tested 24 h later (Fig. 4B, Retention 1), the mice injected with kynurenic acid showed significantly less freezing upon presentation of the CS alone compared with the control mice (Fig. 4C; \( P = 0.043 \), unpaired t-test). However, when the kynurenic acid group of mice was retested 2 d after infusion of vehicle (Fig. 4B, Retention 2), they showed normal learning in response to photostimulation and exhibited robust CS-induced freezing during the retention test (Fig. 4D; two-way repeated-measures ANOVA, Drug \( \times \) CS interaction: \( F_{(1,4)} = 18.82, P = 0.0123 \). Bonferroni post hoc test between pre-CS and CS: \( P = 0.104 \) for Retention 1, \( P = 0.002 \) for Retention 2). These results establish that fear conditioning was mediated by photostimulation of presynaptic glutamatergic auditory inputs to the LA. Taken together, our results revealed that activation of auditory projections to neurons can serve as a CS that, when paired with unconditioned footshock, can produce fear conditioning and form a long-term associative memory.

**Discussion**

A large amount of evidence supports the notion that the LA is a key site for encoding and storing the associative memory for Pavlovian fear conditioning. This hypothesis predicts that activating only the synaptic inputs that deliver CS information to LA neurons should be sufficient both to form fear memory and to
allow its subsequent retrieval. However, this prediction has never been tested directly. Here we have used selective photostimulation of auditory presynaptic inputs in the LA as a CS for fear conditioning, thereby presenting a CS directly to the LA rather than through upstream sensory brain regions such as the thalamus and the cortex. After learning, the same photostimulus alone could activate retrieval of the fear memory. Thus, upon photostimulation, mice exhibited strong freezing that corresponded to the normal fear response formed by natural auditory cues. This artificial fear memory trace was formed by the associative activation of auditory inputs and the US pathway; fear memory did not occur if the CS photostimulus was presented alone or was temporally uncoupled from the US.

The electrical stimulation methods that have been used in many previous studies to activate brain regions suffer from a lack of specificity. For example, although it has been reported that strong stimulation of the MgM/PIN causes an unconditioned freezing response (Cruikshank et al. 1992; Kwon and Choi 2009), it is possible that this response was caused by activation of axonal projections, including nociceptive inputs that pass through this region (Lanuza et al. 2004). In contrast, the ChR2-mediated photostimulation technique that we have used allowed us to activate auditory inputs in a highly specific manner. Indeed, we observed that photostimulation alone did not cause any freezing behavior, indicating that we were selectively activating the auditory CS inputs rather than mixed and undefined inputs from thalamic and cortical projections.

In our experiments, we simultaneously stimulated both thalamic and cortical auditory pathways. In the auditory fear conditioning circuit, auditory information reaches the LA through both of these pathways and both are thought to be important for encoding fear conditioning. It has been suggested that the thalamic pathway delivers a rapid but simple pattern of auditory information, whereas the cortical pathway encodes slow but detailed information (LeDoux 1996). In addition, some studies have suggested that the interaction between thalamic and cortical inputs may be advantageous for inducing plastic synaptic changes within the LA (Shin et al. 2006; Cho et al. 2012). Therefore, by simultaneously stimulating both thalamic and cortical projections, we attempted to closely mimic the processes that deliver natural sensory information. Nevertheless, it is possible that activating a single auditory pathway, either thalamic or cortical, could be sufficient to form and support long-term fear memory. Further experiments will be needed to test this possibility.

