Convergent coding of recent and remote fear memory in the basolateral amygdala

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Abstract

Animals must learn to anticipate recently encountered threats as well as dangers experienced long ago. In both rodents and humans, the basolateral amygdala (BLA) is essential for the encoding and retrieval conditioned fear memories. Although the BLA is a putative storage site for aversive memory, recent evidence suggests that these memories undergo time-dependent reorganization and no longer require the BLA after the passage of time. To explore this question, we systematically examined the role for the BLA in recent and remote fear memory using optogenetic, electrophysiological, and calcium imaging methods in male and female Long-Evans rats. Critically, we used a behavioral design that permits within-subjects comparison of recent and remote memory at the same time point. We found that BLA c-Fos expression was similar after the retrieval of recent (1 day) or remote (2 weeks) fear memories. Extracellular recordings in awake, behaving animals revealed that the majority of BLA neurons encoded both recent and remote memories, suggesting substantial overlap in the allocation of temporally distinct events. Fiber photometric recordings of BLA principal neurons also revealed similar patterns of CS-evoked activity to recent and remote CSs. Consistent with these results, continuous or CS-specific optogenetic inhibition of BLA principal neurons impaired conditioned freezing to both recent and remote CSs. Collectively, these data reveal that single BLA neurons encode both recent and remote fear memories. This may underlie the broad generalization of fear memories across both space and time. Ultimately, these results provide robust evidence that the BLA is a long-term storage site for emotional memories.

Introduction

Post-traumatic stress disorder (PTSD) is characterized by the intrusive re-experiencing of pathological memories that can persist for years after traumatic events (1, 2). In the laboratory setting, traumatic memories are studied using aversive learning and memory procedures such as Pavlovian fear conditioning in rats (3-6). Decades of work has led to the discovery that fear memories are initially encoded by a network of key brain regions, such as the prefrontal cortex, hippocampus and amygdala, but are later distributed to other sites for long-term storage, such as the neocortex (7-11). This time-dependent reorganization of memory storage is known as systems consolidation (12-14). A complete understanding of which brain regions mediate long-term memory storage is essential to therapeutically targeting pathological memories.

The amygdala is a critical site of plasticity underlying associative learning and memory in Pavlovian fear conditioning (6). Within the amygdala, the basolateral complex (BLA) is particularly important for the synaptic encoding of associations between a conditioned stimulus (CS), such as an auditory tone, and unconditioned stimulus (US), such as a footshock (15-21). We and others have previously shown that recent (24 hour) fear memories are encoded at the level of single neurons in the BLA such that individual neurons show increased spike firing in response to auditory CSs during memory retrieval (22-27). However, it is unknown if remote memories are similarly coded in the BLA. Early work showed that lesions or pharmacological inactivation of the BLA prevents both the acquisition and retrieval of both recent and remote (up to 1-year-old) fear memories (18, 28, 29), suggesting that the BLA is a permanent site of fear memory storage. Conversely, more recent work suggests BLA neurons are not required for retrieving long-term fear memories and that fear memory networks reorganize to include neocortical (10) and prefrontal-thalamic circuits (7, 30). Despite decades of work, the role for BLA neurons in the storage and retrieval of long-term fear memories remains controversial.

To directly compare recent and remote memories in the BLA, we used a within-subjects behavioral design whereby rats retrieved separately conditioned recent and remote auditory CSs while employing electrophysiological, optogenetic, and fiber photometry methods. We found that BLA neurons were equally activated upon retrieval of recent and remote fear memories as measured by c-Fos immunoreactivity, CS-evoked firing of single-units, and CS-evoked fluorescence. Additionally, we found that the majority of CS-responsive BLA neurons encoded both recent and remote memories, suggesting a large overlap in memory allocation. Suppression of BLA principal neuron activity via optogenetic inhibition of CaMKII-positive neurons, or excitation of inhibitory neurons, impaired the retrieval of both recent and remote fear memories, including when optogenetic inhibition occurred only during the CS. We thus conclude that the BLA similarly encodes recent and remote memories at the single-cell level and that BLA principal neuron activity is indispensable for the retrieval of both recent and remote fear memories.

Materials and methods

Animals

Adult naïve male and female Long-Evans Blue Spruce rats (200 - 240 g upon arrival) were obtained from Envigo (Indianapolis, IN). Rats were individually housed in clear plastic cages on cylindrical racks in a climate-controlled vivarium with a fixed 14:10 hour light:dark cycle (lights on at 7:00 AM). All experiments were conducted during the light phase. Rats were given access to standard rodent chow and water ad libitum. Upon arrival, all rats were handled by the experimenter (~30 sec/rat/day) for a minimum of 5 days prior to the start of any surgical or

behavioral procedures. All experimental procedures were conducted in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Texas A&M University Institutional Animals Care and Use Committee (IACUC).

Viruses

AAV8-CaMKII-Jaws-KGC-GFP-ER2-WPRE-SV40 (AAV8-CaMKII-Jaws-GFP) was purchased from University of Pennsylvania Vector Core. AAV8-EF1a1.1-FLEx-tdTomato (AAV8-FLEx-tdTomato) and AAV8-CaMKII-GFP were purchased from UNC vector core. AAV9-CaMKII-GCaMP6f-WPRE-SV40 (AAV8-CaMKII-GCaMP6f), AAV8-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-HGHpA (AAV8-DIO-hChR2-mCherry), AAV9-mDlx-GFP-Fishell 1 (AAV9-mDlx-GFP), and AAV9-mDlx-ChR2-mCherry-Fishell 3 (AAV9-mDlx-ChR2-mCherry) were purchased from Addgene (Watertown, MA). Viruses were diluted to particular titers with sterilized 1x DPBS. The final titers of viruses injected into the BLA were 5.8×10^{12} GC/mL for AAV8-CaMKII-Jaws-GFP, 4.2×10^{12} GC/mL for AAV8-CaMKII-GFP, 5.0×10^{12} GC/mL for AAV9-CaMKII-GCaMP6f, 4.8×10^{12} GC/mL for AAV8-DIO-tdTomato, 5.6×10^{12} GC/mL for AAV8-DIO-hChR2-mCherry, 4.5×10^{12} GC/mL for AAV9-mDlx-GFP, and 8.0×10^{12} GC/mL for AAV9-mDlx-ChR2-mCherry.

Surgery

Rats were anesthetized with isoflurane (5% for induction, 1-2% for maintenance) and placed into a stereotaxic frame (Kopf Instruments). The hair on the scalp was shaved, povidine-iodine was applied to the skin, and a small incision was made in the scalp to expose the top of the skull. The skull was leveled by placing bregma and lambda in the same horizontal plane. Small holes were drilled in the skull to affix three to four jeweler's screws.

For optogenetic and fiber photometrics experiments, rats received bilateral infusions of viruses into the BLA (0.5 µl/ side; AAV8-CaMKII-Jaws-GFP and AAV8-CaMKII-GFP for optogenetics and AAV9-CaMKII-GCaMP6f for fiber photometry). Viruses were infused at a rate of 0.1 µL/min and injector tips were left in the brain for ten additional minutes to allow for diffusion. The coordinates for BLA viral injection were: AP: -2.85 mm, ML: -4.85 mm, DV: -8.7 mm (relative to bregma surface). Immediately after viral injection, optical fibers with ceramic ferrules were implanted into the BLA bilaterally (0.3 mm above the viral injection sites). For optogenetics, fibers with white ceramic ferrules were used [200-µm core; 10-mm length; Thorlabs (Newton, NJ)]. For fiber photometry, fibers with black ferrules were used [200-µm core; 10-mm length; Neurophotometrics Ltd. (San Diego, CA)].

For awake-behaving electrophysiology, a 16-channel microelectrode array (Innovative Neurophysiology, Durham, NC) was chronically implanted targeting the BLA unilaterally in the right hemisphere. The microelectrodes were of comprised sixteen 10.5-mm long tungsten wires in a 4x4 array with 200 µm center-to-center spacing of adjacent wires (50 µm diameter). The ground and reference channels were joined together and a single, silver ground wire was wrapped around a skull screw above the cerebellum and affixed with conductive silver paint. Coordinates for targeting the BLA were: AP: -2.9 mm; ML: + 4.5 mm; DV: -8.55 mm (relative to bregma skull surface). The AP coordinate was based on the center of the array and the ML coordinate was based on the leftmost wire of the array. Dental cement was used to secure optical fibers and microelectrode arrays to the skull. Topical antibiotic (Triple Antibiotic Plus; G&W Laboratories) was applied to the surgical site and one chewable carprofen tablet (2 mg; Bio-Serv)

was provided for post-operative pain management. Rats were given a minimum of one week to recover prior to the beginning of behavioral testing.

Behavioral apparatus

Recent and remote fear conditioning were conducted in two distinct rooms within the laboratory. Each room housed 8 identical rodent fear conditioning chambers (30 × 24 × 21 cm; Med Associates, St. Albans, VT). Each chamber consisted of two aluminum sidewalls and a Plexiglas ceiling and rear wall, a hinged Plexiglas door, and a grid floor. The grid floor consisted of 19 stainless steel rods that were wired to a shock source and solid-state grid scrambler for delivery of the footshocks (Med Associates). A speaker for delivering auditory stimuli, a ventilation fans, and house lights were installed in each chamber. Fear retrieval sessions were conducted in similar chambers (30 × 24 × 21 cm; Med-Associates) equipped with either a red laser (Dragon Lasers, Changqun, China), electrophysiology system (Plexon, Dallas, TX), or fiber photometry system (Neurophotometrics Ltd., San Diego, CA).

For all conditioning sessions and optogenetic experiments, locomotor activity was acquired online by a load-cell system positioned underneath the behavioral chamber which converted chamber displacements into voltages via Threshold Activity software (Med-Associates). Freezing was defined as less than 10 a.u. for at least one second. In the electrophysiology experiment, the load-cell activity was recorded directly via the electrophysiological recording software (OmniPlex, Plexon). For the fiber photometry experiment, locomotor activity was recorded by a camera and analyzed by Video Freeze software (Med-Associates). For all systems, freezing was defined as immobility that lasted at least 1 s. Therefore, all freezing behavior was automatically recorded and analyzed, providing unbiased measurements.

Distinct contextual environments (contexts A, B, and C) were used during conditioning and retrieval. For context A, the house light was turned off and the overhead white light and ventilation fan were turned off. The cabinet door remained open for the duration of each session. The chamber was wiped with 1.0 % ammonium hydroxide prior to each behavioral session. Rats were transported to context A in black plastic boxes. For context B, the house light was turned on, the fan was turned on, and the room was dimly lit by overhead fluorescent red lights. The cabinet door remained closed for the duration of each behavioral session. A black Plexiglas floor was placed over the grid (except during fear conditioning) and the chamber was wiped down with a 3.0 % acetic acid solution prior to each behavioral session. Rats were transported to context B in white plastic boxes with a clean layer of bedding. For context C, both the house light and overhead white light were turned on, the fan were turned off, and the cabinet door remained closed. Black and white stripped wallpapers were taped on the chamber walls. A clear plastic floor was placed over the grid. The chamber was wiped with 70% ethanol prior to each behavioral session and rats were transported to context C in white plastic boxes with a clean layer of bedding.

Behavioral procedures

Overviews of each behavioral experiment are provided in the figures. Two fear conditioning procedures were used in the study: standard auditory fear conditioning using a single CS and within-subjects auditory fear conditioning using distinct recent and remote CSs.

Standard auditory fear conditioning. For the c-Fos experiment (Fig. 1) and single CS continuous inhibition experiment (Sfig. 3), rats underwent a standard auditory fear conditioning procedure in which an innocuous auditory tone (conditioned stimulus; CS) was paired with an aversive footshock (unconditioned stimulus; US) in context A. This conditioning session was comprised of a 3-min baseline period and five CS (10 s, 80 dB, 8 kHz)-US (1.0 mA, 2 s) pairings with 60-s intertrial intervals (ITIs), and additional 60-s post-shock period. For the c-Fos experiment, home cage control rats were handled daily and were never exposed to the conditioning chamber or the CS. Conditioned rats underwent fear retrieval either 1 day (recent retrieval) or 14 days later (remote retrieval) in context B. This retrieval session was comprised of a 3-min baseline period followed by 5 CS-alone presentations (10s, 80 dB, 30s ITI). For the c-Fos experiment, 1 day or 14 days later (half and half), no retrieval rats were exposed context B (380 s; matched to retrieval groups) with no CS presentations. Rats were then perfused (90 min after behavioral testing) at 9:00-11:00am on the same day to minimize variations.

Within-subjects auditory fear conditioning. For experiments 2 to 5, rats underwent a within-subjects auditory fear conditioning procedure that permitted a direct comparison neuronal and behavioral responses to recent and remote CSs in the same subjects. On the first day, rats were conditioned in context A for remote conditioning [3-min BL, five tone (CS^{remote}; 10 s, 80 dB, 8 kHz)-footshock (US; 1.0 mA, 2 s) pairings with 60-s intertrial intervals (ITIs), and additional 60 s post-shock period]. Thirteen days later, rats were conditioned in context B for recent conditioning [3-min BL, five tone (CS^{recent}; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) pairings with 60-s intertrial intervals (ITIs), and additional 60-s post-shock period].

For retrieval testing in electrophysiology experiment (experiment 2), all rats were unilaterally implanted with a 16-channel microwire array targeting the BLA one week prior to behavioral testing (see *Surgeries* section for details). Conditioned rats (Conditioned) underwent the within-subjects auditory fear conditioning, whereas control rats (Control) were presented an equal number of CSs without footshock during conditionings. One day after recent conditioning (Fig. 2A), the rat was placed in the recording chamber (context C) and underwent CS^{recent} and CS^{remote} retrieval tests [both with 3-min BL; 5 x 10 s tones (2 kHz or 8 kHz); 30-s ITIs], which were separated by 15 min. Pips (500 ms, 1 Hz, 500-ms interpip-interval) were used in place of the continuous 10-s CS to increase the number of CS-evoked trials for data analysis. The CS testing order was counterbalanced (half of the animals were tested with CS^{recent} first, the other half with CS^{remote} first). During the 15-min period between the two test sessions, the recording system was paused, and the rat was temporarily placed in a 5-gallon buck with bedding (the recording cable remained connected); the experimenters quickly cleaned the recording chamber during this pause. The rat was then placed back into the recording chamber and underwent the second retrieval session. This dual testing procedure enabled us to record CS-elicited activity of the same single-units during the retrieval of both recent and remote fear memories.

