Optical activation of lateral amygdala pyramidal cells instructs associative fear learning
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Humans and animals can learn that specific sensory cues in the environment predict aversive events through a form of associative learning termed fear conditioning. This learning occurs when the sensory cues are paired with an aversive event occurring in close temporal proximity. Activation of lateral amygdala (LA) pyramidal neurons by aversive stimuli is thought to drive the formation of these associative fear memories; yet, there have been no direct tests of this hypothesis. Here we demonstrate that viral-targeted, tissue-specific expression of the light-activated channelrhodopsin (ChR2) in LA pyramidal cells permitted optical control of LA neuronal activity. Using this approach we then paired an auditory sensory cue with optical stimulation of LA pyramidal neurons instead of an aversive stimulus. Subsequently presentation of the tone alone produced behavioral fear responses. These results demonstrate in vivo optogenetic control of LA neurons and provide compelling support for the idea that fear learning is instructed by aversive stimulus-induced activation of LA pyramidal cells.

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cells/section, n = 3 animals) whereas there was minimal CFOS activation in the nonstimulated side (average of 29 ± 4 cells/section, n = 3 animals) (Fig. 2 C and D). Proportionally, this was a substantial number of activated neurons as the total number of CaMKII+ cells per LA section was 402 ± 37. Thus in vivo, 20-Hz laser stimulation resulted in activation of large numbers of LA cells, further validating the use of the 20-Hz stimulation protocol.

Behavioral conditioning experiments were then used to determine whether direct activation of LA pyramidal neurons as an US, when paired with an auditory CS, produces fear learning. For these experiments a chronic guide cannula was targeted just dorsal to 10 d after surgery, a term fear memories in ChR2/paired animals, freezing was assessed in a separate context 24 h after training (Fig. 3B). A one-way ANOVA comparing CS-evoked freezing by group (ChR2/paired vs. GFP/paired vs. ChR2/unpaired) found a significant main effect (F_{2.21} = 5.41, P = 0.013) and post hoc analyses revealed that the ChR2/paired group froze significantly more than both the GFP/paired (P = 0.017) and the ChR2/unpaired (P = 0.017) groups. Because using constant illumination to stimulate LA neurons produced similar levels of action potential firing as that evoked by eyelid shocks (Fig. S3), we also used 2-s constant laser illumination as an US (instead of 10-ms laser pulses at 20 Hz described above) and found that this stimulation protocol produced similar levels of fear conditioning (Fig. S5) to that evoked by the 20-Hz laser US. Thus light activation of LA pyramidal cells as an US produced long-term fear memories.

Discussion
Our results show that specific activation of LA pyramidal cells as an US, in the absence of a peripheral shock US, produced fear conditioning. ChR2 was expressed preferentially in LA pyramidal cells as an US and this produced fear learning and memory formation. Finally, this process is associative (i.e., requires the close temporal contiguity of the CS and laser US and that the laser US occurrence is contingent upon the CS preceding it, ref. 26) as it

![Image of LA pyramidal cells with ChR2/YFP expression](https://www.pnas.org/cgi/doi/10.1073/pnas.1002418107)

**Fig. 1.** Tissue-specific expression of ChR2 in LA pyramidal neurons. (A) Example of ChR2/YFP expression in the LA. LA boundaries are outlined in yellow. (B) Percentage (y-axis) of ChR2+ cells that were also CaMKII+ (black bar, n = 3) and percentage of ChR2+ cells that were also GABA+ (white bar, n = 2). (C) Immunolabeled ChR2+ cells and neuropil (green, Left), CaMKII+ cells (red, Right), and an overlay of the two (Bottom). Individual cell examples are indicated by yellow (ChR2/YFP+) and white (CaMKII+ cells) arrows.