Although our optogenetic strategy specifically targeted auditory inputs projecting to the LA, it is possible that the fear memory trace is represented in regions in addition to LA. Specifically, other subdivisions of the amygdala, such as BA and CE, and still other brain regions downstream from LA, such as the periaqueductal gray matter, are involved in memory representation (Wilensky et al. 2006). For example, recent studies show that fear learning induces long-term synaptic potentiation in the CE (Ciocchi et al. 2010). It is very unlikely that direct activation of BA or CE through the optical stimulation of axons projecting to the BA or CE mediates fear memory formation in our experiments. Our analysis showed highly restricted expression of ChR2 in the LA, whereas the BA showed very weak, if any, ChR2-expressing auditory projections (see Fig. 1D,E). Moreover, although some anatomical studies have reported that CE also directly receives sensory projections (Turner and Herkenham 1991; McDonald 1998), these would not have been activated in our experiments (Fig. 1E). In addition, photostimulation produced no fear memory during local block of glutamatergic synaptic transmission in the LA. Thus, we conclude that a fear memory trace can be formed in the LA by the association of photostimulated LA-projecting auditory inputs along with the US. However, the LA serves as an interface for the amygdala, by receiving initial sensory inputs, and has strong interconnections to the downstream BA and CE (Sah et al. 2003; Ehrlich et al. 2009). It is, therefore, possible that there are learning-related changes occurring downstream from LA, but in our experiments these would secondarily result from activation of LA neurons. More generally, our findings do not mean that circuit components other than those we manipulated have no role for memory formation. Rather, broader neural circuits, encompassing sensory perception to behavioral expression, may be necessary to shape different aspects of the memory such as its strength, maintenance, specificity, and other features.

In conclusion, we demonstrated for the first time that the activity of synaptic inputs to the LA is sufficient to serve as a CS to produce long-term associative memory for fear conditioning. Our study strongly supports the idea that activation of sensory inputs to LA synapses may be a crucial and sufficient step to form the memory for association of the CS and the US during natural fear conditioning and that this specific brain site is the place where the memory is formed, providing important new insights into the role of specific circuit elements within the broader neural circuitry for associative memory formation.

Materials and Methods

Mice

129/C57Bl/6 hybrid background mice (2–3 mo old, 23–35 g) were group-housed on a 12-h light/dark cycle at a constant temperature of 22 ± 1°C with 40%–60% humidity. Food and water were available ad libitum throughout the experiment. All procedures were consistent with the animal ethics guidelines of the institutions where the experiments were performed (KAIST and KIST).

Virus production

For ChR2 gene delivery, we produced recombinant adeno-associated virus vectors. DNA plasmids coding AAV-hSyn-ChR2-Venus or AAV-hSyn-EGFP were amplified and purified using a Maxiprep kit (Qiagen). The purified plasmids were mixed into a CaCl2 solution with the DNA plasmid coding AAV 2/1 and pAAVE and cotransfected into HEK293T cell using calcium phosphate precipitation. Cells were harvested 72 h after transfection and the relevant virus was purified in an ultracentrifuge on an iodixanol gradient. The viral concentration was determined by quantitative PCR, and the final titer was 0.5–1 × 1012 vg/mL.

Brain surgery

Mice were anesthetized by intraperitoneal injection of pentobarbital (83 mg/kg) and placed in a stereotaxic frame. A glass micro-pipette containing an AAV virus solution was positioned on the targeted AuV and MgM brain region (AuV: AP = −2.7 mm, ML = +4.4 mm, DV = −3.2 mm, MgM: AP = −3.2 mm, ML = +1.9 mm, DV = −3.5 mm from bregma). AAV solutions were unilaterally injected at a rate of 0.1 μL/min for 8 min (total, 0.8 μL/region). Micropipettes were left in place for an additional 10 min to ensure diffusion. The pipette was removed, head skin was clipped, and mice were then allowed to recover on a heat pad and moved back to their home cage. Experiments were conducted at least 4 wk later, to allow sufficient time for expression of ChR2 in axons projecting to the LA. Three weeks after AAV injection, mice used for behavioral experiments were implanted with a guide cannula for optical fiber insertion. The guide cannula was positioned 1 mm above the LA (AP = −1.8 mm, ML = −3.4 mm, DV = −3.0 mm from bregma) and fixed with dental cement.