In the fiber photometry experiment (experiment 3), a within-subject auditory conditioning procedure was used. Three groups of rats were used: one group (CS^{recent} and CS^{remote} conditioned) was conditioned to both CSs; the other two groups (CS^{recent} conditioned only; CS^{remote} conditioned only) were only conditioned to either CS^{recent} or CS^{remote} (rats still presented with both CSs, but only one was associated with footshock). These group assignments allowed us to determine whether stimulus generalization drove neuronal responses to the CSs. For all groups, 8 kHz and 2 kHz tones were used as CS^{recent} and CS^{remote} in a counterbalanced manner. For the retrieval test conducted one day after CS^{recent} conditioning (or CS^{recent} exposure),

rats underwent a 2-day testing procedure (the order of CS^{recent} and CS^{remote} testing was counterbalanced and all testing occurred in context C). On each day, the rat underwent a 3-min BL followed by 5 tone (CS^{recent} or CS^{remote})-alone presentations (30-s ITIs).

For the retrieval test in the optogenetic experiments (experiments 4 and 5) conducted one day after the recent conditioning, rats underwent a counterbalanced 4-day testing procedure (context C). During these tests, animals were tested to a single CS with either the laser on or off (counterbalanced order). For each test session, the rats underwent a 3-min BL followed by 5 tone (CS^{recent} or CS^{remote})-alone presentations (30-s ITIs).

Optogenetics

Rats expressing Jaws or control GFP in BLA principal neurons were bilaterally illuminated using a red laser (635 nm laser; Dragon Lasers, Changqun, China). Rats expressing ChR2 or control GFP in BLA PV neurons, interneurons (mDlx promoter), and their controls were bilaterally illuminated using a blue laser (450 nm laser; Dragon Lasers, Changqun, China). Laser power was set to 6-7 mW at the tips of optical fibers. A fiber-optic rotary joint (Doric Lenses Inc; Québec, QC, Canada) and a bundled patch cord (Thorlabs; Newton, NJ) were used to allow bilateral optostimulation. The laser was controlled by Med Associates software via a TTL adaptor. In the constant Jaws inhibition experiments (Fig. 4 and Sfig. 4), laser light was delivered 10 s before the first tone onset and persisted to the end of testing session. In the CS-specific Jaws inhibition and ChR2 experiments (Fig. 5 and Sfigs. 7 and 8), laser light was delivered 10 s before each tone onset and turned off at tone offset.

Immunohistochemistry

Rats were transcardially perfused 90 minutes after behavior and brains were sliced as described in the *Histology* section(31). The following steps were performed on a plate shaker. Brain slices (30 μm) were first washed 3 × 10 min in 1× PBS and then blocked in 3% normal donkey serum (NDS) in PBS with 0.1% Triton-X (pH 7.4) for one hour at room temperature. Slices were then incubated with rabbit anti-c-Fos primary antibody [1:500 in PBST (1X PBS with 0.1% Tween 20); Abcam, Cambridge, MA, USA] at 4 °C for overnight. The next day, slices were washed with PBST for 3 × 10 min. Slices were then incubated with Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody (1:500 in 1× PBS) for 2 h at room temperature. After a final wash with PBS (3 × 10 min), stained brain sections were then wet-mounted on slides, coverslipped with DAPI-containing fluoromount mounting medium (Invitrogen) and photographed using a Zeiss microscope (Axio Imager). An experimenter who was blind to the experimental design counted the c-Fos positive neurons and analyzed the data. c-Fos positive cells were counted manually with 10x magnification images (ImageJ software). Four to six images from distinct rostrocaudal levels of BLA (-2.5 to -3.8 mm from bregma) was averaged and divided by the surface area (standardized to 0.1 mm^2) for each rat.

Awake-behaving electrophysiology

Extracellular single-unit activity and freezing behavior were automatically recorded by a multichannel neurophysiological recording system (OmniPlex, Plexon, Dallas, TX) as previously described(32). Wideband signals recorded on each channel, amplified (2000×), digitized (40-kHz sampling rate), and saved for offline sorting and analysis. For each animal, one of the 16 in-brain wires was chosen to serve a reference to optimize signal quality for single-unit detection. Waveforms that exceeded a threshold of 3 standard deviations below baseline noise were

selected for unit sorting. Units were sorted manually using 2D principal component analysis after bandpass filtering (600-6000 Hz; Offline Sorter, Plexon); only well-isolated units were included in analysis. Sorted waveforms and their timestamps were then imported to NeuroExplorer (Plexon) for further analysis. Data were analyzed during a 1.0-s period (0.25 s before, 0.5 s during and 0.25 s after each CS pip). After the experiment, rats were anesthetized with sodium pentobarbital (Fatal Plus; 100 mg/mL, 0.5 mL, i.p.) and a small current was passed through four wires at the corners of the 4x4 array (1 mA pulse; 10 s). Rats were then perfused transcardially with physiological saline followed by 10% formalin for histology.

Acute electrophysiology

Acute electrophysiological recordings were performed to validate the Jaws inhibitory opsin (AAV8-CaMKII-Jaws-GFP). Three weeks before recording, 3 male rats were injected bilaterally with the Jaws virus and 2 male rats were injected with the GFP control virus as previously described targeting the BLA (AP: -2.85 mm, ML: -4.85 mm, DV: -8.7 mm). Isoflurane was used throughout the recordings as the anesthetic agent (5 % induction, 1-2 % maintenance). Animals were placed into a stereotaxic system, the scalp was excised, small burr holes were drilled bilaterally above the BLA and unilaterally anterior of bregma, and a small jeweler's skull was secured in the burr hole anterior of bregma. An optrode consisting of a 16-channel microelectrode array attached to an optic fiber (Opto MEA, Microprobes) was used for simultaneous extracellular recording and optogenetic stimulation. The electrode array was comprised of platinum/iridium wires arranged in a circle with no more than 250 μm of lateral spacing from the optic fiber. A silver ground wire was wrapped around the skull screw to ground the electrode and the pre-amplifier was directly grounded to the stereotaxic frame.

Recordings consisted of a 10-s baseline followed by at least ten trials of 10-s continuous red-laser illumination (635 nm) with 20-s intertrial intervals. Recordings were made in a track beginning ~1.5 mm above the viral injection coordinates (DV: -7.2 mm) and ending ~0.5 mm beyond the placement of the viral injections (DV: -9.2 mm) in the BLA. After the target depth was reached and neurons isolated on the array, a recording was made and the electrode was then lowered ~250 μm or until new units were observed. All animals were perfused following anesthetized recordings as described above. All data were hand sorted, exported, and analyzed as described above.

Single-unit analysis

All single-unit analyses were performed using custom-written Python scripts. Single-unit firing rates were estimated using peristimulus time histograms (PSTHs) computed in 25-ms bins. To analyze CS-evoked activity, *z*-scores were computed with a baseline period of -0.25 to 0 s before pip onset and averaged across all 50 pips (10 per CS trial). A single-unit was considered to be significantly modulated by the CS if at least one bin exceeded ±3 *z*-scores within 300-ms on pip onset.

Fiber photometry

A fiber photometry system (FP3002; Neurophotometrics; San Diego, CA) was used to record GCaMP6f activity in the BLA (see the *Surgeries* section for viral injection). The 470 nm and 410 nm LEDs were used as light sources for illuminating GCaMP6f to record calcium-dependent and isosebestic activities. The light intensities of the source LEDs were set at particular levels in order to obtain ~ 50 μW power at the tips of optical fibers. Sampling rate was set at 40 Hz for

410 nm and 470 nm LEDs (470 nm LED turned on first). Raw fiber photometry data were then transferred to ΔF/F values by using the open-source tool pMAT for further analysis(33). One rat in the remote condition group was excluded due to an absence of a calcium signal.

Histology

Upon completion of the experiment, rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/mL, 0.5 mL, i.p.) and perfused transcardially with physiological saline followed by 10% formalin. Brains were extracted and stored about 16-18 h (at 4° C) in 10 % formalin after which they were transferred to a 30 % sucrose solution for a minimum of 5 days. Brains were then sliced using a cryostat (Leica Microsystems) at -20° C. Viral expression was verified with a Zeiss microscope (Axio Imager). Brain slices were also stained with thionin (0.25%) for verifying fiber and microelectrode array placement.

Statistics

All data are represented as means ± SEM. Data were analyzed using Prism GraphPad 9.0. For conditioning, two-way repeated-measures (RM) analysis of variance (ANOVA) with trial as the within-subjects factor and virus (or group) as the between-subjects factor was conducted. For the retrieval test, three-way RM ANOVA with laser and memory age as within-subjects factors and virus as between-subjects factor was conducted first. Recent memory and remote memory were then separately analyzed with two-way RM ANOVA with laser as within-subjects factor and virus as between-subjects factor. Two-way RM ANOVA was conducted for firing z-score data, with CS-type as within-subjects factor and conditioned as between-subjects factor. Significant two-way ANOVA was followed by post hoc Bonferroni's multiple comparisons test. One-way ANOVA was conducted for c-Fos data followed by post hoc Turkey analysis. Chi-squared test was used to analyze data of the percentage of neurons. Group sizes were determined based on prior work and what is common in the field. $P < 0.05$ was considered statistically significant.

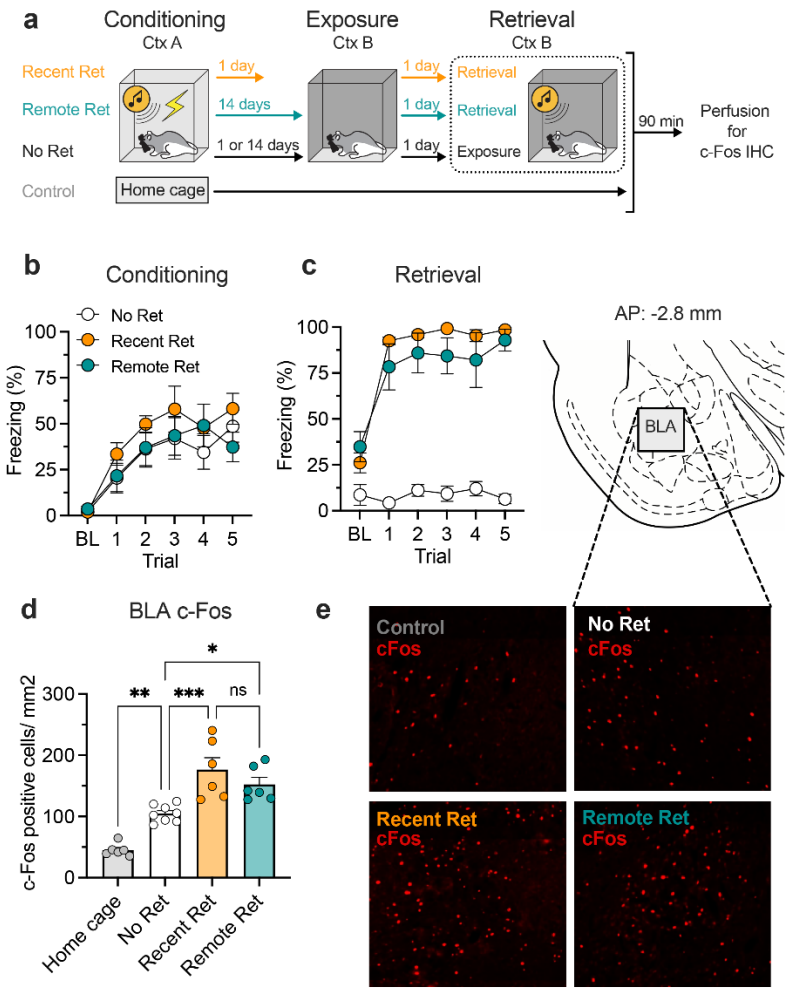

Figure 1. Both recent and remote fear retrieval induce c-Fos expression in the BLA. a, Schematic representation of experimental design. **b,** Percentage of freezing during fear conditioning. **c,** Percentage of freezing during retrieval testing. Recent (Recent Ret) and remote (Remote Ret) CSs evoked similar and high levels of freezing compared to the no-retrieval (No Ret) control. **d,** Recent and remote fear retrieval induced similar and high levels of c-Fos expression in the BLA. Context exposure alone (No Ret) activated BLA c-Fos at a lower level. **e,** Representative images showing c-Fos immunofluorescence in the BLA from all groups and schematic atlas template showing the area within the BLA that was imaged for c-Fos counting. Data are shown as mean ± SEM. *$p < 0.05$, $n = 6$ per group. CS, conditioned stimulus; Ret, retrieval; IHC, immunohistochemistry; BL, baseline; BLA, basolateral amygdala; ns, no significant difference.

Results

Recent and remote fear retrieval induce similar levels of c-Fos activation in the BLA.

It has previously been shown that auditory CSs increases immediate early gene expression in the BLA, however direct comparisons of IEG expression to recent and remote CSs are limited (Pollack/Bergstrom). To address this question, we first examined the expression of c-Fos (a neuronal activity marker) in the BLA after recent and remote fear retrieval. In this experiment (Fig. 1A), rats were fear conditioned to an auditory CS (2 kHz tone; 5 CS × US associations; day 1) and then underwent fear retrieval sessions at either a recent (Recent Ret; day 2) or remote (Remote Ret; day 15) timepoint. A control group was conditioned but then merely exposed to the retrieval context at same recent or remote times (No Ret). All conditioned rats were pre-exposed

to the retrieval context one day before the retrieval test to minimize possible c-Fos expression induced by novelty or fear generalization. An additional control group remained in the home cage on the day of retrieval testing.