![Image of laser stimulation and freezing responses](https://www.pnas.org/cgi/doi/10.1073/pnas.1002418107)

**Fig. 2.** Laser stimulation produces robust activation of LA neurons. (A) Example peristimulus time histogram (PSTH) plot (bin size = 50 ms) and spike rasters from a single LA neuron shows average firing rate (y axis, in Hz) in response to laser stimulation at 20-Hz stimulation frequency (10-ms laser pulse duration) for 1 s (thick blue lines denote each laser pulse during the 1-s stimulation period). (B) Population averaged firing rate in response to 20-Hz (Upper) and 50-Hz (Lower) laser stimulation at 10-ms (black lines), 5-ms (broken lines), and 2-ms (gray lines) laser pulse durations. Total n = 15 cells (from four animals) for 20-Hz stimulation, nine of which also received 50-Hz stimulation. Blue bar beneath graphs denotes the duration of the 1-s train at the different frequencies. (C and D) Examples of CFOS-immunolabeled cells (brown dot labeling) in the LA from the laser-stimulated (D) and nonstimulated (C) hemispheres.
Fig. 3. Laser stimulation in the LA produces fear conditioning. (A) Percent freezing (of the total 18 s CS, y axis) across the four conditioning trial blocks (four trials/block, 16 total CS–US pairings or 16 CS presentations for the unpaired group) in the ChR2/paired (squares, n = 8), GFP/paired (triangles, n = 8), and ChR2/unpaired (open circles, n = 8) groups. (B) Percent freezing (y axis) during the LTM test in the different groups (x axis). For A and B, * and # indicate significant differences between the ChR2/paired group and the GFP/paired and ChR2/unpaired groups.

A large number of studies have provided convincing evidence that associative plasticity in the LA contributes to fear memory formation (4, 6, 7). It is widely believed that LA plasticity underly fear learning occurs as a result of a Hebbian mechanism whereby the shock US directly depolarizes LA pyramidal cells that are concurrently activated by weaker CS inputs, resulting in potentiation of the CS input synapses (5, 6, 9, 31). This model suggests that depolarization of LA pyramidal cells by the US provides an instructive signal for LA plasticity and fear learning. However, it is possible that LA plasticity underlying fear learning occurs through a non-Hebbian process, which is independent of US-evoked postsynaptic depolarization of LA neurons. For example, US-induced activation of neuromodulatory systems and subsequent intracellular signaling cascades could by itself serve to potentiate coactivated CS input synapses (a possibility, which is supported by some experimental evidence, refs 32, 33). Although various studies provide indirect evidence that fear learning occurs through a Hebbian mechanism (8, 9, 13–15, 34–36), other work (37) and interpretational issues make it difficult to determine whether this is in fact true. To adequately test whether depolarization of LA pyramidal neurons by the US instructs fear conditioning, it is necessary to manipulate neural activity (either increase or decrease depolarization) directly in this cell population specifically during the US period and examine the effects of these manipulations on fear memory formation. In the present study we produced strong depolarization and action potential firing in LA pyramidal neurons and showed that this stimulation, when used as an US, reinforces fear learning, offering compelling support for the idea that Hebbian mechanisms are involved in fear memory formation.

Although stimulation of LA neurons supported fear learning, freezing scores during the long-term memory (LTM) test were relatively low compared with results typically obtained with an electric shock US. One reason for this may have been that either a small number of neurons were activated by laser stimulation or because laser stimulation produced too little (or too much) depolarization of LA neurons compared with a shock US. However, the CFOS experiments reported here demonstrate that large numbers of LA neurons were activated by laser stimulation and the electrophysiology experiments provide evidence that 20-Hz laser stimulation caused more action potential firing in LA cells than a shock US, suggesting that these were not the reasons for lower levels of fear learning. Furthermore, the low levels of freezing were not the result of too much action potential firing (compared with a shock US) because using a constant illumination US, which produced similar amounts of action potential firing as a shock US (Fig. S3), resulted in comparable levels of fear learning (Fig. S5). Relatedly, the unilateral nature of the laser stimulation may have resulted in low fear learning. This is unlikely, however, as other studies have shown that robust fear memories are formed when LA plasticity is targeted to only one LA using unilateral eyelid shock (24, 25). In fact, using the same fear conditioning protocol, we also found substantial levels of fear conditioning induced by a unilateral eyelid shock US (see Fig. S6 for comparison of shock vs. laser stimulation US-induced learning). This occurred despite the fact that the eyelid shock US used in this conditioning protocol produced weaker or similar levels of action potential firing in LA neurons compared with the two laser stimulation US used in the current study. Together, the previous results and the present data suggest that the low freezing levels obtained using the laser-stimulation US were not the result of unilateral stimulation.

