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Abstract

The ability to predict the occurrence of an aversive outcome based on available cues requires associative learning and plastic changes in the amygdala. When the predictive cue and aversive shock outcome are separated in time as in trace fear conditioning, additional circuitry is needed, including the prelimbic (PL) area of the prefrontal cortex. We have previously shown that neuronal firing in the PL during the trace interval separating the cue and shock is required for trace cued fear memory formation, but whether this mnemonic

signal is conveyed to the amygdala is unknown. Here we show in males that silencing PL activity during the trace interval reduces Arc protein in the basolateral amygdala (BLA) of trace-conditioned rats. Then, using pathway-specific optogenetic and chemogenetic silencing, we show a role for direct PL-BLA communication in trace cued fear learning under weak training conditions, but not standard training. These results suggest that PL input to the BLA may serve to promote cued learning when the cue-shock relationship is most ambiguous and that other trace fear circuitry can compensate for the loss of this connection with additional training. This also highlights the challenge to studying how emotional memories are formed and stored within a distributed network and suggests that the function of individual connections within such a network may best be determined using weak training conditions.

Keywords

Memory formation, Fear conditioning, Optogenetics, Chemogenetics, DREADDs

1. Introduction

Learning to anticipate threat is crucial for mounting adaptive defensive responses. The associative learning required for the successful anticipation of threat based on predictive cues requires synaptic plasticity in the amygdala (Pare et al., 2004, Rodrigues et al., 2004). When the predictive cue terminates several seconds before the aversive reinforcer, as in trace fear conditioning, additional circuitry is recruited into memory formation, including the prelimbic (PL) medial prefrontal cortex (Gilmartin and Helmstetter, 2010, Gilmartin et al., 2014, Guimarais et al., 2011, Runyan and Dash, 2004, Runyan et al., 2004). The PL is thought to maintain a representation of the conditional stimulus (CS) across the empty trace interval that separates this auditory cue from the aversive foot shock unconditional stimulus (UCS). PL neurons show learning-related increases in firing to the CS that persists until shock delivery (Baeg et al., 2001, Gilmartin and McEchron, 2005), and optogenetic silencing of PL activity during the trace interval prevents cued fear learning (Gilmartin, Miyawaki, Helmstetter, & Diba, 2013). Thus, the PL provides a neuronal signal needed for memory formation, but how this signal is incorporated within the broader fear learning network and the amygdala in particular is unknown. The basolateral amygdala (BLA) is necessary for the acquisition and consolidation of trace fear conditioning (Gilmartin et al., 2012, Guimarais et al., 2011, Kochli et al., 2015, Kwapis et al., 2011), which places the BLA as a key memory storage site. Moreover, trace conditioning enhances the excitability of a subset of BLA-projecting intrinsically bursting principal neurons in the prefrontal cortex (Song, Ehlers, & Moyer, 2015). Such bursting cells are important for reliably conveying information and promoting plasticity (Lisman, 1997). Here, we test the hypothesis that direct input to the BLA from the PL during the acquisition of trace fear conditioning is necessary for the formation of trace cued fear memory.

The PL and BLA are reciprocally connected via direct glutamatergic projections (Brinley-Reed et al., 1995, Hoover and Vertes, 2007, McDonald, 1987, McDonald, 1996, McDonald et al., 1996, Vertes, 2004) which allowed us to use pathway-specific optogenetic and chemogenetic tools to inhibit PL input to the BLA. First, we examined the consequence of local optogenetic silencing of PL neurons on plasticity-related Arc immunoreactivity in the BLA and found that silencing reduced the expression of Arc (Guzowski et al., 2000, Link et al., 1995, Lyford et al., 1995). Next, we used pathway-specific optogenetics and chemogenetics to inhibit PL-BLA communication during training. We found that silencing this direct connection impaired the expression of fear in some animals but did not prevent the acquisition of fear. Finally, we made acquisition more difficult by presenting only two pairings with a weaker shock and found that inhibiting PL input to the BLA impaired learning. Together, the findings suggest that the direct connection between the PL and BLA is needed for the rapid acquisition of conditional fear during early training when the cue-shock relationship is most ambiguous.