All groups of conditioned rats acquired similar levels of fear as indicated by time spent freezing during the conditioning session [a significant main effect of trial ($F_{5, 85} = 14.83$, $p < 0.01$), but no significant main effect of virus ($F_{1, 17} = 1.86$, $p = 0.19$) or virus × trial interaction ($F_{10, 85} = 0.46$, $p = 0.91$); Fig. 1B]. For the retrieval test, the ANOVA revealed significant main effects of virus ($F_{2, 17} = 64.31$, $p = 0.01$) and trial ($F_{5, 85} = 62.09$, $p < 0.01$), and a virus × trial interaction ($F_{10, 85} = 18.57$, $p < 0.01$). Post hoc analyses showed that both recent and remote retrieval rats showed higher freezing than no retrieval rats (all t's < 0.05), but did not differ from one another (all t's > 0.05; Fig. 1C). All rats were perfused 90 min after memory retrieval (recent and remote retrieval rats) or context exposure (no retrieval rats) along with the home cage controls.

After c-Fos immunohistochemistry, the BLA was imaged and c-Fos positive neurons were counted (Fig. 1E). An ANOVA on these data revealed a significant main effect ($F_{3, 22} = 25.83$, $p < 0.01$). Post hoc analysis indicated that animals exposed to a recently or remotely conditioned CS showed higher c-Fos immunoactivity in the BLA than rats not exposed to a CS (Recent Ret vs. No Ret: $t_{12} = 6.71$, $p < 0.01$; Remote Ret vs. No Ret: $t_{12} = 4.44$, $p = 0.02$). No difference was found between recent retrieval and remote retrieval rats ($t_{10} = 2.12$, $p < 0.45$; Fig. 1D). These results indicated that recent and remote fear retrieval elicited similarly high levels of c-Fos activity in the BLA. Interestingly, rats in the no-retrieval condition showed significantly higher BLA c-Fos expression than home cage controls ($t_{12} = 5.57$, $p < 0.01$; Fig. 1D). This suggest that generalization might increase BLA Fos in the absence of substantial freezing behavior. Collectively, these results indicate that recent and remote fear memory retrieval induce similar levels of Fos expression in the BLA.

Conditioning-related spike firing in the BLA during the retrieval of recent and remote fear memory

Although cFos expression correlates with neuronal activity, it has poor temporal resolution and does not inform whether individual neurons encode recent or remote CSs. Decades of work have shown that neurons in the lateral amygdala exhibit CS-evoked firing, a neuronal correlate of fear memory (5, 22, 25), but the nature of CS-evoked firing to recent and remote CSs has not been explored. To address this question, we conducted extracellular recordings of BLA spike firing in awake, behaving rats retrieving recently or remotely conditioned fear memories. We employed a within-subjects fear conditioning procedure in which two independent CSs underwent conditioning either 1 day (CS^{recent}) or 2 weeks (CS^{remote}) prior to the electrophysiological recording session (Fig. 2A). Notably, this procedure allowed us to examine neuronal and behavioral responses to recent and remote CS without confounding memory age and recent exposure to footshock, for example.

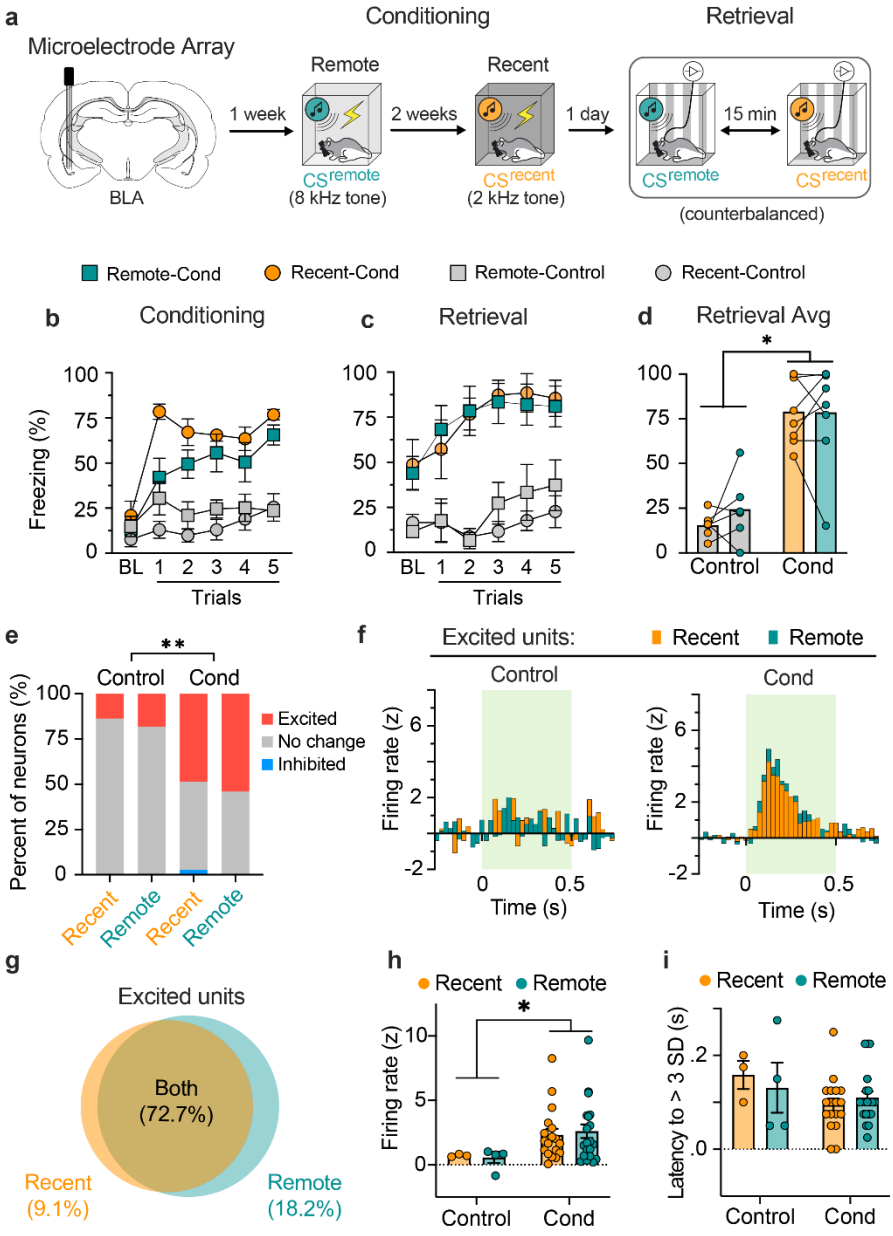

Figure 2. BLA neurons exhibit excitatory responses to both recent and remote conditioned stimuli. a, Schematized experimental design of the electrophysiology experiment. Conditioned rats received two fear conditioning sessions with distinct conditioned stimuli (CS^{remote} and CS^{recent}) separated by two weeks; control rats were presented with the same CSs without foot shocks. **b,** Freezing data showing that conditioned, but not control, animals acquired fear to both the recent and remote CS**. c-d,** Trial (**c**) and average (**d**) data showing that conditioned animals, and not control, show similarly high levels of fear to the recent and remote CS (n = 6-8 per group). **e**, Stacked bar plots illustrate the percentage of BLA neurons showing excitatory or inhibitory responses to CS^{remote} and CS^{recent} in conditioned and control rats. Both recent and remote conditioning increased the proportion neurons showing excitatory CS responses during fear retrieval. Only one neuron (2.7 %) showed an inhibitory response to CS. **f,** Perievent time histograms illustrating the responses of BLA neurons with excitatory responses to the CSs. Both the CS^{remote} and CS^{recent} induced similarly high firing rates among BLA neurons in conditioned rats. **g**, Venn diagram showing that the majority (72.7%) of CS-responsive BLA neurons encode both recent and remote fear memory. **h,** Average firing rates (z-scores) of all recorded neurons. Both recent and remote fear retrieval induced higher firing rates of BLA neurons. **i,** The average latency at which conditioned CS-responsive neurons showed

increased firing relative to baseline (first bin of *z*-score > 3 SD). Data are shown as mean ± SEM. *$p < 0.05$. CS, conditioned stimulus; BLA, basolateral amygdala.

Conditioned rats received 5 CS-US associations during conditioning, whereas control rats were only exposed to CSs without footshock. For recent fear conditioning, two-way RM ANOVA showed significant main effects of trial ($F_{5, 60} = 12.86$, $p < 0.0001$) and footshock ($F_{5, 60} = 12.86$, $p < 0.0001$) and an interaction of trial × footshock ($F_{5, 60} = 1.28$, $p = 0.28$) (Fig. 2B). For remote fear conditioning, two-way RM ANOVA revealed significant main effects of trial ($F_{5, 60} = 4.88$, $p = 0.0008$) and footshock ($F_{5, 60} = 8.71$, $p = 0.01$) and an interaction of trial × footshock ($F_{5, 60} = 2.59$, $p = 0.03$). For retrieval, three-way RM ANOVA revealed main effects of trial ($F_{5, 60} = 6.49$, $p < 0.0001$) and footshock ($F_{5, 60} = 32.80$, $p < 0.0001$) and an interaction of trial × footshock ($F_{5, 60} = 2.93$, $p = 0.02$), but not significant main effect of memory age ($F_{1, 12} = 0.27$, $p = 0.61$) or interaction of trial × footshock × memory age ($F_{5, 60} = 0.55$, $p = 0.74$) (Fig. 2B). These data indicated that rats showed similar levels of recent and remote fear retrieval. During the retrieval test sessions, conditioned rats exhibited similar levels of freezing to both the recent and remote CSs (Fig. 2C and 2D). This observation was confirmed by a significant main effect of conditioning ($F_{1, 12} = 38.06$, $p < 0.01$), but no significant main effect of CS-type ($F_{1, 12} = 0.57$, $p = 0.47$) or interaction conditioning × CS interaction ($F_{1, 12} = 0.48$, $p = 0.43$); Fig. 2C and 2D]. We recorded from a total of 58 neurons: 37 neurons in conditioned rats ($n = 8$) and 21 neurons from the controls ($n = 6$). The average baseline firing rate of these neurons was 2.92 ± 0.39 Hz in the conditioned rats and 3.49 ± 0.77 Hz in the controls, consistent with the firing properties of BLA principal neurons(34). Firing rates were unimodally distributed, suggesting that principal neurons constituted the majority of neurons sampled in our recordings, though we cannot rule out the possibility that interneurons were among the recorded cells.

To determine the responsiveness of BLA neurons to CS onset, spike firing rates in each neuron were normalized to its pre-CS baseline firing rate. Normalized CS-evoked single unit data for every unit is shown in Sfig. 1. As illustrated in Fig. 2E, the percentage of neurons showing increases or decreases in spike firing ($z > 3$ SDs in at least one post-CS bin) to either the recent or remote CS was calculated. Among all of the units recorded, only one exhibited an inhibitory response to CS onset; this cell was excluded from further analysis. Presentation of the recent or remote CS produced robust increases in spike firing in the majority of BLA neurons; 22 neurons (59.4%) responded to one or both of the CSs. Overall, CS-evoked firing rates were similar to the recent (2 kHz) and remote (8 kHz) tone CSs (Fig. 2F). Not surprisingly, there was a higher percentage of BLA neurons that exhibited excitatory responses to the CS^{recent} in conditioned (18/37; 48.65%) compared to control (3/22; 13.64%) rats ($\chi^2 = 7.82$, $p < 0.01$); this was similar for responses to the CS^{remote} in conditioned (20/37; 54.05%) compared to control (4/22; 18.18%) rats ($\chi^2 = 7.36$, $p < 0.01$) (Fig. 2E).

Importantly, the behavioral design allowed us to characterize the response of each unit to both the recent and remote CSs. In particular, we examined whether CS-responsive neurons ($n = 22$) in conditioned rats exhibited preferential firing to recent or remote CSs. As shown in Fig. 2G, the majority of CS-responsive neurons (72.7%) responded to both the recent and remote CSs. Only six neurons (27.3%) showed selective responses to the CSs: two neurons (9.1%) responded selectively to the recent CS and four neurons (18.2%) showed selective responding to the remote CS. This reveals that there is considerable overlap in the population of BLA neurons representing recent and remote conditioning memories. It is also possible that BLA firing among CS-responsive cells is influenced by generalization between the auditory stimuli, though the selective firing of some of the neurons in the sample argues against this possibility.

As shown in Fig. 2F, the average normalized firing rate of excited neurons was far greater in conditioned compared to controls rats [main effect of group: ($F_{1, 41} = 4.28$, $p = 0.045$)]. In conditioned rats, post-hoc analyses revealed no difference in the percentage of excited neurons ($\chi^2 = 0.12$, $p = 0.73$) or the average normalized firing rate of all CS-responsive neurons ($n = 22$; $t_{57} = 1.27$, $p = 0.38$; Fig. 2H) to CS^{recent} vs CS^{remote}, suggesting that the BLA is equally excited to both recent and remote cued fear. In addition, perievent histograms indicate that the latency and magnitude of CS-evoked responses was similar to recent and remote CS (Fig 2I). The average latency at which conditioned CS-responsive neurons showed increased firing relative to baseline was 96 ± 13 ms to the recent CS and 108 ± 12 ms to the remote CS (Fig. 2I); the latency of peak responding was also similar to recent (139 ± 16 ms) and remote (142 ± 14 ms) CSs (Sfig. 1). There was no main effect of conditioning ($p > 0.5$) or memory age ($p > 0.2$) for either metric. Among neurons ($n = 16$) that showed excitatory responses to both the recent and remote CSs, firing to the recent and remote CSs was similar (paired *t*-test: $t_{15} = 1.70$, $p = 0.11$; Sfig. 1). Importantly, these findings are unlikely to be due to generalization of the two CSs insofar as a subset of excited neurons in conditioned rats were selectively excited by either the CS^{recent} (5.41%) or CS^{remote} (10.81%; Fig. 2G). This suggests that CS^{recent} and CS^{remote} memories are encoded by distinct, but highly overlapping neuronal ensembles.