It is more likely that fear learning was limited because additional mechanisms (in addition to depolarization of LA pyramidal neurons) are required to modulate the strength of fear memory formation. One possibility is that plasticity in other parts of the fear circuit is required during fear conditioning (38–42). Although stimulation of LA pyramidal neurons may be sufficient to produce some fear learning by activating local LA plasticity mechanisms, plasticity in other parts of the circuit may be required as well for the full expression of fear conditioning. It is also possible that more specific cell targeting is necessary to direct plasticity to cells involved in generating the fear response (as opposed to other potentially competing responses). In addition to being essential for averantly motivated learning, plasticity in the LA is also required for several forms of apperently motivated learning (43–45) and a number of studies have found that separate populations of amygdala neurons mediate appetive and apperitive processing (46–48). Thus separate, intermixed populations of amygdala neurons are likely to participate in producing different behavioral responses (i.e., averantly vs. apperently motivated). The laser conditioning could have induced plasticity indiscriminately in both cell populations and this may have interfered, at least partially, with the expression of the conditional freezing response. A final possibility is that coactivation of neuromodulatory systems, in addition to depolarization of LA pyramidal neurons, may be required for maximal long-term memory formation (9, 49–51). In fact it has been suggested that coactivation of noradrenergic β receptors and subsequent G protein-coupled signaling pathways in conjunction with depolarization of LA neurons cooperatively regulates LA plasticity and fear conditioning (50). This multiprocess model may be required for maximal fear learning to occur. Although it is clear from the present results that activation of LA neurons can produce fear memories, it will be important in future
studies to determine the additional factors that contribute to robust fear memory formation.

**Materials and Methods**

**Subjects.** Male Sprague-Dawley rats (Hilltop) weighing 275–300 g on arrival were individually housed on a 12-h light/dark cycle and given food and water ad libitum. All procedures were approved by the New York University Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals.

**Vector Construction.** The AAV construct carrying EGFP under the control of the CaMKII promoter (termed pAAV-CaMKII-GFP) was kindly provided by P. Osten (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The backbone vector, pAAV-MCS (Stratagene) has been modified to contain the 1.3-kb mouse α-CaMKII promoter, a cDNA for EGFP, and woodchuck hepatitis posttranscriptional regulatory element (WPRE) from FCK(1.3G) (SW). pAAV-CaMKII-ChR2/EYFP construct was made by replacing EGFP in pAAV-CaMKII-GFP with a cDNA for ChR2/EYFP from pCDNA3.1/hChr2(1H34R)-EYFP.

**Virus Production and Purification.** To combine the advantages of AAV1 and AAV2 in relation to tissue tropism and ease of virus purification, we made the chimeric virus, which contains both AAV1 and AAV2 capsid proteins (53). Viruses were made by standard method of CaPO4 triple transfection of 293T cells with an expression vector, either of pAAV-CaMKII-ChR2/EYFP or pAAV-CaMKII-GFP and helper plasmids, pDP1 and pDP2 (both provided by J. Kleinschmidt, German Cancer Research Centre, Heidelberg, Germany; ref. 54). Cells were harvested at 48–72 h after transfection, resuspended in 150 mM NaCl buffer containing 50 mM Tris, pH 8.4, subjected to three cycles of freeze/thaw, and treated with 50 U/mL of benzonase (Sigma) for 30 min at 37 °C. After brief centrifugation and filtration, clarified supernatants containing virus were subjected to HiTrap heparin column chromatography (GE Healthcare). Peak virus fractions were collected, concentrated by Amicon Ultra-15 (Millipore), and washed with PBS containing 1 mM MgCl2 and 2.5 mM KC1 in the same filtration unit. The virus genomic titer was quantified by real-time PCR using Icyycler and IQ SYBR reagent (Bio-Rad Laboratories). Before real-time PCR, virus samples were pretreated with DNase I (Roche). The DNase I was then heat inactivated for 10 min at 70 °C. After the heat inactivation, the samples were treated with proteinase K (Invitrogen) at 50 °C for 60 min, and the proteinase K was subsequently heat inactivated at 95 °C for 20 min at 95 °C. A plasmid DNA standard curve was set up using 0.0001–100 ng of DNA. Primer sequences are EGFP-F: 5′-GGAGGCACG-GATCTTCTTCA-3′ and EGFP-R: 5′-AGGGTGTCGCCCTCGAA-3′. Final virus titers were 1.8 × 1012 genomic copies (GC/mL) for AAV-CaMKII-ChR2/EYFP and 5.2 × 1012 GC/mL for AAV-CaMKII-GFP.