2. Methods

2.1. Subjects

Adult male Long-Evans rats (325–450 g; Envigo, Indianapolis, IN) were housed individually in an AAALAC-accredited vivarium and maintained on a 14 h:10 h light:dark cycle. Rats received food and water *ad libitum* throughout the experiment. Seventy-eight rats were used in this study. All procedures were in accordance with the National Institutes of Health guidelines and approved by the Marquette University Institutional Animal Care and Use Committee.

2.2. Viral constructs

The adeno-associated viral vector containing the light-sensitive archaerhodopsin from *Halorubrum* strain TP009 (ArchT; rAAV9/CAG-ArchT-GFP; titer 6.6×10^{12} molecules/ml) was prepared by Dr. R. Jude Samulski and the University of North Carolina Vector Core (Chapel Hill, NC) from material provided by Dr. Ed Boyden and the Massachusetts Institute of Technology. The control vector (GFP; AAV9/CAG-GFP; titer 2×10^{12} molecules/ml) and the retrograde CRE-expressing vector (retroAAV2-CAG-CRE; titer $5.3–8.1 \times 10^{12}$ molecules/ml) were also produced and packaged by the University of North Carolina Vector Core. CRE-dependent inhibitory HMDi4-expressing DREADD vector (AAV8-hSyn-DIO-hM4D(Gi)-mCherry; titer $4.4–19 \times 10^{12}$) and the corresponding inactive control virus (AAV8-hSyn-DIO-mCherry; titer: 4.1×10^{12}) were obtained from Addgene (Watertown, MA).

2.3. Surgical procedures

After a minimum of three days of handling, rats underwent intracranial surgery under isoflurane anesthesia (1%–2% isoflurane in 100% O_2). Peri-operative pain was managed with daily administration of carprofen in an edible supplement (5 mg/kg in MediGel® CPF; ClearH2O, Westbrook, ME) the day before, the day of, and the day after surgery (Experiments 1–2) or by sub-cutaneous administration of 5 mg/kg carpofen the day of and the day following surgery (Experiments 3–4). During surgery, the rat was secured in a stereotaxic instrument (David Kopf, Tujunga, CA) and a midline incision was made in the scalp. The fascia was retracted to expose the skull and small craniotomies were drilled above the target sites. All viral injections were made using a stereotax-mounted UMP3 UltraMicroPump injector (World Precision Instruments, Sarasota, FL) and injected at a rate of 0.05 µL/min using a 10 µL syringe and 34 gauge needle. Injectors were left in place for 10 min after injection to allow the virus to diffuse away from the injector.

2.4. Optogenetic virus injection and optic fiber implantation

For experiments 1 and 2, 0.3 µL of ArchT or GFP control virus was injected into the prelimbic (PL) region of the prefrontal cortex at a 14° angle (AP + 3.2, ML ± 1.6, DV −3.7 mm from the skull). For Experiment 1, optic fibers were implanted in the PL bilaterally a 14° angle (AP + 3.2, ML ± 1.6, DV −3.0 mm from the skull). For Experiment 2, optic fibers were implanted in the BLA (AP-2.8, ML ± 5.0, DV-7.8) between 6 and 7 weeks after virus injection in PL. Each optic fiber consisted of a 200 µm diameter, 0.39NA fiber secured in a 2.5 mm ceramic ferrule. Fibers were secured to the skull with skull screws and dental cement. Training occurred 10–14 days (Expt 1) or 8 weeks (Expt 2) weeks following virus injection to allow for appropriate expression in PL cell bodies or terminals, respectively.