Conditioning-related calcium signals in BLA principal neurons during the retrieval of recent and remote fear memory

The previous experiment suggests that single neurons in the BLA are engaged during the retrieval of recent and remote fear memories. Although the electrophysiological data are consistent with a role for BLA principal neurons in this effect, we sought to clarify this question using fiber photometric methods to asses BLA population activity. Because the majority of CaMKII-positive neurons in the BLA are principal cells(35), we bilaterally injected a virus encoding GCaMP6f under the CaMKII promotor (AAV8-CaMKII-GCaMP6f) into the BLA and chronically implanted optical fibers (Sfig. 2 for histology). Two weeks after viral injection, rats underwent the within-subject fear conditioning procedure described above (Fig. 3A), except that 10-sec tones rather than pips were used as the CSs. Three groups of rats were used in this experiment: Recent-Only, Remote-Only, and Dual-conditioned (Dual). We included recent- and remote-only groups to determine the extent to which a recent conditioning generalized to a remote tone (and vice versa). All rats underwent the within-subject fear conditioning procedure, however only the Dual group was conditioned to both CS^{recent} and CS^{remote}. The Recent-Only and Remote-Only groups were conditioned to the CS^{recent} and CS^{remote}, respectively, and were merely exposed to the other CS. This was to control for the possibility of generalization between the two CSs. Fear retrieval and fiber photometric recordings began one day after recent conditioning (Fig. 3A).

Analysis of conditioning data revealed a significant main effect of trials ($F_{5, 150} = 19.94$, $p < 0.01$), group ($F_{5, 30} = 15.44$, $p < 0.01$), and a trial × group interaction ($F_{25, 150} = 3.45$, $p < 0.01$], demonstrating that rats equally acquired fear to conditioned stimuli but not unpaired stimuli. During fear retrieval, the CS^{recent} and CS^{remote} induced equivalent freezing behavior in the Dual-Cond rats ($t_5 = 0.34$, $p = 0.75$; Fig. 3D), whereas Recent-Only (Fig. 3B) and Remote-Only (Fig. 3C) rats froze exclusively to CS^{recent} and CS^{remote} presentations, respectively (paired *t* test; Recent Only: $t_5 = 2.91$, $p = 0.03$; Remote Only: $t_5 = 2.84$, $p = 0.04$; Fig. 3E). Importantly, this suggests that freezing to the CS^{remote} in dual conditioned rats in this and the previous experiment is not due to generalization.

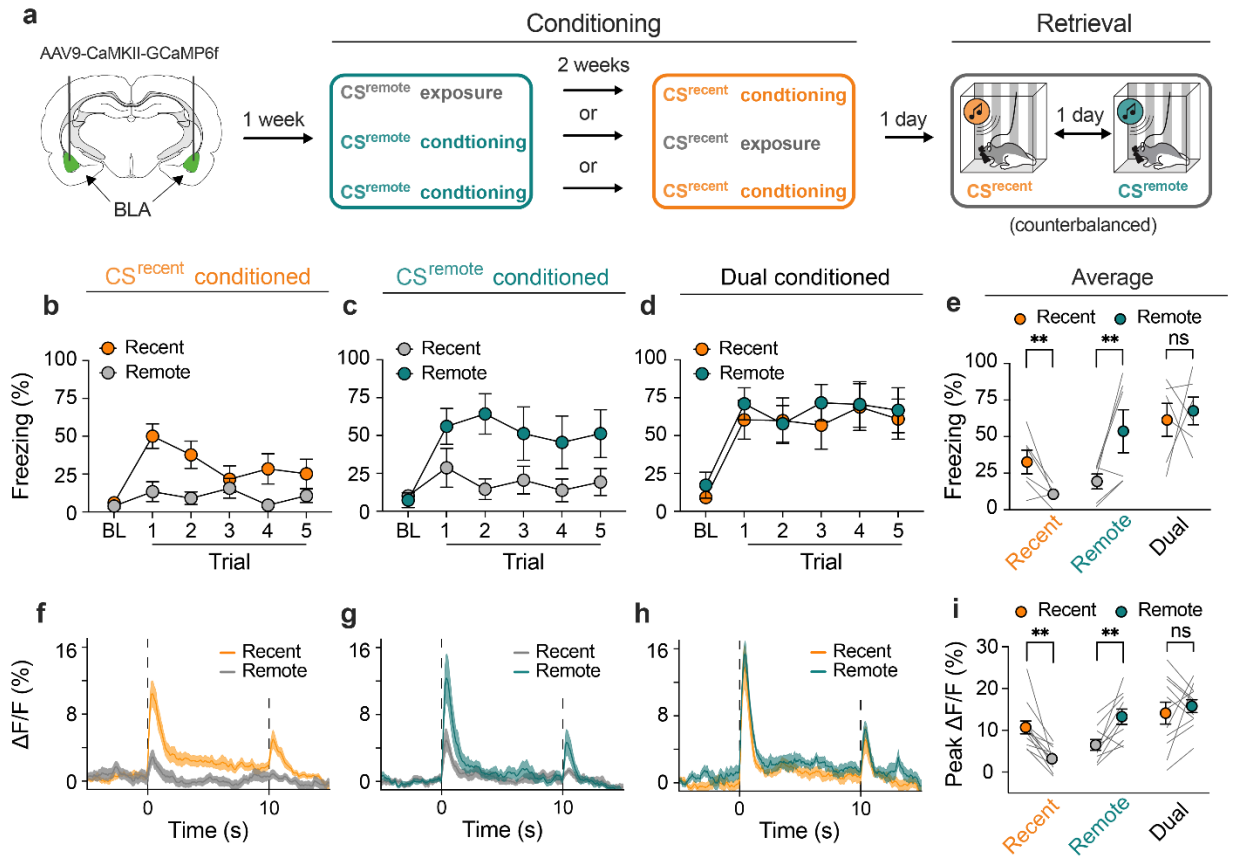

Figure 3. BLA principal neurons response to both recent and remote fear cues. a, Schematic procedure of fiber photometry experiment. **b-d,** Retrieval freezing data by trials for CS^{recent}-only (**b**), CS^{remote}-only (**c**), and Dual conditioned animals (**d**). **e,** Average freezing data showing that animals only exhibit high freezing levels to their respective conditioned CS and do not generalize to non-conditioned stimuli. **f-h,** Average CS-evoked traces of BLA calcium signal (ΔF/F) in CS^{recent}-only (**f**), CS^{remote}-only (**g**), and Dual conditioned animals (**h**). **i,** As illustrated by average peak calcium signal, conditioned stimuli triggered higher calcium signal than non-conditioned stimuli across groups. Data are shown as mean ± SEM. *$p < 0.05$, n = 6 animals per group. BL, baseline; BLA, basolateral amygdala; CS, conditioned stimulus.

Importantly, CS-evoked calcium dynamics tracked these behavioral outcomes. As illustrated in the fiber photometry data (Fig. 3F-H) and heatmaps (Sfig. 2), CSs elicited a short latency increases in BLA fluorescence, which did not differ between CS^{recent} (540 ± 192 ms) and CS^{remote} (386 ± 15 ms). The latency of the CS-evoked calcium signal was slightly longer than that of CS-evoked spike firing, consistent with the slower response of calcium indicators to neuronal activity. In addition, we observed a marked offset response in the calcium signal after the termination of the CS (Fig. 3F-H), which we previously observed electrophysiologically using a continuous CS(22). Importantly, the CS^{recent} and CS^{remote} elicited similar levels of peak fluorescence in the BLA of dual conditioned rats (paired *t* test; $t_{10} = 0.66$, $p = 0.53$; Fig. 3H and 3I); the latency of these peaks was also similar (data). In contrast, Recent-Only rats displayed significantly higher peak fluorescence to the CS^{recent} ($t_{11} = 4.81$, $p = 0.0005$; Fig. 3F and 3I), whereas Remote-Only rats exhibited higher peak fluorescence to the CS^{remote} ($t_9 = 3.68$, $p = 0.005$; Fig. 3G and 3I) compared to their non-conditioned counterparts. Collectively, these data

indicate that BLA principal neurons exhibit CS-evoked activity to both recent and remote CSs. This activity cannot be accounted for by fear generalization between the two CSs in the dual conditioning paradigm.

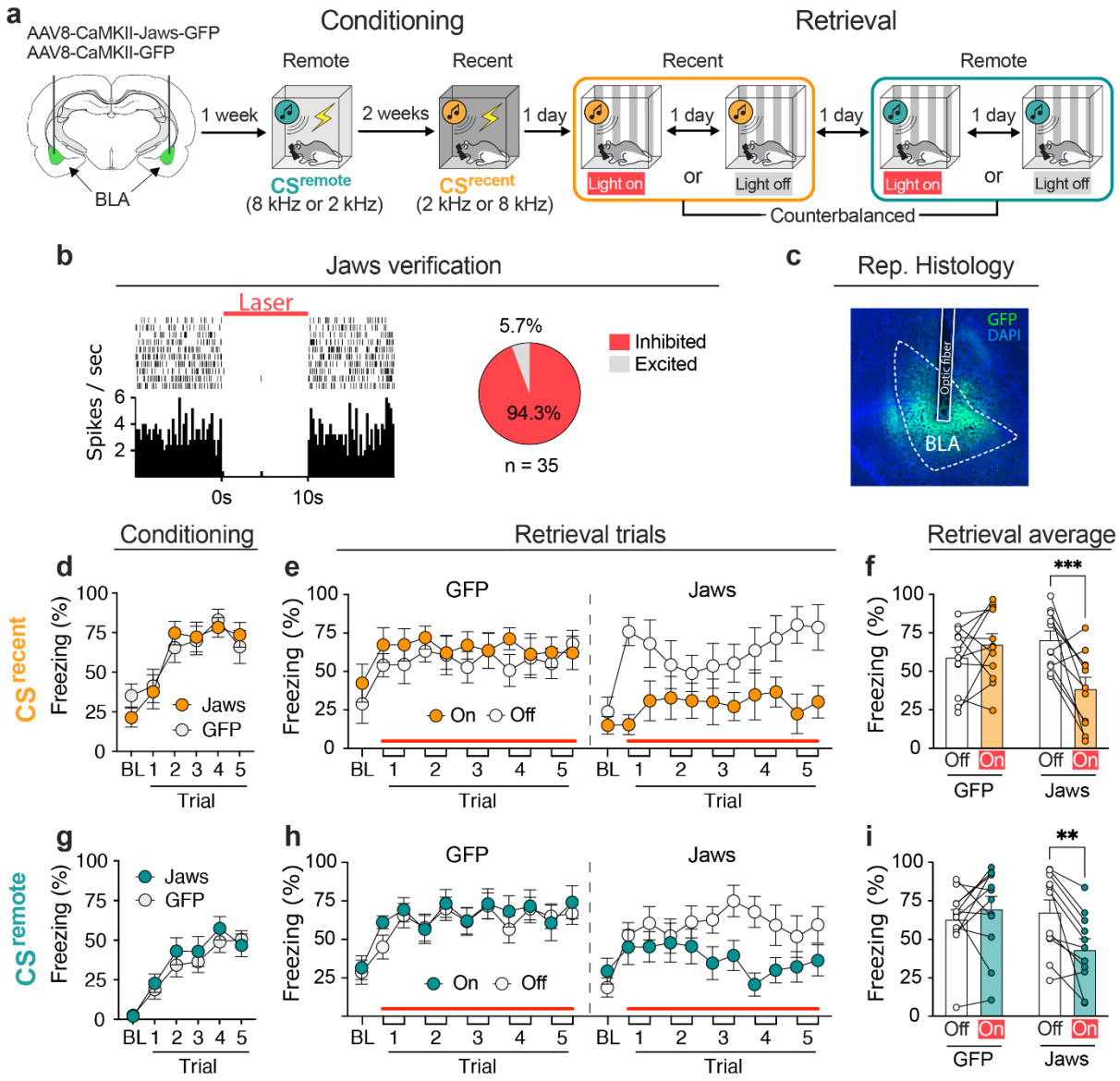

Figure 4. Optoinhibition of the BLA reduces freezing to both recent and remote CSs in a within-subjects fear conditioning procedure. a, Schematic representation of experimental design. 8 kHz and 2 kHz tones were used as remote (CS^{remote}) and recent (CS^{recent}) cues, respectively. Continuous laser illumination started 10 s before the first tone onset and persisted to the end of testing session. **b,** Jaws silencing decreased spontaneous firing rate of BLA neurons. **c,** Micrograph showing viral expression and fiber placement in the BLA. **d,** Recent fear conditioning. **e,** Optoinhibition of the BLA dramatically reduced CS^{recent}-induced freezing. Each trial consisted of a 10 s CS and a 30 s post-CS period. **f,** Average freezing during recent fear retrieval. **g,** Remote fear conditioning. **h,** Optoinhibition of the BLA also decreased CS^{remote}-induced freezing. **i,** Average freezing during remote fear retrieval. Data are shown as mean ± SEM. *$p < 0.05$, n = 11-12 per group. BL, baseline; BLA, basolateral amygdala; CS, conditioned stimulus.

Constant optogenetic inhibition of BLA principal neurons impairs retrieval of recent and remote fear memories

The BLA has been reported to have an enduring role in the retrieval of long-term fear memories. However, a recent report suggested that optogenetic silencing of BLA principal neurons produced an impairment in short-term (6 hours) but not long-term (1 week) fear memory retrieval (7). To compare the causal role of BLA in recent and remote fear memory retrieval in the same animals, we optogenetically silenced BLA principal neurons using Jaws, a red-shifted inhibitory opsin, during fear retrieval (Fig. 4A). We first validated Jaws *in vivo* by performing acute electrophysiological recordings in the BLA of anesthetized rats. Rats were injected AAV8-CaMKII-Jaws-GFP (Jaws) or its control virus AAV8-CaMKII-GFP (GFP) into the BLA three weeks before electrophysiological recording. As shown in Fig. 4B and Sfig. 3, red laser illumination (635 nm) dramatically reduced the overall spontaneous firing rate of BLA neurons in Jaws-, but not GFP-expressing, rats.

To examine the behavioral effects of BLA optoinhibition, we microinjected the same Jaws virus or control GFP virus into the BLA and implanted fibers bilaterally (Fig. 4C and Sfig. 4) one week prior to behavioral experimentation (Fig. 4A). All rats then underwent two independent cued fear conditioning sessions (CS^{recent} and CS^{remote} in different contexts) separated by two weeks as previously described. Recent and remote memories were retrieved in a counterbalanced, four-day testing procedure with light-on and light-off testing counterbalanced across day and CS. Retrieval testing began the day after the second conditioning session such that CS^{remote} was tested 15-18 days after conditioning and CS^{recent} was tested 1-4 days after conditioning. Each memory was tested only twice under laser on and off conditions, which minimized potential extinction effects.