**Stereotaxic Cannula Implantation and Virus Injection.** For electrophysiological and double-labeling immunocytochemistry experiments, animals were injected with a mixture of ketamine (100 mg/kg) and xylazine (6 mg/kg) intraperitoneally and placed in a stereotaxic apparatus (David Kopf Instruments). Supplemental doses were given to maintain the anesthesia. The tip of a micropipette (tip diameter: ∼12 μm) was targeted to the LA (stereotaxic coordinates from Bregma, anterior–posterior: −3.0 mm, dorsal–ventral: −8.0 mm, medial–lateral: 5.4 mm). Injections were made through the glass micropipette, which was attached to a 30-mL syringe by polyethylene tubing at a rate of 0.5–40 mW laser power. The electrodeable apparatus was targeted to the amygdala and just dorsal to the LA, recordings began and periodic laser stimulation was given as the electrode was advanced in 1-μm steps until single laser responsive cells were isolated. Electrophysiological signals were amplified (×1,000) and filtered (300 Hz low/20 kHz high) using an AM Systems amplifier (model 1800) and then monitored and stored online using the Spike 2 acquisition system. Single neuron waveforms were then isolated offline and had to exhibit a refractory period of at least 1 ms and mean spike amplitude of at least 80 μV to be included in the study.

**In Vivo Electrophysiological Recording and Laser Stimulation.** Animals were anesthetized as described in Stereotaxic Cannula Implantation and Virus Injection and placed in a stereotaxic apparatus. A glass microelectrode (1 M Ω impedance filled with NaCl) was mounted alongside a fiber optic cable (200 μm core diameter, 0.37 numerical aperture), which extended slightly beyond the tip of the electrode (−0.3 mm). The fiber optic cable was attached to a 473-diode pumped solid state laser (Laserlight), which output 30–40 mW of the fiber output. The electrodeable apparatus was targeted to the amygdala and just dorsal to the LA, recordings began and periodic laser stimulation was given as the electrode was advanced in 1-μm steps until single laser responsive cells were isolated. Electrophysiological signals were amplified (×1,000) and filtered (300 Hz low/20 kHz high) using an AM Systems amplifier (model 1800) and then monitored and stored online using the Spike 2 acquisition system. Single neuron waveforms were then isolated offline and had to exhibit a refractory period of at least 1 ms and mean spike amplitude of at least 80 μV to be included in the study.

**Stimulus-evoked responses were analyzed by plotting peristimulus time histograms (PSTHs) triggered by the stimulus onset using Neurolynx data analysis software. For each cell, raw spike counts in each bin of the PSTH were converted to firing rates using the equation \( R_i = S_i/N_i \), where \( R_i \) is the firing rate for the \( i \)-th bin of the PSTH (in Hz), \( S_i \) is the raw spike count in the bin, \( N_i \) is the number of trigger events for the PSTH, and \( \Delta t_i \) is the PSTH bin size in seconds. For population averaging of neural responses, each cell’s PSTH was converted to a normalized scale. The response in the \( i \)-th bin of the normalized PSTH was given by \( Y_i = (S_i - \mu)/\sigma \), where \( S_i \) is the raw spike count in the \( i \)-th bin, \( \mu \) is the expected spike count in each bin at baseline, and \( \sigma \) is the spike count SD at baseline. The expected spike count was for all analyses presented here, \( \mu \) was 50 ms bins and the expected spike count at baseline (\( \mu \)) was obtained by concatenating all of the 50-ms bins within the 1.2-s prestimulus period from every trial of the session.