2.5. Chemogenetic virus injection

To achieve pathway-specific chemogenetic attenuation of PL to BLA input, a combinatorial dual-virus approach was used (Tervo et al., 2016). A retrograde CRE-expressing vector (retroAAV2-CAG-CRE; titer: 8.1×10^{12} (Expt 3) or 5.3×10^{12} (Expt 4) molecules/ml) was injected into the BLA bilaterally (0.4 µL/side; AP-2.8, ML ± 5.0, DV-8.6). The CRE-dependent inhibitory HMDi4-expressing DREADD vector (AAV8-hSyn-DIO-hM4D(Gi)-mCherry; titer: 4.4×10^{12} (Expt 3) and 1.9×10^{13} (Expt 4)) or inactive control virus (AAV8-hSyn-DIO-mCherry (titer: 4.1×10^{12},

Addgene) was injected bilaterally in the PL (0.4 µL/side at a 14° angle; AP + 3.2, ML ± 1.6, DV −3.7 mm). An additional five rats were injected with *retro*-CRE in the PL and CRE-dependent inactive virus in the BLA. These were used to test the effects of 5 mg/kg CNO on behavior independent of Gi expression. Training occurred at least 4 weeks following surgery to allow for virus expression in BLA-projecting PL cells.

2.6. Fear conditioning and testing

Fear conditioning took place in Med Associates (St. Albans, VT) conditioning chambers (internal dimensions: 30.5 × 24.1 × 29.2 cm), housed in a sound attenuating outer chamber and illuminated with a 7.5-W white incandescent lamp (Expts 3–4) or LED light strip (Expts 1–2) attached to the outer chamber. After a 6-min baseline period, rats received 6 pairings of a 10-s white noise conditional stimulus (CS; 72 dB) and a 1-s footshock unconditional stimulus (UCS) at 1.0 mA (Expts 1–2) or 0.8 mA (Expt 3). For trace conditioning, the CS offset and UCS onset were separated by an empty 20-s trace interval and an intertrial interval (ITI) of 240 ± 20 s separated UCS offset with the next CS onset. For delay conditioning (Expt 2), the UCS was delivered at CS offset and an ITI of 260 ± 20 s was used. In Experiment 4, a weak training protocol was used in which rats received 2 trace conditioning trials (6-min baseline, ITI of 240 ± 20 s) and a reduced 0.5-mA shock level. The white noise CS was delivered through a speaker centered in one sidewall of each conditioning chamber. Stainless steel bars (4 mm diameter, spaced 12 mm apart) on the floor of each chamber served to deliver the footshock UCS. The chambers were cleaned with a 70% ethanol (Expts 1–2) or 5% ammonium hydroxide (Expts 3–4) between rats.

During training, rats learn to associate both the auditory CS and the training context with the shock UCS. Rats were tested for memory of each association separately the day after training. Conditional freezing to the CS was assessed in novel chambers (internal dimensions: 30.5 × 24.1 × 29.2 cm) housed in sound attenuating outer chambers with 55–60 dB background noise. These chambers were located in a separate room and differed from the training chambers in illumination (infrared house lamp), texture (solid, textured floor), and odor (5% acetic acid solution). In addition, transport between the colony room and the testing room was altered from that used for training: rats were covered during transport and an alternate route was used. For Experiment 2 in which PL input to the BLA was silenced just during the trace interval using optogenetics, we used a CS retention test previously used to measure rats' fear to both the CS and CS-offset (Gilmartin et al., 2013b, Kirry et al., 2018). This test consisted of a 2-min pre-CS baseline period followed by a single 10-s CS. This CS was followed by a 2-min stimulus-free period (SFP) to assess freezing in response to CS-offset. After this 2-min period, the CS was presented for 5 min to assess freezing in response to the CS. For all subsequent experiments, CS memory was assessed with 8 CS-alone trials (30-s CS, 60-s inter-trial interval following a 2-min pre-CS baseline period) (Kirry, Durigan, Twining, & Gilmartin, 2019). Two to 3 h after the CS test, contextual fear memory was assessed by measuring conditional freezing during a 10-min re-exposure to the original training chamber.