For conditioning, Jaws and GFP rats acquired fear similarly to both CS^{recent} [main effect of trial ($F_{5, 105} = 14.08$, $p < 0.0001$), no significant main effect of virus ($F_{1, 12} = 0.006$, $p = 0.94$) or virus × trial interaction ($F_{5, 10} = 0.61$, $p = 0.69$); Fig. 4D] and CS^{remote} [main effect of trial ($F_{5, 105} = 23.71$, $p < 0.0001$), no significant main effect of virus ($F_{1, 12} = 0.45$, $p = 0.51$) or interaction of virus × trial ($F_{5, 105} = 0.42$, $p = 0.83$); Fig. 4G]. As shown in the trial by trial retrieval data in Fig. 4E and 4H, laser illumination in the Jaws rats, but not GFP controls, reduced freezing during both recent and remote retrieval. We then analyzed the average freezing during retrieval by using three-way repeated-measures ANOVA, which revealed significant main effect of laser ($F_{1, 21} = 8.60$, $p = 0.008$) and virus × laser interaction ($F_{1, 21} = 25.93$, $p < 0.0001$), but no main effects of virus ($F_{1, 21} = 3.39$, $p = 0.07$), memory age ($F_{1, 21} = 0.99$, $p = 0.77$), or virus × laser × memory age interaction ($F_{1, 21} = 0.43$, $p = 0.52$). These data suggest that memory age did not influence the effects of BLA inhibition on fear retrieval. For the CS^{recent} retrieval test, planned comparisons revealed significant main effect of laser ($F_{1, 21} = 4.88$, $p = 0.04$) and an virus × laser interaction ($F_{1, 21} = 14.40$, $p = 0.01$), but no significant main effect of virus ($F_{1, 21} = 1.36$, $p = 0.26$). Post hoc analysis showed that laser illumination reduced freezing in the Jaws rats ($t_{10} = 4.16$, $p < 0.01$), but not GFP controls ($t_{11} = 1.15$, $p = 0.53$; Fig. 4F). For the CS^{remote} retrieval, the ANOVA revealed a trend toward significant main effect of laser ($F_{1, 12} = 3.81$, $p = 0.06$) and a virus × laser interaction ($F_{1, 12} = 11.85$, $p < 0.01$), but not a main effect of virus ($F_{1, 12} = 1.39$, $p = 0.25$). Post hoc analysis showed that laser illumination only disrupted fear retrieval in the Jaws rats ($t_{10} = 3.73$, $p < 0.01$) but not control GFP rats ($t_{11} = 1.08$, $p = 0.59$; Fig. 4I). These data reveal that BLA optoinhibition similarly disrupts the retrieval of recent and remote fear memories.

A previous report showed that optogenetic inhibition of BLA disrupted the retrieval of a 6-h but not 7-day old auditory fear memory. In this study, retrieval testing at the remote

timepoint was conducted after retrieval testing at the recent timepoint a procedure that might have influenced the remote test. To determine whether this procedure would moderate the effects of BLA optoinhibition, we next examined the role of BLA in fear retrieval procedure in which a single CS was tested sequentially at recent and remote time points. After waiting three weeks for optimal viral expression, all rats underwent conditioning (context A; day 1), recent (context B; day 2 & 3), and remote (context C; day 7 & 8) retrieval tests. Consistent with our findings in within-subjects fear conditioning procedure, constant laser illumination disrupted both recent and remote cued fear retrieval in rats expressing Jaws in the BLA relative to GFP control rats (Sfig. 5). Taken together, these data reveal that constant optoinhibition of the BLA reduced retrieval of both recent and remote fear memories.

CS-specific optogenetic inhibition of the BLA impairs retrieval of recent and remote fear memories

In the previous experiment, we demonstrated that constant inhibition of BLA principal neurons reduces conditioned freezing behavior to both recent and remote auditory CSs. However, others have shown that constant optogenetic inhibition differentially affects memory retrieval compared to acute inhibition (36). Moreover, as experiment 2 and 3 demonstrate that CS-evoked firing is the neuronal correlate signaling fear memory in the BLA, inhibition of BLA activity during only the CS should be sufficient to suppress fear retrieval. To assess this possibility, we used the same within-subjects experimental design as experiment 4 (Fig. 5A), except inhibited BLA principal neurons only during the CS period (laser illumination started 10 s before each CS onset and turned off at each CS offset; Fig. 5B and Sfig. 4). The Jaws and GFP rats acquired similar levels of CS^{recent} fear [significant main effect of trial ($F_{5, 75} = 5.41$, $p = 0.0003$); no significant main effect of virus ($F_{1, 15} = 2.41$, $p = 0.14$) or interaction of virus × trial ($F_{5, 75} = 0.60$, $p = 0.70$); Fig. 5C] and CS^{remote} fear [significant main effect of trial ($F_{5, 75} = 17.28$, $p < 0.0001$); no significant main effect of virus ($F_{1, 15} = 1.70$, $p = 0.21$) or interaction of virus × trial ($F_{5, 75} = 0.51$, $p = 0.77$); Fig. 5F].

As shown in Fig. 5D and 5G, CS^{recent} and CS^{remote}-elicited freezing in Jaws rats was attenuated by BLA inhibition during CS presentations in the retrieval test. For average freezing during the retrieval tests, three-way repeated-measures ANOVA showed a significant main effect of laser ($F_{1, 15} = 8.11$, $p = 0.01$), but no main effects of virus ($F_{1, 15} = 0.02$, $p = 0.88$) or memory age ($F_{1, 15} = 2.24$, $p = 0.16$); there was also a virus × laser interaction ($F_{1, 15} = 7.88$, $p = 0.01$), but no interaction of virus × laser × memory age ($F_{1, 15} = 0.35$, $p = 0.56$). An additional two-way RM ANOVA analysis of CS^{recent}-induced freezing data revealed a significant main effect of laser ($F_{1, 15} = 10.75$, $p < 0.01$) and an interaction of virus × laser ($F_{1, 15} = 7.36$, $p = 0.02$), but no significant main effect of virus ($F_{1, 15} = 0.91$, $p = 0.36$). Post hoc analysis indicated that CS-specific inhibition of BLA attenuated freezing in the Jaws rats ($t_8 = 4.47$, $p < 0.01$) but not control GFP rats ($t_7 = 0.39$, $p = 0.91$; Fig. 5E). For CS^{remote}-induced freezing, a two-way RM ANOVA revealed a trend toward to significant main effect of laser ($F_{1, 15} = 3.48$, $p = 0.08$) and an interaction of virus × laser ($F_{1, 15} = 4.58$, $p = 0.049$), but not significant main effect of virus ($F_{1, 15} = 0.31$, $p = 0.59$). Post hoc analysis showed that laser illumination decreased freezing in the Jaws rats ($t_8 = 2.92$, $p = 0.02$) but not GFP control rats ($t_7 = 0.19$, $p = 0.98$; Fig. 5H). These data demonstrate that selectively inhibiting BLA principal neurons during CS presentation is sufficient to disrupt the retrieval of both recent and remote cued fear memories.

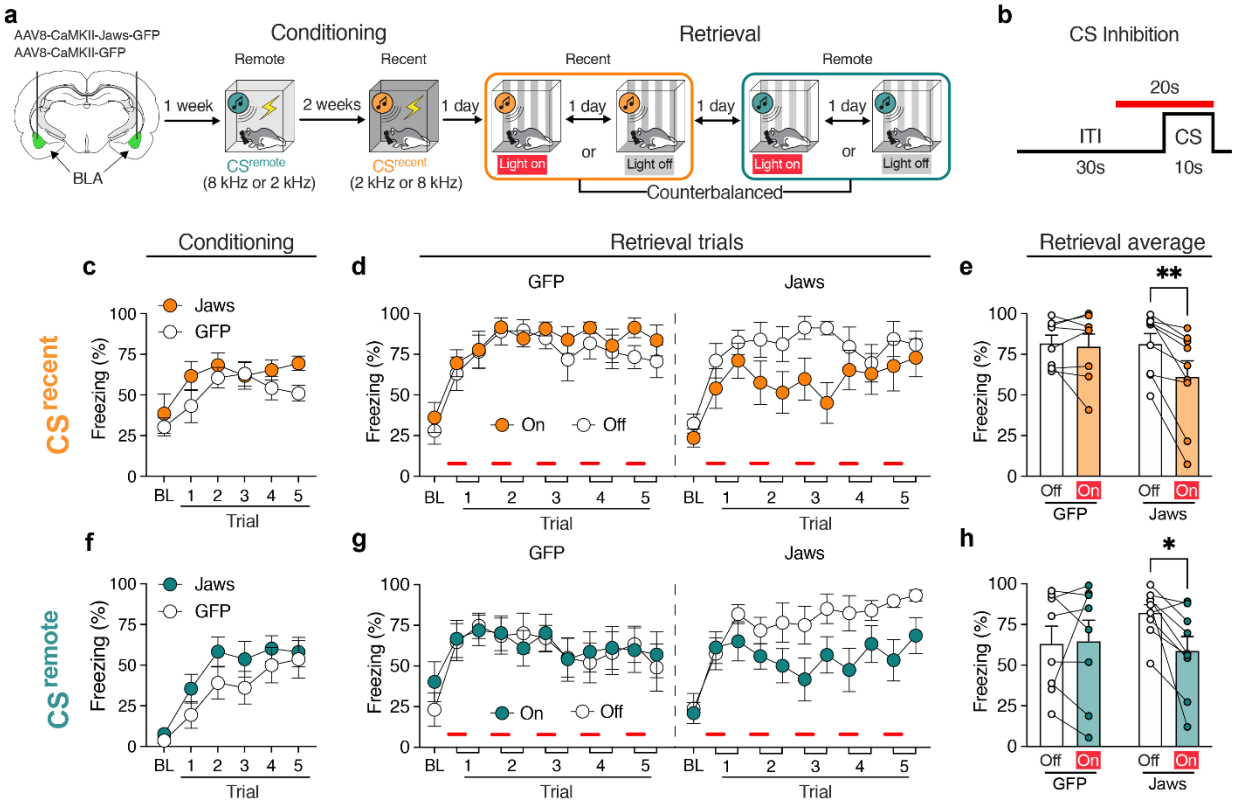

Figure 5. CS-specific optoinhibition of the BLA attenuates freezing to both recent and remote cues in the within-subjects fear conditioning procedure. a, Experimental design. **b,** Laser illumination was started 10 s before each tone onset and turned off at each tone offset to precise inhibit BLA activity during CS presentations. **c,** Recent fear conditioning. **d,** Precise optoinhibition of the BLA decreased CS^{recent} fear retrieval. **e,** Average freezing of CS^{recent} fear retrieval. **f,** Remote fear conditioning. **g,** Precise optoinhibition of BLA attenuated the retrieval of CS^{remote} memory. **h,** Average freezing of CS^{remote} fear retrieval. Data are shown as mean ± SEM. $*p < 0.05$, n = 7-8 per group. BL, baseline; BLA, basolateral amygdala; CS, conditioned stimulus; ITI, inter-trial interval.

Besides principal neurons, the BLA contains interneurons that comprise about 10~20% of the total population of neurons. Although previous studies revealed a crucial role of BLA interneurons in regulating fear conditioning (26, 37), it remains unknown whether they contribute differentially to recent and remote fear retrieval. To explore this, we expressed Channelrhodopsin-2 (ChR2) in BLA interneurons. This was accomplished by injecting a virus encoding ChR2 under the mDlx enhancer that restricted the expression of ChR2 in interneurons into the BLA (AAV8-mDlx-ChR2-mCherry) (38). Four weeks after viral injection, rats underwent conditioned (day 1), recent retrieval (days 2&3), and remote retrieval (days 7&8). We observed that laser illumination (450 nm) reduced retrieval of both recent and remote cued fear in rats expressing ChR2 but not its control virus in BLA interneurons (Sfig. 6). In addition, optostimulation of BLA PV neurons also reduced remote cued fear retrieval (Sfig. 7). Taken together, these data further confirm that BLA participates in the retrieval of both recent and remote cued fear.

Discussion

Understanding how and where memories are stored in the brain has been a long-standing problem in neuroscience. Although early work suggested that BLA was a critical site for the long-term storage of fear memories, more recent work challenged this notion. Moreover, it is unknown if similar coding schemes are used to signal recent and remote memory. Here we investigated if and how recent and remote memories are encoded in the BLA of male and female rats. We demonstrate for the first time that the majority of CS-responsive BLA neurons encoded both recent and remote memories. BLA population activity, assessed with either c-Fos immunohistochemistry or calcium imaging, also revealed similar engagement of BLA neurons by recent and remote fear memory retrieval. Moreover, optogenetic inhibition of BLA principal neurons or excitation of BLA inhibitory interneurons similarly attenuated the retrieval of both recent and remote fear memories, and was specific to the CS. These results indicate that BLA neurons similarly encode recent and remote memory, and that their activity is critical to memory retrieval at both recent and remote time points.

Previous chemical lesion studies showed that the BLA is involved in the persistence of long-term fear memory. Maren and colleagues (1996) showed that NMDA lesions of the BLA 1, 14, or 28 days after fear conditioning prevented cued fear retrieval (18). In a dual-conditioning procedure, Gale and colleagues (2004) showed that chemical lesion of BLA disrupted both recent and remote fear retrieval, where 16 months elapsed between the recent and remote conditioning procedures (28). This suggests that the BLA is indeed important for the retrieval of life-long remote cued fear memory (28). Recent recording studies with semi-naturalistic predators also suggest that BLA neurons have a general role in threat detection that spans many sensory modalities and time scales (39, 40). Consistent with this, the present study showed that optoinhibition of the BLA disrupted both recent and remote memory retrieval. We found that either continuous BLA inhibition (during both CSs and ITIs) or phasic inhibition during the CS alone produced robust impairments in conditioned freezing. Interestingly, these deficits were similar for recent and remote CSs, although constant BLA inhibition did produce greater inhibition of freezing to the recent CS during the earliest test trials (trials 1 and 2) of the five-trial test. Nonetheless, freezing during the later test trials (trials 3-5) was uniformly disrupted by optoinhibition of the BLA. This suggests that the BLA may have a more critical role in the maintenance of conditioned fear once retrieved.