The number of spikes elicited per laser pulse (with a pulse defined as each single laser stimulation during a 1-s stimulation trial) was determined by perfused transcardially with PBS/heparin (40 U/mL) followed by 4% PFA (0.1% glutaraldehyde 7–10 d after virus injection. The brains were then removed and blocks containing the amygdala (2 mm thick) and postfixed in 4% PFA. Brains were then cut into 40-μm-thick sections on a vibratome and immersed in 1% sodium borohydride and then 1% hydrogen peroxide. For double labeling of ChR2 and CaMKII, brain sections (every fourth section) were labeled with mouse monoclonal anti-CaMKII (1:200; Millipore/Upstate) followed by the fluorescent secondary Alexa 555 goat anti-mouse (1:200; Invitrogen) and then reacted with rabbit polyclonal anti-GFP primary antibody (1:500 with 0.2% Triton X; Invitrogen) followed by Alexa 488 goat anti-rabbit (1:200; Invitrogen). All blocking steps were done in 1% BSA. Sections were then mounted and coverslipped with antifade (Invitrogen). Infection efficacy was quantified using a confocal microscope and counting the number of ChR2+ (EYFP) cells that were also CaMKII+ or GABA* (i.e., number double labeled) as well as the total number of ChR2+ positive cells within a counting frame (510 μm x 507 μm). This analysis was applied to each amygdala section in which ChR2 label was present. The proportion of double-labeled cells was calculated as the number of ChR2+/GABA+ cells divided by the total number of ChR2+ cells. For behavioral experiments, sections were processed as described above and an experimenter blind as to animal and treatment group assessed whether ChR2 was expressed in the LA neurons and whether the tip of the guide cannula was dorsal and proximal to the LA. Three ChR2/paired and 2 ChR2/ unpaired were not included in the final analysis as these criteria were not met.

For COFS immunocytochemistry experiments, perfusion, postfixation brain slicing were identical to above. Brain sections (every fourth section) were then reacted with rabbit polyclonal anti COFS primary antibody (1:20,000; Calbiochem) followed by goat anti-rabbit biotynilated secondary (1:200; Vector Labs) and then incubated in avidin–biotin complex (Vector Labs). This was followed by reaction with diaminobenzidine nickel reaction to reveal immunoreactivity. For double-staining COFS–labeled sections were counterstained with DAPI, and double-labeled sections were in sections throughout the amygdala. For total CaMKII+ cell counts, fluorescent immunocytochemistry was as described above (but for CaMKII alone) and the CaMKII+ cell counting procedure was identical to that used for COFS counting. Cell counts were then expressed as counts/section by dividing the total number of cells counted by the number of sections analyzed.
The Amygdala

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References

Fig. S1. ChR2 is expressed at low levels in LA GABAergic neurons. Immunolabeled ChR2\(^+\) cells (green, Left), GABA\(^+\) cells (red, Middle), and an overlay of the two (Right), with examples of single-labeled ChR2\(^+\) (blue) and GABA\(^+\) (white) cells. Individual cell examples are indicated by yellow (ChR2/YFP\(^+\) cells) and blue (GABA\(^+\) cells) arrows.

Fig. S2. Parametric analysis of laser stimulation of LA neurons with different stimulation frequencies (20 Hz and 50 Hz) and laser pulse durations (10, 5, and 2 ms). The spike probability (y axis) of each single laser pulse (at different pulse durations, x axis) within the 1-s stimulation period of 20 Hz (open triangles) or 50 Hz (circles) or to a single laser pulse (squares) to produce an action potential in the 9 cells that received all stimulation parameters (only 9 of the 15 total cells received all types of stimulation). * indicates significant differences between 50-Hz stimulation and both 20-Hz and single-pulse stimulation.