2.7. Behavioral procedures

2.7.1. Experiment 1: Arc immunoreactivity in BLA following PL silencing

Rats were trained 10–14 days after virus injection and optic fiber implantation surgery. They received three days of handling and three additional days of acclimation to transport from their home cage to the procedure room (Gilmartin et al., 2012, Kirry et al., 2018). During this time, the rats were acclimated to gentle restraint in a towel that would occur during optic fiber patch cord (200 µm diameter, 0.39NA) hook-up to the implanted fibers. On the third day of transport the rats were attached to the patch cord in the training context for 5 min. The following day, rats received 6 trace fear conditioning trials as described above. Green laser light (532 nm wavelength, 12mW) was delivered to the PL during the 20 s trace interval on each trial (Gilmartin, Miyawaki et al., 2013). Rats were euthanized 30 min following training. Home cage control rats were euthanized time-matched to trained rats. These untrained control rats were injected with ArchT, implanted with optic fibers, and were handled identically to trained rats, but were not exposed to the conditioning chambers.

2.7.2. Experiment 2: Optogenetic silencing of PL terminals in BLA.

Eight weeks after virus injection in the PL and at least one week after optic fiber implantation in the BLA, rats were acclimated and trained as in Experiment 1, with laser light delivered to PL terminals in BLA during the 20-s trace interval. An additional group of rats was trained with delay fear conditioning with laser light delivered during the 10-s CS. All rats were tested for CS and contextual fear memory the day after training. No laser light was delivered during testing.

2.7.3. Experiment 3. Chemogenetic inhibition of PL input to BLA.

Four weeks after virus injection, rats were trained with trace fear conditioning. Prior to training rats received three days of handling and three additional days of acclimation to transport from their home cage to the procedure room. In addition, rats received mock intraperitoneal (i.p.) injections in their colony room for three days prior to training: rats were manipulated to expose their abdomen and gently scratched near the injection site. On the day of training, rats were injected with freshly made clozapine N-oxide (1 mg/kg or 5 mg/kg, i.p., clozapine N-oxide; Tocris) or saline vehicle 20–30 min before trace fear conditioning. All rats were tested for CS and contextual fear memory the following day in the absence of drug.

2.7.4. Experiment 4: Chemogenetic inhibition of PL input to BLA using a weak training procedure

Four weeks after virus injection, rats were trained with trace fear conditioning using a modified protocol in which rats received two pairings with a weaker 0.5 mA shock. Pre-training handling and acclimation was the same as in Experiment 3. The following day, rats received a neutral cue exposure session in the test chambers (6 min baseline, 4 30-s 6 kHz tones; 60-s ITI), a CS retention test in the test chambers (2 min baseline, 8 30-s CS-alone trials; 60-s ITI), and a context retention test (10 min) in the training chambers. Each session was separated by 2 h. Following these test sessions, rats received two more paired training trials and another round of testing the following day. This was repeated for 3 days such that all rats received 6 total pairings across 3 days (2 trials/day; procedure shown in Fig. 5A). As in Experiment 3, freshly made clozapine N-oxide (5 mg/kg, i.p.; clozapine N-oxide hydrochloride; Tocris) or saline vehicle was injected 20–30 min prior to the training session on each day (1–3). All tests occurred in the absence of drug.