These findings contrast with a recent study that found the fear memory retrieval becomes less dependent on the BLA with the passage of time (7). In this case, optogenetic inhibition of BLA principal neurons disrupted conditioned freezing to an auditory CS when delivered 6 hours, but not 7 days, after conditioning (7). Moreover, conditioning-related increases in c-Fos expression in the BLA was only observed after recent (6-24 hours), but not remote (7 days), retrieval of the fear memory. Based on this work, the authors concluded that the BLA does not mediate the retrieval of remote fear memories. Rather, they argued that the BLA may have a time-limited role in fear expression and that other neural circuits, including the medial prefrontal cortex (mPFC) and paraventricular nucleus of the thalamus (PVT), have an enduring role in this process. It is important to note, however, that these experiments were conducted using conditioned suppression procedures in food-restricted rats. Instrumental conditioning procedure may limit the involvement of the BLA (41) and engage mPFC-PVT circuits to arbitrate motivational conflicts between approach and avoidance (42). It is also possible that the differential involvement of the BLA in recent and remote retrieval in this study was due to the test procedures, which were conducted with optoinhibition of the BLA during only two CSs. We

found that BLA inhibition produced more robust deficits in conditioned freezing after at least two test trials.

The role for the BLA in encoding recent and remote memory was reflected by significant increases in spike firing to both the recent and remote CS among the majority of BLA neurons. We have previously established that conditioning-induced increases in BLA spike firing represent the associative properties of the CS, rather than the behavioral state of fear the CS engenders (22). Nonetheless, the similar engagement of BLA neurons by recent and remote CSs might suggest that, rather than convergently encoding of these memories, BLA neurons show generalized responding to the two auditory CSs. Indeed, generalization between auditory CSs increases with time (43). Specifically, rats conditioned to a 5 kHz tone generalized to 3, 8, and 12 kHz tones (but not to 2 kHz tone) during a remote retrieval test (30 days after conditioning) (43). However, in the present study, we used two tones frequencies (2 kHz and 8 kHz) that were discriminable at a remote time point (43). This is supported by our fiber photometry data in which rats conditioned to only one CS (either CS^{recent} or CS^{remote}) showed behavioral and neuronal responses to the conditioned CS, but not the non-conditioned CS. Moreover, we observed small populations of BLA neurons that showed selectivity for recent and remote CSs. Nevertheless, in the within-subjects fear conditioning procedure, both CSs were associated with the same aversive stimulus, i.e., footshock (28). Rats showed higher freezing in the second (recent conditioning) compared to the first (remote) conditioning, which suggests that non-associative shock sensitization may have contributed to the freezing observed during the second conditioning session.

The propensity for remote conditioning to generalize to recent conditioning in our experiments may have increased the excitability of BLA neurons (22) thereby biasing these cells for "coallocation" in the neural network representing the remote conditioning experience (44, 45). Consistent with this, Rashid and colleagues (2016) have shown that fear memories become coallocated among neurons in the lateral nucleus of the amygdala (LA) when conditioning experiences occurred closely in time (within 6 hours), but not when separated by 24 hours, under conditions in which there is little generalization of freezing between the two experiences (44). However, artificial activation of the LA during the second fear conditioning experience did result in coallocation, even over a 24-hour interval separating the two conditioning experiences (7). This suggests that memory coallocation results from increases in neuronal excitability in the LA that bias particular fear neurons during fear conditioning. Although recent and remote fear conditioning were separated by two weeks in our procedure, generalization of fear from the remote conditioning episode to the recent conditioning experience may have forced coallocation of these two memories. Consistent with this, we observed that individual BLA neurons encoded both recent and remote fear memories. Interestingly, convergent neural coding of recent and remote memory among individual BLA neurons suggests that animals might have difficulty discriminating recent from remote fear memories. Although we are not aware of any studies that have directly examined this question, it is consistent with the subjective reports of individuals with PTSD whose memories of past traumas is re-experienced vividly as if the trauma had just occurred.

Although our data indicate that the BLA is required for both recent and remote cued fear memory, considerable work indicates that there are unique neural circuits dedicated to remote memory retrieval. For example, lesions of the secondary sensory cortices disrupted remote fear memories, whereas recent memories were left intact (10). Further work has shown that the secondary auditory cortex (Te2) drives the BLA activity during remote cued fear retrieval (46).

Moreover, midline thalamic projections from the nucleus reuniens to the BLA have a unique role in extinction of remote fear memories (47). Clearly, future studies are needed to understand shifts in the neural circuits representing fear memory over time. In conclusion, the present study indicates that the BLA neurons encode both recent and remote fear memories, and that optogenetic inhibition of these networks impairs fear memory retrieval independent of the age of those memories. This suggests that at least some aspects of emotional experience may be permanently stored in the BLA.

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Author contributions
JL, MT, and SM designed electrophysiological experiments; JL and SM designed the other experiments. MT and JL performed electrophysiological experiments and analyzed the data. LD performed c-Fos immunization and counting. JL performed all other experiments and analyzed the data. JL, MT, and SM wrote the manuscript.

Disclosures
The authors declare no competing interests.

Data availability
The data from these experiments are available from the corresponding author upon request.

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Supplemental information

Convergent coding of recent and remote fear memory in the basolateral amygdala

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Supplemental materials and methods

Supplemental experiments

Supplemental Exp. 1. Electrophysiological recording in anesthetized rats (related to Sfig. 3).
Rats were bilaterally injected AAV9-CaMKII-Jaws-GFP or AAV9-CaMKII-GFP into the BLA [(AP: -2.85 mm, ML: -4.85 mm, DV: -8.7 mm (relative to bregma surface)] three weeks before recording. On the day of electrophysiological recording, rats were anesthetized with isoflurane (5% for induction, 1-2% for maintenance) and placed into a stereotaxic frame (Kopf Instruments). The hair on the scalp was shaved, povidine-iodine was applied to the skin, and a small incision was made in the scalp to expose the top of the skull. The skull was leveled by placing bregma and lambda in the same horizontal plane. A small hole was drilled in the skull to affix one jeweler's screw that were connected to grounding channel of the optrode. The optrode (Microprobes) was slowly implanted into the BLA [(AP: -2.85 mm, ML: -4.85 mm, DV: -8.2 to -8.7 mm (relative to bregma surface)]. After the optrode was lowered to the target and single-units were isolated, the optrode was left in place for XX minutes to allow the recordings to stabilize. Once stabilized, laser light (635 nm; 6-7 mW at the tip of the optrode; Dragon Lasers, Changchun, China) was applied to the optrode (20-s duty cycle; 10 sec on and 10 sec off).

Supplemental Exp. 2. Effects of constant optoinhibition of BLA on fear retrieval in a standard fear conditioning procedure (Sfig. 5).
AAV9-CaMKII-Jaws-GFP or AAV9-CaMKII-GFP was bilaterally microinjected into the BLA [(AP: -2.85 mm, ML: -4.85 mm, DV: -8.7 mm (relative to bregma surface)]. Optical fibers (200 μm core) were implanted to 0.3 μm above the viral injection sites. Three weeks after viral injection, rats underwent a standard auditory fear conditioning procedure. On the first day (day 1), rats underwent fear conditioning that consisted of a 3-min stimulus-free baseline (BL), five tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) pairings with 60-s intertrial intervals (ITIs), and additional 60 s post shock period. Twenty-four hours after fear conditioning, the rats underwent two retrieval tests separated by 24 hours (e.g., days 2 and 3). These tests were conducted in the optogenetic chamber to measure recent cued memory (context B). One week later (days 8 & 9), rats underwent fear retrieval in the optogenetic chamber to measure remote cued fear (modified to context C). In each memory retrieval, rats received a 3-min BL followed by 5 tone-alone presentations (30 s ITIs), with counterbalanced laser on and off in the 2-day testing sessions (one day with laser on and the other day with laser off; half rats with laser on first and the others with laser off first). Laser was illuminated 10 s before the first tone onset and persisted to the end of testing session. Freezing was recorded via Threshold Activity software automatically (Med-Associates).

Supplemental Exp. 3. Effects of optostimulation of BLA interneurons on fear retrieval (related to Sfig. 6).
Rats were bilaterally injected AAV8-mDlx-ChR2-mCherry or AAV8-mDlx-tdTomato (n = 4/group) into the BLA [(AP: -2.85 mm, ML: -4.85 mm, DV: -8.7 mm (relative to bregma surface)]. Optical fibers (200 μm core) were implanted to 0.3 μm above the viral injection sites. Three weeks after viral injection, rats then underwent fear conditioning (day 1), recent retrieval (days 2&3), and remote retrieval (days 15&16). Laser was illuminated 10 s before each CS onset and turned off at the offset of each CS presentation. Freezing was recorded via Threshold Activity software automatically (Med-Associates).

Supplemental Exp. 4. Effects of optostimulation of BLA PV neurons on fear retrieval (related to Sfig. 7).

Breeding pairs of PV-Cre line [LE-Tg (Pvalb-iCre)2Ottc] was purchased from the Rat Resource and Research Center (RRRC#: 773). Hemizygous PV-Cre rats were bred with wildtype Long-Evans Blue Spruce rats. Male and female hemizygous PV-Cre rats were used in this study. PV-Cre rats (n = 9/group) were bilaterally injected AAV8-DIO-ChR2-mCherry (5.6×10^{12} GC/mL) or AAV8-DIO-tdTomato (4.8×10^{12} GC/mL) into the BLA [(AP: -2.85 mm, ML: -4.85 mm, DV: -8.7 mm (relative to bregma surface)]. Optical fibers (200 μm core) were implanted to 0.3 μm above the viral injection sites. Three weeks after viral injection, rats then underwent fear conditioning (day 1), recent retrieval (days 2&3), and remote retrieval (days 8&9). No recent fear retrieval was conducted in this experiment. Laser was illuminated 10 s before each CS onset and turned off at the offset of each CS presentation. Freezing was recorded via Threshold Activity software automatically (Med-Associates).

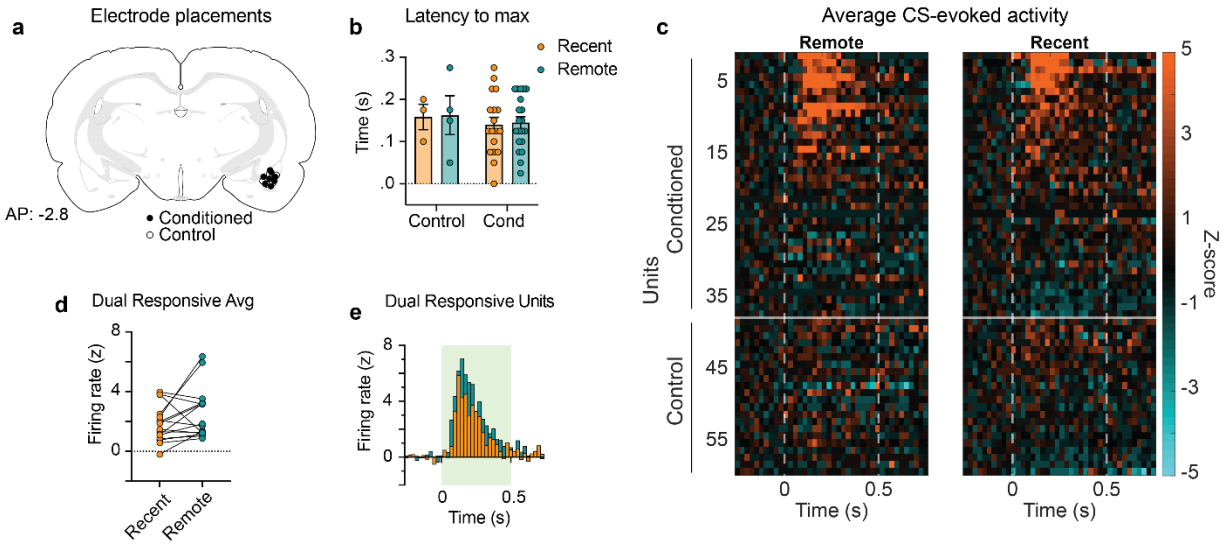
Histology

Upon completion of the experiment, rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/mL, 0.5 mL, i.p.) and perfused transcardially with physiological saline followed by 10% formalin. Brains were extracted and stored about 16-18 h (at 4° C) in 10 % formalin after which they were transferred to a 30 % sucrose solution for a minimum of 5 days. Brains were then sectioned using a cryostat (Leica Microsystems) at -20° C. Viral expression and fiber tips was verified with a Zeiss microscope (Axio Imager). One Jaws rat in Supplemental Exp. 2 as well as four ChR2 rats and one tdTomato control rat in Supplemental Exp. 4 were excluded due to unilateral or no expression of virus in the BLA.