Fig. S3. Comparison of neural responses evoked by 20-Hz laser stimulation (20 Hz; \(n = 15\) cells), constant laser illumination (constant Illumin; \(n = 9\) cells), and eyelid shock (Shock; 2 ms, 2.5-mA shocks at 6.66 Hz; \(n = 25\) cells) during a 1-s stimulation period. Firing rate (frequency, y axis) during a 1-s baseline period before stimulus onset (white columns) and during the 1 s in which the stimulus was on (black columns). A repeated-measures ANOVA revealed a significant interaction (\(F_{2,46} = 5.83, P = 0.006\)) between stimulus (baseline and stimulus on) and group (20 Hz, constant illumination and eyelid shock), and post hoc comparisons demonstrated that evoked firing rate during the 20-Hz laser stimulation on period was significantly higher than firing rate during both the constant laser stimulation (*\(P = 0.005\)) and the eyelid shock (**\(P = 0.008\)). The eyelid shock dataset was gathered using tetrode recordings in awake, behaving rats that had previously undergone conditioning and were receiving random, unpredicted shocks interspersed with paired shocks. The data presented here is the response to the random, unpredicted shocks, exclusively. Single-unit recordings were obtained using a 32-channel data acquisition system (Neuralynx). Offline cluster cutting was performed manually using Neuralynx SpikeSort 3D software. To be included in the study, spike trains had to exhibit a refractory period of at least 1 ms and a mean spike amplitude of at least 80 \(\mu\)V over background noise of \(\pm 20\ \mu\)V. The experimenter visually inspected spike waveforms and cluster boundaries to make sure that they remained stable throughout the recording session for cells to be included in the data analysis. For data analysis, peristimulus time histograms (PSTH) were constructed as described in Materials and Methods, except that spike counts during a 1-s bin before stimulus onset and a corresponding 1-s bin during the stimulus period for each trial was calculated. Baseline firing rate and stimulus-evoked firing rate for each cell was then determined by averaging across all baseline and stimulus on trials to give frequency during the two conditions for individual cells. Baseline and stimulus-evoked firing rates for the population were then calculated by averaging across all cells.
Fig. S4. Laser stimulation produced a freezing unconditioned response. To avoid any freezing elicited by the CS, freezing (y axis) was measured in the ChR2/unpaired group (i.e., they received laser stimulations uncoupled from the auditory CS, n = 6) during the 2-s “laser on” period and during a corresponding baseline period before laser onset (“laser off”). *Significant difference between stimulus on and off conditions. Behavioral ratings were collected as described in Materials and Methods during the 2-s laser stimulation period and the 2-s period preceding laser onset.

Fig. S5. Optical stimulation as an US (using a 2-s constant illumination US) produced behavioral fear conditioning, percent freezing (y axis) during the LTM test in the ChR2/paired (black column, n = 8), GFP/paired (gray column, n = 8), and ChR2/unpaired (white column, n = 8) for the different groups (x axis). A one-way ANOVA comparing CS-evoked freezing by group (ChR2/paired vs. GFP/paired vs. ChR2/unpaired) found a significant main effect ($F_{2,21} = 9.81$, $P = 0.001$) and post hoc analyses revealed that the ChR2/paired group froze significantly more than both the GFP/paired ($P = 0.002$) and the ChR2/unpaired ($P = 0.001$) groups. * and ** indicate significant differences between the ChR2/paired group and the GFP/paired and ChR2/unpaired groups.

Fig. S6. Comparison of shock vs. laser US-induced fear conditioning. Percent freezing (y axis) during the LTM test in the eyelid shock US conditioned group (shock, black column, n = 6), 20 Hz laser stimulation US conditioned group (20 Hz, gray column, same as ChR2/paired in Fig. 3B) and 2-s laser stimulation US conditioned group (2 s, white column, same as ChR2/paired in Fig. S5).