2.8. Immunohistochemistry:

The effect of prefrontal silencing on BLA Arc protein was analyzed in Experiment 1. Thirty minutes after the end of training, rats were deeply anesthetized with isoflurane and transcardially perfused with 0.1 M phosphate-buffered saline followed by 10% buffered formalin. This timepoint was chosen based on previous reports of learning-related Arc protein expression in the BLA and hippocampus (Lonergan et al., 2010, Lyford et al., 1995, Wallace et al., 1998, Young and Williams, 2013). The brains were removed, post-fixed 24 h in 10% buffered formalin, and then transferred to a 30% sucrose solution (in 0.1 M phosphate buffer) for 48 h for cryoprotection. The brains were embedded in OCT compound (Fisher Scientific, USA) prior to freezing (−80 C). Coronal sections (40-µm thick) of the prefrontal cortex and amygdala were collected with a cryostat (Leica Microsystems). Every other section in the PL was mounted on one set of slides for cresyl violet staining to identify optic fiber placement with light microscopy. The adjacent sections were mounted on a separate set of slides with anti-fade UltraCruz mounting medium (Santa Cruz Biotechnology) for epifluorescent imaging of virus expression in PL. Every fourth section through the amygdala was collected for Arc immunohistochemistry. Free-floating sections were rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4; 3 × 5 min), in 0.1 M PBS with 0.3% hydrogen peroxide (10 min), and then again in 0.1 M PBS (3 × 5 min). The sections were then incubated in a blocking solution containing 3% normal goat serum and 0.3% Triton-X in 0.1 M PBS for 60 min. Incubation in rabbit polyclonal anti-ARC (1:500; Santa Cruz, sc-15325) occurred in blocking solution for 40 hr at 4 °C (Pandey et al., 2008, Tanimizu et al., 2017). Following a brief rinse in 0.1 M PBS (3 × 5 min), the sections were incubated in biotinylated goat anti-rabbit (1:500; Vector Labs, BA-1000) for 2 hr. Sections were then rinsed in 0.1 M PBS (3 × 5 min) and incubated in Avidin-biotin complex reaction (Vector Labs, PK-6100) for 60 min. Following another

rinse in PBS (2 × 10 min) and a rinse in 0.05 M Tris buffer (pH 7.4; 10 min), the sections were placed in a DAB solution (0.05% DAB, 0.05% Nickel Sulfate, and 0.015% hydrogen peroxide in Tris buffer, pH 7.4) to reveal the immunoreactivity. The sections were rinsed in Tris buffer (2 × 10 min) and mounted on gelatin-coated slides. The mounted sections were cleared with ethanol and xylene and coverslipped with Permount mounting medium (Fisher Scientific, USA). The following negative controls were used; anti-Arc antibody omission, anti-rabbit antibody omission, and combined omission. Staining was not observed in these controls.

2.9. Arc immunoreactivity quantification

Color brightfield images of DAB reactions in the amygdala complex were acquired using a 10x objective on a Nikon 80i epifluorescence microscope fitted with a Retiga 2000R digital camera (QImaging, Surrey, BC, Canada) using NIS Elements-D software (Nikon Instruments, Melville, NY, USA). Images were filtered via a liquid crystal RGB filter and an auto-white balance was performed on each slide using areas lacking tissue (QImaging RGB-HM-S-IR) (Gasser, Hurley, Chan, & Pickel, 2017). Coronal slices from the planes of 2.1 mm to 3.2 mm posterior to bregma were analyzed for each rat (6–8 slices per rat). Regions of interest (ROIs) were drawn for the lateral (LA), basolateral (BLA), and central (CEA) nuclei of the amygdala based on anatomical landmarks (Paxinos & Watson, 2007). An ROI was also drawn around the PL terminal field in BLA. The boundaries of this ROI were based on our images of GFP-positive terminals in the BLA 8 weeks after AAV/CAG-GFP injection in the PL, and consistent with previous reports (McDonald et al., 1996, Vertes, 2004). The dorsal border was the lateral amygdala, the medial border was the intermediate capsule, and the most dorsal-lateral point along the external capsule was triangulated medially to just dorsal of the main intercalated cell cluster where it converged with the termination of the medial border (Pinard, Mascagni, & McDonald, 2012). Representative transcribed ROIs are shown in Fig. 1c. An experimenter blind to the training condition of each rat quantified Arc immunoreactivity in each ROI in a single hemisphere across the rostral-caudal extent using ImageJ (Analyze Particles tool; National Institutes of Health). All images were converted to 8-bit grayscale. The signal intensity threshold within each ROI was adjusted until only cells with immunostaining were highlighted above background tissue intensity with no saturation beyond the defined pixels. A cell was defined as Arc-positive if the highlighted pixels were in a cluster with a minimum size of 40^2 pixels. This criterion was chosen because the cell counts obtained with this supervised automatic detection method matched cell counts of cells in a subset of LA, BLA and CeA ROIs hand-counted by the experimenter. Arc expression was expressed as the density of Arc-positive cells per 100 μm^2. ROI area was calculated using a Micrometer Scale slide (Ted Pella, INC) to convert total pixels in an individual ROI to mm^2. The average density of Arc was calculated across all slices analyzed for each rat.