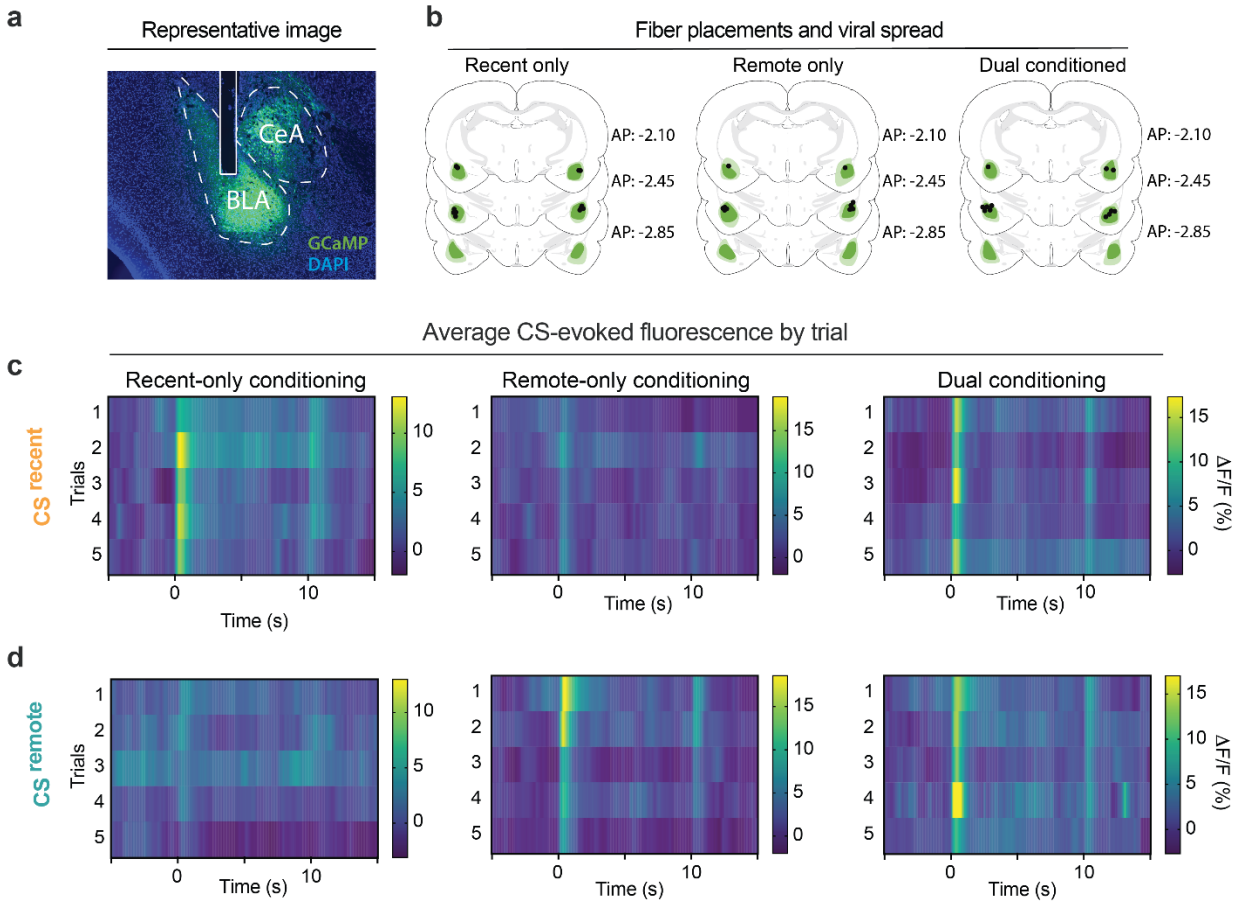
Statistics

All data were represented as means ± SEM. Data were analyzed using Prism Graphpad 9.0. For conditioning, two-way repeated-measures (RM) analysis of variance (ANOVA) with trial as the within-subjects factor and virus (or group) as the between-subjects factor was conducted. For the retrieval (average freezing), two-way RM ANOVA with laser as within-subjects factor and virus as between-subjects factor. Significant two-way ANOVA was followed by post hoc Bonferroni's multiple comparisons test. Group sizes were determined based on prior work and what is common in the field. $P < 0.05$ was considered statistically significant.

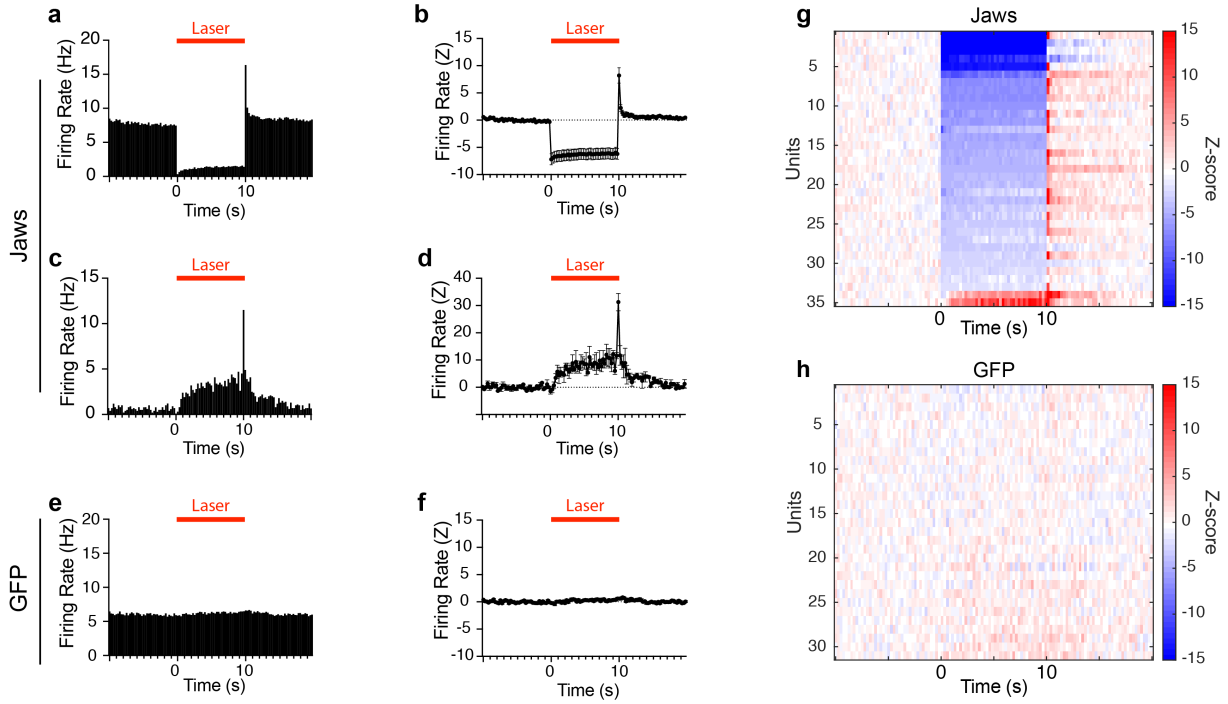
Supplemental figures



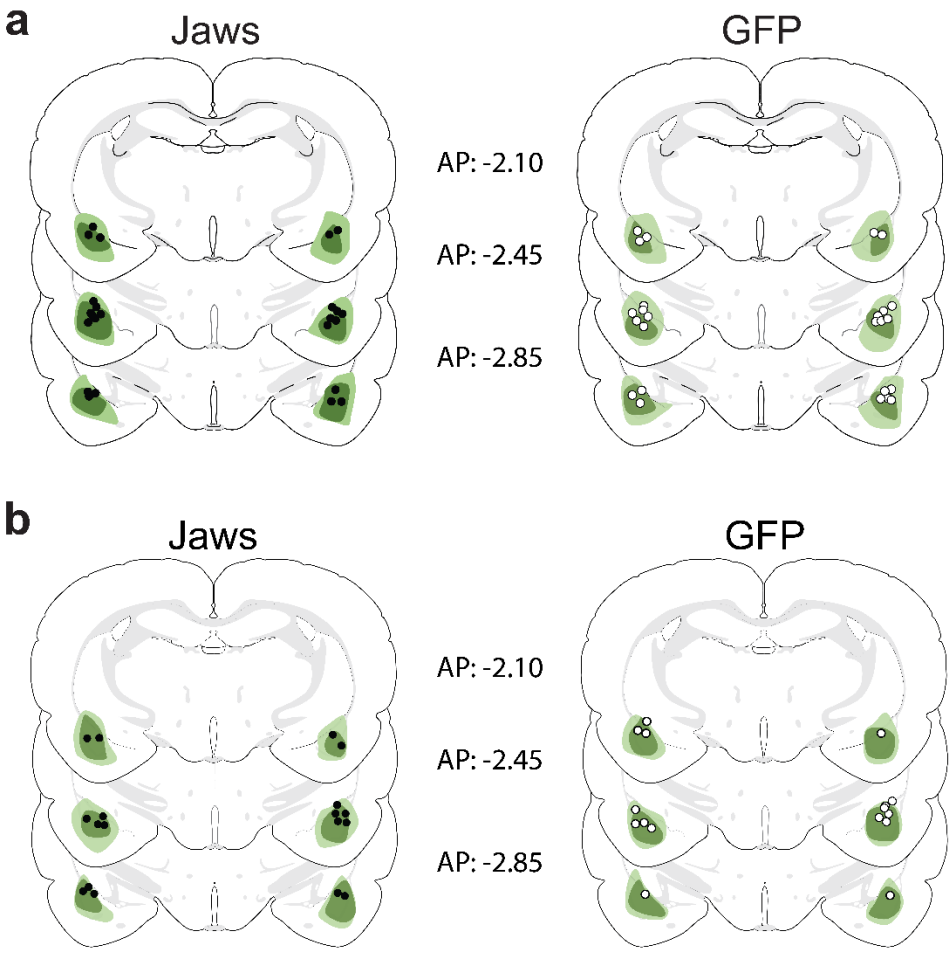
Sfig. 1. Supplemental information of electrophysiological recording data, related to Fig. 2. **a,** Schematic illustration showing electrode placements in the BLA. **b,** The latency of peak responding was similar to recent (139 ± 16 ms) and remote (142 ± 14 ms) CSs. **c,** Heatmaps of firing rate of BLA neurons. **d,** Among neurons (n = 16) that showed excitatory responses to both the recent and remote CSs, firing to the recent and remote CSs was similar (paired t-test: t_{15} = 1.70, p = 0.1). **e,** Peri-event histograms of neurons that showed excitatory responses to both the recent and remote CSs. Data are shown as mean ± SEM. *p < 0.05, n = 6-8 per group. BL, baseline.



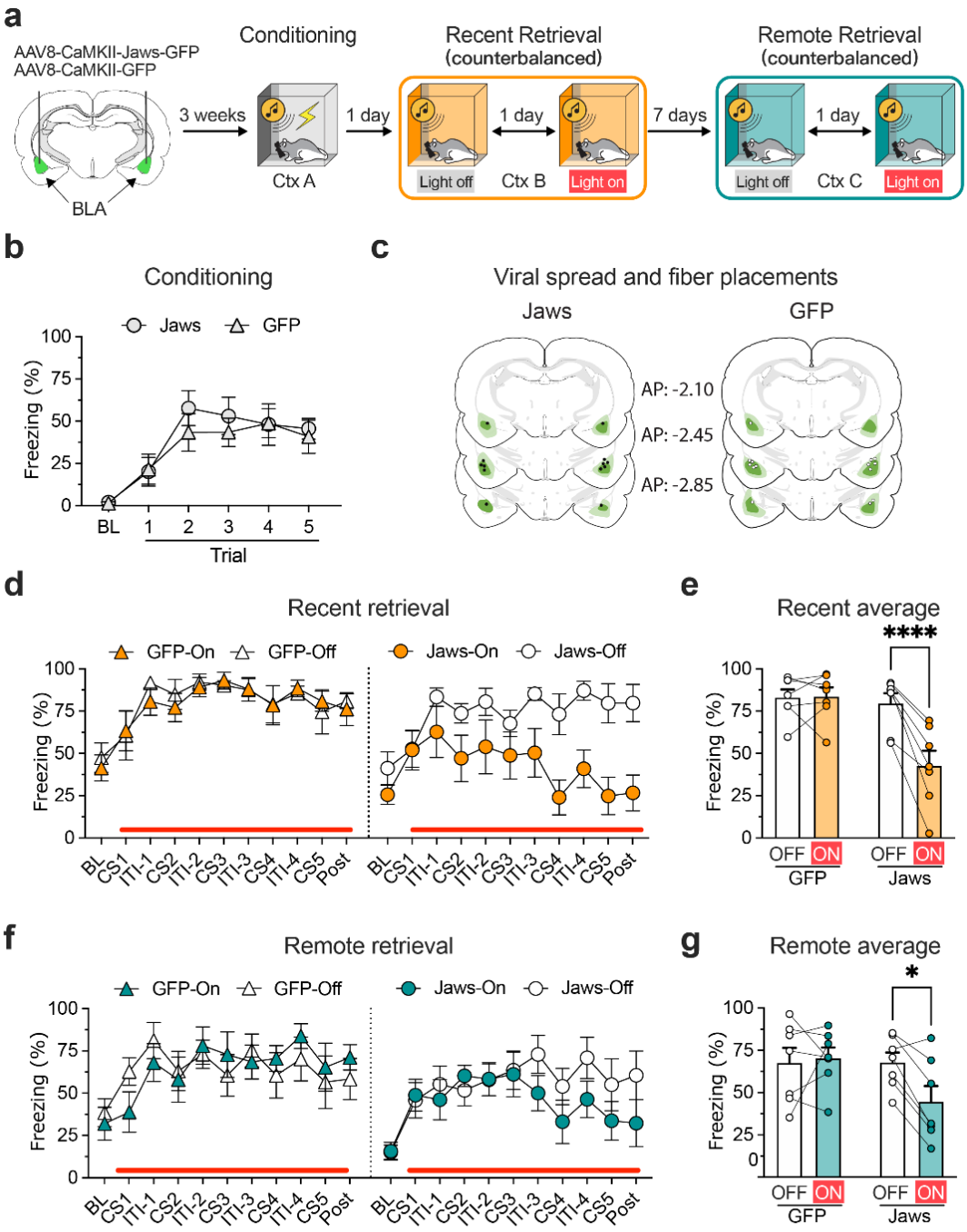
Sfig. 2. CS-evoked fluorescence is observed across retrieval trials, related to Fig. 3. a, A representative micrograph showing viral spread and optic fiber tip placement within the BLA. **b**, Average viral spread and fiber tip placements for all animals. **c**, heatmaps displaying average CS^{recent}-evoked fluorescence by trial for Recent-only, Remote-only, and Dual conditioned groups. **d**, heatmaps displaying average CS^{remote}-evoked fluorescence by trial for Recent-only, Remote-only, and Dual conditioned groups.