To quantify ChR2-Venus expression, mice were perfused and fixed with 4% PFA, and the brain was then sliced into 40-μm thick sections. Brain sections corresponding to −1.7, −1.9, and −2.1...
mm posterior from bregma were then selected for analysis. The amygdala was subdivided into dorsal/ventral LA, BA, and CE (Fig. 1D; Franklin and Paxinos 2008). Under a fluorescence microscope, fluorescence intensity values were measured in the four subdivisions using NIS imaging software (Nikon). The adjacent pinfrom cortex of the same sections, which did not express ChR2-venus, was selected for measurement of background fluorescence. Image acquisition conditions were adjusted to keep the intensity value of the background fluorescence constant across all slices. The mean intensity value for each region was calculated by averaging the three sections from a single mouse, and mean fluorescence intensity values were averaged for each region to determine whether ChR2-Venus expression was successful or was a failure for each mouse.

**Acute brain slice recordings**

Coronal brain slices (1.6 mm posterior from bregma) were obtained from mice 1 mo after AAV injection into Mgm and AuV and were prepared using conventional methods (Petit and Augustine 2000; Nakajima et al. 2004; Wang et al. 2007). In brief, a Vibratome (VT-1200, Leica) was used to slice brains into 350-μm-thick coronal sections in a solution containing high sucrose. This solution was composed of (in mM): 87 sodium chloride, 75 sucrose, 25 NaHCO3, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 1.25 Na2HPO4, and 25 d1+1-glucose. pH was maintained at 7.4 by gassing the solution with 95% O2 and 5% CO2. Slices were transferred to an incubation chamber filled with oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 25 d1+1-glucose, 2.5 CaCl2, 1 Mgl2, 3 sodium pyruvate, and 1 ascorbic acid. Slices were incubated at 36°C for 30 min and at least 30 min at room temperature prior to use.

Whole-cell patch-clamp recordings were performed at 32°C in an upright microscope (FV1000MP, Olympus) in a recording chamber perfused with 95% O2/5% CO2 aerated ACSF. To characterize photostimulation of AuV or Mgm neurons (Fig. 2A, top), coronal sections 3.0 mm posterior from the bregma were used and recordings were made from somata of neurons within these regions. For analysis of LA responses to synaptic photostimulation (Fig. 2A, bottom), coronal sections from bregma ~1.6 mm were used; these do not contain somata of AuV and Mgm but do contain ChR2-expressing axonal fibers coming from the injected site and projecting to LA excitatory neurons (Fig. 1D). Occasionally, kynurenic acid (2 mM, Sigma) was added to the ACSF to block excitatory synaptic transmission. Pyramidal neurons in the LA or AuV, or LA were identified with intracellular differential interference contrast (IR-DIC) optics in combination with a digital video camera (MCE-B013-U, Microtech). Whole-cell patch-clamp recordings were made from these neurons using glass pipettes (5–12 MΩ) filled with internal solution containing (in mM): 130 K-gluconate, 2 NaCl, 4 MgCl2, 20 HEPES, 4 Na2ATP, 0.4 Na3GTP, 0.5 EGTA, and 10 Na2phosphocreatine, pH adjusted to 7.25 with 1 M KOH. Osmolality of this solution was 290–295 mosm. Current measurements were made under voltage clamp, at a holding potential of ~60 mV. Electrical responses were detected with an amplifier (Multiclamp 700B, Molecular Devices), digitized 20 kHz via an A–D converter (Digitdata 1440A, Molecular Devices), acquired using pClamp software (Molecular Devices), and analyzed using Clampfit software (Molecular Devices). A mercury arc lamp (USH-1030L, Olympus) filtered by a band-pass filter (470–495 nm) was used to activate ChR2. Photostimuli were applied through a 25× (1.05 NA) water-immersion objective lens and the entire width of the microscope field (~500 μm diameter) was illuminated. During synaptic photostimulation, this light spot surrounded an LA neuron and was used to photostimulate ChR2-expressing axonal fibers projecting from AuV and Mgm. Pulse duration was controlled by an electronic shutter (Uniblitz VS25, Vincent). For direct photostimulation of AC and MG neurons, light pulses of 0.7-mW/mm² luminance and 10-msec duration were applied at 5 Hz for 2 sec. For evoking synaptic responses in the LA, slightly brighter single light pulses (1.2-mW/mm², 10-msec duration) were delivered every 30 sec.