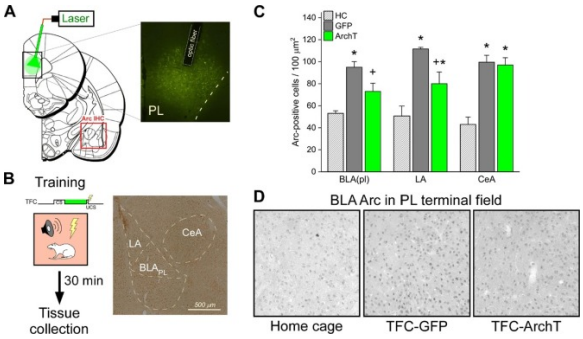

Fig. 1. Photoinhibition of PL cells during training attenuates training-induced Arc expression in the basolateral amygdala. (A) AAV9/CAG-ArchT-GFP or inactive virus was injected bilaterally into PL. Arc protein immunoreactivity was assessed within subregions of the amygdala. (B) The brain was extracted 30 min after trace fear conditioning (TFC). The micrograph image from a TFC-GFP rat shows the ROIs used to analyze Arc immunohistochemistry in the amygdala. (C) Bars depict the average Arc-positive cells per 100 μm^2 in each group: home cage controls (HC, n = 4), GFP-trained (n = 6), ArchT-trained (n = 7). (D) Representative micrograph

images (20×) of Arc immunoreactivity in the PL terminal field of the BLA (BLA_{PL}) for each group. The brightness and contrast of these images were adjusted for optimal display in print, using identical parameters for each image. PL = prelimbic cortex, BLA(pl) = basolateral amygdala subregion directly innervated by PL terminals, LA = lateral amygdala, CeA = central amygdala. *$p < 0.05$ compared with home cage controls, $^{+}p < 0.05$ compared with GFP controls. Diagrams adapted from Paxinos & Watson, 2007, with permission from Elsevier.

2.10. Unit recording

To verify functional disruption of PL input to BLA by combinatorial DREADD expression in this pathway, BLA activity was recorded in the absence and presence of CNO in one rat from Experiment 3. After the completion of Experiment 3, one Gi-DREADD-expressing rat (from the 1 mg/kg CNO group) was implanted with a 16-channel silicon probe (NeuroNexus, Ann Arbor, MI) into the BLA. Two months later, the rat was injected with saline (1 ml/kg, i.p.) and placed in the recording chamber 20 min later. After a 2-minute baseline period, 6 CS-alone presentations were delivered (ITI 60 s) followed by a single trace conditioning trial and an additional 6 CS-alone presentations. The rat was then removed from the chamber, injected with CNO (5 mg/kg, i.p.), and placed back in the chamber. Neuronal activity was monitored for 20 min, after which the same CS procedure was repeated. Extracellular single-unit activity in the BLA was recorded using a 32-channel neurophysiological recording system (RZ2 and Synapse Suite, Tucker-Davis Technologies, Alachua, FL) via a flexible recording cable attached to a quiet, electrically shielded motorized commutator to allow free movement. Activity for each probe site, referenced to a common average signal was collected and stored for offline unit isolation and analysis. Discrimination of individual waveforms corresponding to a single neuron was accomplished using a supervised template and principal component analysis in Offline Sorter (Plexon 4.4.0, Dallas TX). Electrolytic lesions were made prior to euthanasia and cresyl violet staining for histological identification of electrode placement.

2.11. Histology

At the end of the study, rats were deeply anesthetized with isoflurane, transcardially perfused with 0.1 M phosphate-buffered saline followed by 10% buffered formalin, and the brains were placed in fixative for two days. The brains were then transferred to a 30% sucrose solution (in 0.1 M phosphate buffer) for cryoprotection. The brains were embedded in OCT compound, frozen, and sectioned coronally. Every other section from PL or BLA was mounted on glass slides and coverslipped with anti-fade UltraCruz mounting medium (Santa Cruz Biotechnology). Adjacent sections were mounted on glass slides and stained with cresyl violet. Images of GFP (Expts 1–2) or mCherry (Expts 3–4) expression were collected with a Nikon epifluorescence microscope and NIS Elements software. Optic fiber placements were confirmed by light microscopy.