Sfig. 3. Optoinhibition of spontaneous single-unit firing in BLA neurons expressing Jaws (related to Fig. 4). a-d, Firing rates of neurons in rats expressing Jaws in the BLA with red laser illumination (10 s; 635 nm laser; 6-7 mw at fiber tips). Representative firing rate and z-scores of BLA neurons showed inhibitory response (**a** and **b**) and excitatory response (**c** and **d**) in Jaws rats. **e-f,** Laser illumination did not change firing rate of BLA neurons in control rats expressing GFP in the BLA. **g-h,** Heatmaps of recorded BLA neurons in Jaws rats (35 cells; **g**) and GFP rats (32 cells; **h**). Data were collected during 10-sec intervals before, during, and after laser illumination.

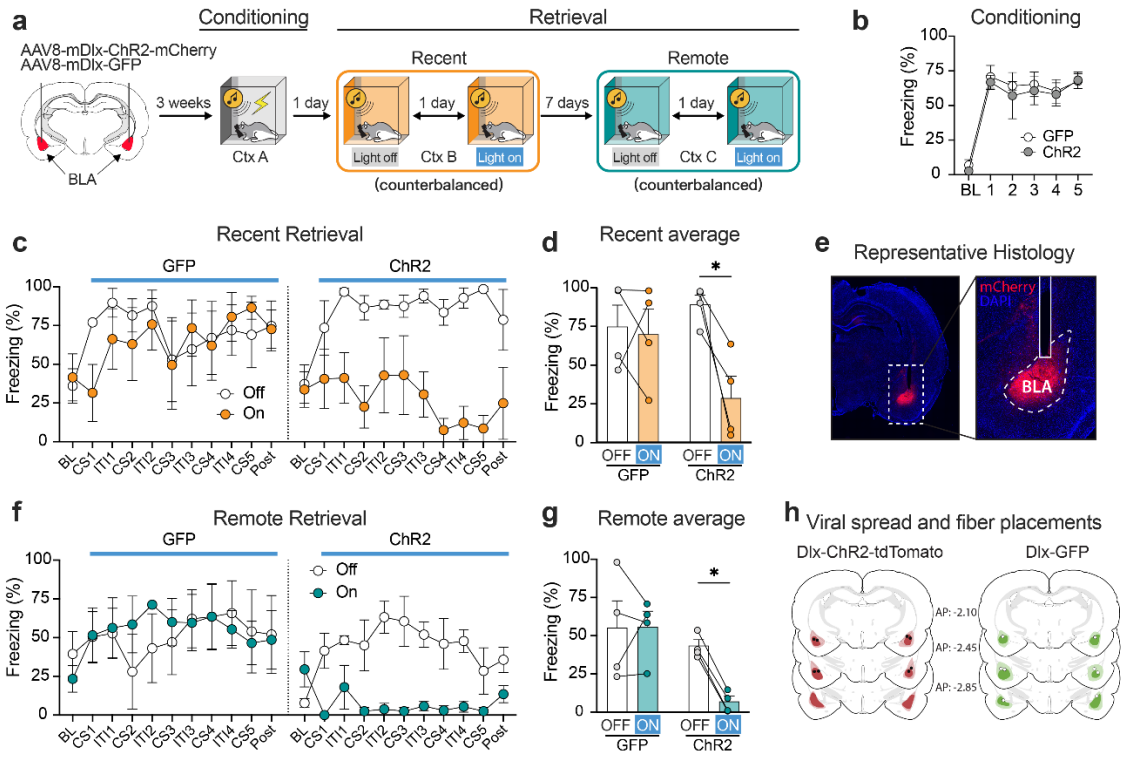


Sfig 4. Viral spread and optic fiber placements for Jaws inhibition experiments, related to Figures 4 (a) and 5 (b).

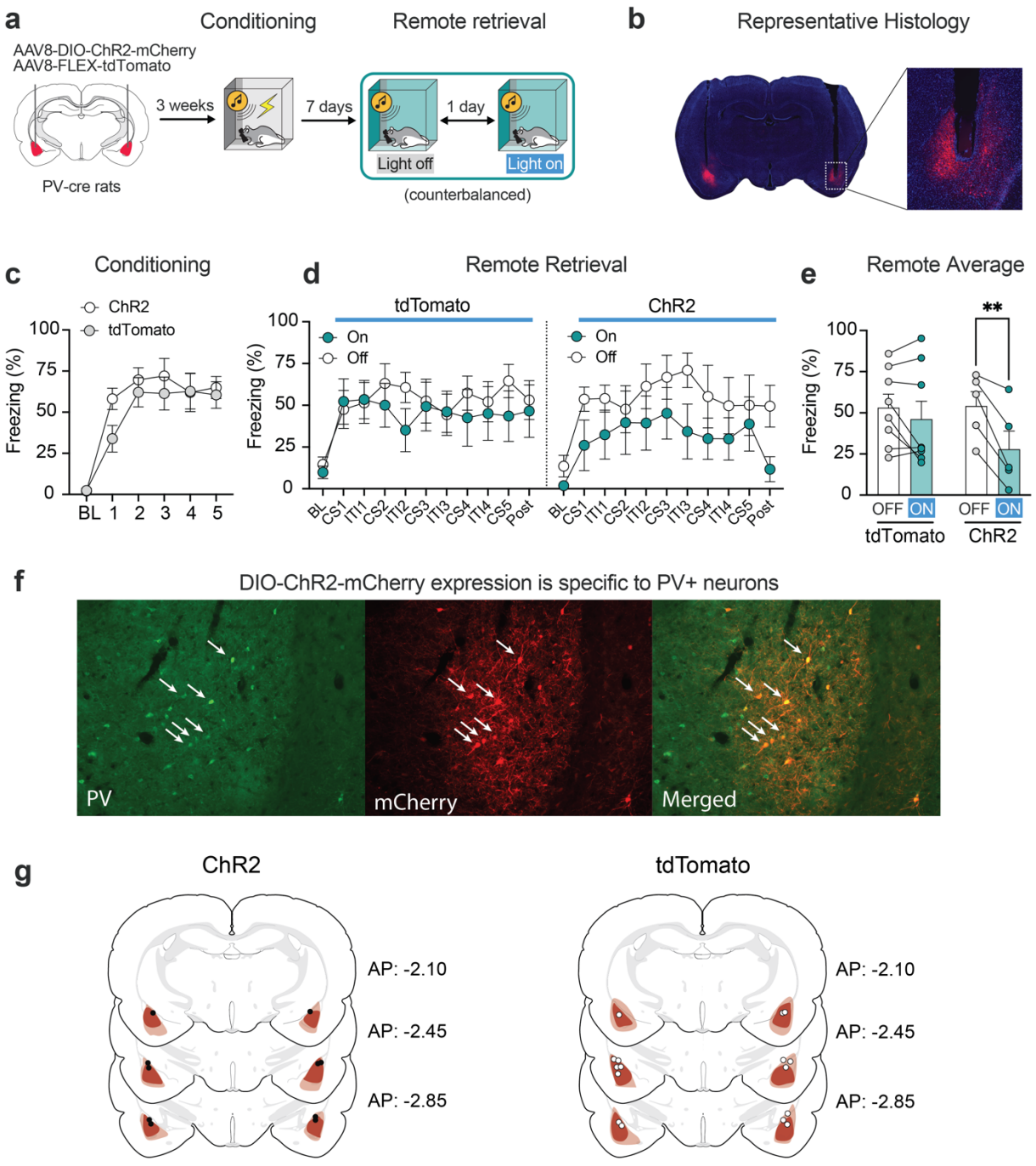


Sfig. 5. Optoinhibition of BLA principal neurons reduces the retrieval of both recent and remote cued fear memories in a repeated fear testing procedure. a, Schematic illustration of the experimental design. Three weeks after viral injections, rats underwent auditory fear conditioning in context A (day 1). Animals were tested for recent (days 2 & 3) and remote (days 8 & 9) memory with laser on and off in two-day testing procedures. Laser illumination (constant 695 nm light; 6-7 mw at fiber tips) started 10 s before the first tone onset and persisted to the end of testing session. **b,** Jaws and GFP control rats conditioned similarly with low freezing during the three-minute baseline period and increased freezing across the conditioning trials [two-way

RM ANOVA: a significant main effect of trial ($F_{5, 60} = 13.46$, $p < 0.01$), but no main effect of virus ($F_{1, 12} = 0.34$, $p = 0.57$) or interaction of trial × virus ($F_{5, 60} = 0.35$, $p = 0.88$)]. **c,** Sterotaxic atlas templates showing maximal and minimal extent of Jaws expression in the BLA. **d,** Optoinhibition of BLA principal neurons reduced freezing during recent fear retrieval (days 2&3) across the 5-trial retrieval test. **e,** CS-induced freezing across the testing session was averaged and analyzed by ANOVA. For the recent retrieval test, the ANOVA revealed significant main effects of laser ($F_{1, 12} = 20.07$, $p < 0.01$) and virus ($F_{1, 12} = 7.47$, $p = 0.02$), and an interaction of virus × laser ($F_{1, 12} = 21.49$, $p < 0.01$). Post hoc analysis showed that laser illumination reduced freezing in the Jaws rats ($t_6 = 6.45$, $p < 0.01$) but not in control GFP rats ($t_6 = 0.11$, $p > 0.99$), suggesting that optoinhibition of BLA impaired recent cued fear memory retrieval. **f,** Optoinhibition of BLA principal neurons reduced freezing during remote fear retrieval testing (days 8&9). **g,** For the average freezing across the remote retrieval test, an ANOVA revealed a trend toward significant main effect of laser ($F_{1, 12} = 4.12$, $p = 0.06$) and an interaction of an interaction of virus × laser ($F_{1, 12} = 6.83$, $p = 0.02$), but not virus ($F_{1, 12} = 1.70$, $p = 0.22$). Post hoc analysis showed that laser illumination only disrupted fear retrieval in the Jaws rats ($t_6 = 3.30$, $p = 0.01$) but not control GFP rats ($t_6 = 0.40$, $p > 0.99$). Data are shown as mean ± SEM. *$p < 0.05$, n = 7 per group. BLA, basolateral amygdala; Cxt, context; BL, baseline; ITI, intertrial interval.



Sfig 6. Optogenetic stimulation of BLA interneurons reduced both recent and remote cued fear. a, Schematic graph of experimental design. AAV9-mDlx-ChR2-mCherry-Fishell-3 or its control virus AAV9-mDlx-GFP-Fishell-1 was bilaterally microinjected into the BLA and fibers were implanted into the BLA immediately after viral injection. After three-week viral expression period, rats underwent fear conditioning (day 1), recent retrieval (days 2&3), and remote retrieval (day 15&16). **b,** ChR2 and GFP control rats showed similar levels of freezing during fear conditioning [two-way RM ANOVA: a significant main effect of trial ($F_{5, 30} = 29.71$, $p < 0.0001$), but no significant main effect of virus ($F_{1, 6} = 0.20$, $p = 0.67$) or interaction of virus × trial ($F_{5, 30} = 0.09$, $p = 0.99$)]. **c & d,** Optogenetic stimulation of BLA interneurons reduced freezing during recent fear retrieval (days 2&3; **c**) and remote fear retrieval (days 15&16; **d**). **e,** Micrographs showing viral expression and placements of fiber tips. **f & g,** Analyzing the average freezing data revealed that optogenetic stimulation of BLA interneurons reduced freezing in both recent retrieval [two-way RM ANOVA: a significant main effect of laser ($F_{1, 6} = 7.52$, $p = 0.03$), no significant main effect of virus ($F_{1, 6} = 0.91$, $p = 0.38$), and a trend toward an interaction of virus × trial ($F_{1, 6} = 5.41$, $p = 0.06$); Post hoc analysis, off vs on: ChR2, $t_3 = 3.58$, $p = 0.02$; GFP, $t_4 = 0.29$, $p > 0.99$; **d**], and remote retrieval [two-way RM ANOVA: a significant main effect of laser ($F_{1, 6} = 7.60$, $p = 0.03$), a trend toward a significant main effect of virus ($F_{1, 6} = 5.15$, $p = 0.06$), and an interaction of virus × trial ($F_{1, 6} = 8.07$, $p = 0.03$); Post hoc analysis, off vs on: ChR2, $t_3 = 3.96$, $p = 0.01$; GFP, $t_3 = 0.06$, $p > 0.99$; **g**]. **h**, Illustrations shows average viral spread and fiber tip placements for all animals. Data are shown as mean ± SEM. *$p < 0.05$, n = 4 per group. BLA, basolateral amygdala; Cxt, context; BL, baseline; ITI, intertrial interval.



Sfig 7. Optogenetic stimulation of BLA PV neurons reduces both recent and remote cued fear. a, Schematic graph of experimental design. AAV8-FLEx-hChR2-mCherry or its control virus AAV8-DIO-tdTomato was bilaterally microinjected into the BLA of PV-cre rats. Fibers were implanted into the BLA immediately after viral injection. After three-week viral expression period, rats underwent fear conditioning (day 1) and remote retrieval (day 8&9). **b,** Micrographs showing ChR2-mCherry expression and fiber placement. **c,** ChR2 and GFP control rats showed similar levels of freezing during fear conditioning [two-way RM ANOVA: a significant main effect of trial ($F_{5, 55} = 33.32$, $p < 0.0001$), but no significant main effect of virus ($F_{1, 11} = 2.33$, $p =$

0.16) or interaction of virus × trial ($F_{5, 55} = 1.28$, $p = 0.28$)]. **d,** Optogenetic stimulation of BLA PV neurons reduced freezing during remote fear retrieval. **e,** Analysis of average freezing data showed that optogenetic stimulation of BLA PV neurons disrupted remote cued fear retrieval [two-way RM ANOVA: a significant main effect of laser ($F_{1, 11} = 16.57$, $p = 0.002$), no significant main effect of virus ($F_{1, 11} = 0.39$, $p = 0.55$), but an interaction of virus × trial ($F_{1, 11} = 5.64$, $p = 0.04$); Post hoc analysis, laser off vs. on: ChR2, $t_4 = 4.11$, $p = 0.004$; GFP, $t_7 = 1.37$, $p = 0.40$]. **f**, Micrographs showing that cre-dependent expression of ChR2-mCherry is specific to PV+ interneurons. **g**, average viral spread and fiber tip placements for all animals. Data are shown as mean ± SEM. *$p < 0.05$, n = 5-8 per group. BLA, basolateral amygdala; Cxt, context; BL, baseline; ITI, intertrial interval.