**In vivo recording**

Photostimulation of neurons in ChR2-expressing mice was tested by recording local field potentials. Mice were unilaterally injected with AAV-ChR2 or AAV-GFP into Mgm and AuV. Four weeks after virus injection, mice were anesthetized with pentobarbital (83 mg/kg, i.p.) and placed in a stereotaxic frame. An optrode, which consisted of a tungsten electrode (5 MΩ) and an optical fiber, was positioned at the point where the LA receives axonal projections from the virus-injected sites. Light was confirmed via postmortem histology. Recordings were band-pass filtered between 10 and 300 Hz using a DAM80 differential amplifier (World Precision Instruments), and digitized at 10 kHz. Signals were processed via custom-made programs written in Labview (National Instruments). Photostimuli were delivered through an optical fiber connected to a 473-nm diode laser (Crystalaser). Light-evoked local field potentials were acquired and analyzed off-line. Five waveforms induced by photostimuli delivered at 1 min intervals were averaged and the baseline-to-negative peak amplitude was calculated from these average signals. For tests of axonal back-propagation, an additional optrode was placed into the Mgm or AuV and EPSPs were measured in Mgm, AuV, and/or LA.

**Fear conditioning with the optogenetic CS**

After 7 d recovery from cannula implantation, mice were handled and an optical fiber insertion site was marked. The fiber was then returned to the target LA through the guide cannula and fixed with cyanoacrylate. Photostimulation of neurons in ChR2-expressing mice was tested with the optogenetic CS and the next day they were subjected to fear conditioning. We minimized light power and the fiber was double coated with a black jacket and with white paint.

During training, the optical fiber was positioned in the target LA through a guide cannula, and placed in a training chamber with video camera monitoring. Two minutes later, mice received six pairings of the optogenetic CS (20 sec duration) followed by a footshock that served as a US (0.4-mA shock for 2 sec). The optogenetic CS consisted of 10-msec light pulses that were repeated at 10 Hz, with 2 sec duration stimulus trains separated by 1 sec interval. Intertrials were randomly given at an average interval of 2 min (Fig. 3A). Mice were kept in the chamber for an additional 30 sec after delivery of the last shock to monitor post-shock behavior and then removed from the chamber. In control experiments, the group receiving the CS alone received the same behavioral training procedure described above for the ChR2-paired group, except for elimination of the footshock during training. Mice in the ChR2-unpaired group were given explicitly unpaired presentations of the CS and the US, with an interstimulus interval longer than 1 min. Retention tests were conducted 24 h after training. For this purpose, mice were placed in a context-shifted test chamber. To clearly distinguish the CS-induced fear responses from general fear responses, the baseline freezing level was recorded after a mouse initiated movement with its hind paw; this was done because mice normally show continuous movement after initiating such a motion. After the 2-min baseline recording, 20 sec of the optical CS was presented three times, with a 10-sec interval, as used for fear conditioning (Fig. 3A). Mice were removed from the test chamber after additional 60 sec following termination of photostimulation. The freezing behavior measured during the 2 min from onset of photostimulation was used as an index of conditioned fear memory for data analysis.

In some experiments, mice were unilaterally infused with 1 μL of kynurenic acid (2 mM dissolved in ACSF) into the LA at a rate of 0.2 μL/min prior to starting training. After allowing 2 min for drug diffusion, these mice were then fear conditioned with the optogenetic CS and the next day they were subjected to retention tests as described earlier. Mice that received the first retention test, mice received the same treatment except for infusion of ACSF instead of kynurenic acid.
Statistical analysis

Comparison of ChR2-Venus expression was performed by one-way ANOVA. Freezing behaviors were analyzed by Student’s unpaired t-test or two-way repeated-measures ANOVA followed by Bonferroni post hoc tests for multiple comparisons. Prism (GraphPad software) was used for all statistical analysis in this study.

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