2.12. Data analysis

Freezing was used as the measure of conditional fear during all training and testing sessions and was defined as the cessation of all movement except that needed for respiration (Fanselow & Bolles, 1979). Freezing was scored automatically using FreezeScan 2.0 (CleverSys; Reston, VA) as described previously (Kirry et al., 2018). All statistical analyses were performed with Statistica version 13 (Tibco, Palo Alto, CA). Group differences in freezing were analyzed using mixed model ANOVAs with repeated measures for acquisition and CS retention or one-way Analysis of Variance (ANOVA)s for post-shock freezing and context retention. Mixed model ANOVAs varied a between-subjects factor of optogenetic or chemogenetic manipulation vs controls, across a within-subjects factor of Trial (acquisition) or Block (CS retention). Block included Baseline and 4-trial CS average or Baseline and 2-min SFP or CS, depending on test design. For Experiment 4, the ANOVA included the 4-trial average CS by Day). Fisher's least significant difference tests were used to make planned pairwise comparisons when ANOVAs revealed an appropriate significant main effect or interaction. The Holm-Sidak procedure was used to contain family-wise alpha level < 0.05. Effect sizes were calculated using Cohen's d and partial eta^2.

3. Results

Prelimbic (PL) neuronal activity during the trace interval separating the cue and shock is needed for the successful formation of trace cued fear memory (Gilmartin, Miyawaki et al., 2013), but it is unknown how this signal influences the consolidation of memory in downstream targets. This study tests the hypothesis that direct communication between the PL and the basolateral amygdala (BLA) during training mediates the integration of a cue-maintenance bridging signal with associative processes in the amygdala. In Experiment 1, we first examined the consequence of local PL silencing during training on the expression of Arc protein in the BLA to determine if plasticity-related immediate early gene expression was reduced downstream of PL. Fig. 1A shows the representative virus expression and fiber placement in PL for a rat injected with ArchT. Fig. 1B shows the anatomical ROIs drawn in the BLA for analysis of Arc expression, and Fig. 1C–D shows Arc immunoreactivity within the BLA 30 min after trace fear conditioning or HC control. Compared with home cage controls (HC), training produced an increase in Arc expression within several amygdala regions of interest (ROI), including the terminal field of PL in the BLA (BLA_{PL}), the lateral amygdala (LA), and the central amygdala (CEA; Fig. 1C). One-way ANOVAs on each ROI revealed a significant effect of group (BLA_{PL}: $F(2,14) = 9.574$; $p = 0.0024$; $\eta_p^2 = 0.578$; LA: $F(2,14) = 11.19$; $p = 0.00125$; $\eta_p^2 = 0.615$; CEA: $F(2,14) = 18.38$; $p = 0.00012$; $\eta_p^2 = 0.724$). In the LA and BLA_{PL}, but not CEA, this increase was attenuated in PL-silenced ArchT rats (Holm-Sidak: FW $\alpha < 0.05$). A complete reduction of training-related Arc to home cage levels was not predicted based on intact contextual fear learning in trace interval-silenced rats (Gilmartin, Miyawaki et al., 2013), and in all regions except BLA_{PL} ($p = 0.053$), Arc expression remained significantly elevated over home cage controls. These data suggest that training-related Arc expression is sensitive to optogenetic silencing of PL activity needed for cued fear learning in trace conditioning and Experiment 2 tested the hypothesis that direct communication between the PL and BLA is necessary for trace cued fear memory.

3.1. Experiment 2: Optogenetic inhibition of prelimbic terminals in the basolateral amygdala

We next sought to determine the extent to which direct prelimbic input to the BLA is necessary for cued fear learning. The BLA serves as a site of memory consolidation for cued and contextual fear memories in trace and delay fear conditioning (Kwapis et al., 2011) and direct communication between these areas is important for early retrieval of learned fear (Do-Monte, Quinones-Laracuente, & Quirk, 2015). In this experiment, we used projection-targeting optogenetics in which we transfected PL neurons with ArchT and delivered laser light to BLA to selectively silence PL input (Fig. 2A–B). Rats injected with ArchT or GFP were trained with trace or delay fear conditioning (n = 5/virus/group), during which light was delivered during the trace interval for the trace groups or during the CS for the delay groups (Fig. 2C). All rats were tested for cued and contextual fear memory retention the following day in the absence of light delivery. Two of the 20 rats in this experiment were excluded from analysis: one rat in the Delay-ArchT group was excluded due to lack of viral expression in one hemisphere and one rat in the Trace-GFP group was excluded due to very high baseline freezing (>80%) during the retention test that exceeded two standard deviations of mean baseline freezing of all trace-conditioned animals. Similar to PL cell body silencing in our previous study (Gilmartin, Miyawaki et al., 2013), optogenetic inhibition of PL terminals in the BLA during the trace interval did not affect the performance of freezing during the paired trials in the trace conditioned group (Fig. 2D, Trace). A mixed model ANOVA on Group (GFP, ArchT) and Period (Baseline, Pairings) revealed a main effect of Period ($F(1,7) = 151.27$; $p = 0.00001$; $\eta_p^2 = 0.956$), but no main effect of Group or a Group × Period interaction (Fs < 1.0). ArchT rats did exhibit a reduction in post-shock freezing (Fig. 2D, Trace), but this was not significant ($F(1,7) = 2.57$; $p = 0.153$). Somewhat surprisingly, silencing PL terminals during the CS in delay-conditioned rats did attenuate freezing during the acquisition session (Fig. 2D, Delay). A mixed model ANOVA on Group and Period revealed a Group × Period interaction ($F(1,7) = 8.17$; $p = 0.024$; $\eta_p^2 = 0.539$), with ArchT rats exhibiting less freezing during paired trials compared with GFP controls

(ps < 0.05, Holm-Sidak). Delay ArchT rats also showed a reduction in post-shock freezing ($F(1,7) = 6.33$; $p = 0.04$; Cohen's $d = 1.688$; Fig. 2D, Delay). However, this within-session effect on freezing in the Delay rats did not affect the formation of memory. During the CS and context retention tests the following day, Delay ArchT rats showed intact freezing to the CS and Context, relative to GFP controls (Fig. 2F, Delay). CS-elicited freezing was assessed in a novel chamber in the absence of laser light delivery. After a 2-min baseline period, a brief 10-s CS was presented followed by a stimulus-free period of 2 min (SFP) to assess freezing in response to CS-offset. A long 5-min presentation of the CS was then presented to assess freezing during the CS itself (Fig. 2E). A mixed model ANOVA on Group (GFP, ArchT) and Period (Baseline, SFP, CS) revealed only a main effect of Period ($F(2,14) = 22.164$; $p = 0.00005$; $\eta_p^2 = 0.760$). No difference in context freezing was observed ($F < 1.0$). Similarly, silencing PL input to BLA during trace conditioning did not impair cued or contextual fear memory (Fig. 2F, Trace). A mixed-model ANOVA revealed a main effect of Period ($F(2,14) = 19.656$; $p = 0.00009$; $\eta_p^2 = 0.737$), but there was no main effect of Group ($F < 1.0$) or Group × Period interaction ($F(2,14) = 2.028$; $p = 0.169$; $\eta_p^2 = 0.225$). These null findings are unlikely to be due to non-specific fear as these shock parameters do not produce sensitized fear in unpaired pseudoconditioned rats (Gilmartin and Helmstetter, 2010, Gilmartin et al., 2013b). Together, these data suggest that direct and time-limited PL input to the BLA has a modest contribution to the expression of freezing in delay conditioning but is not crucial for the acquisition of associative fear to the cue or context in either conditioning paradigm.

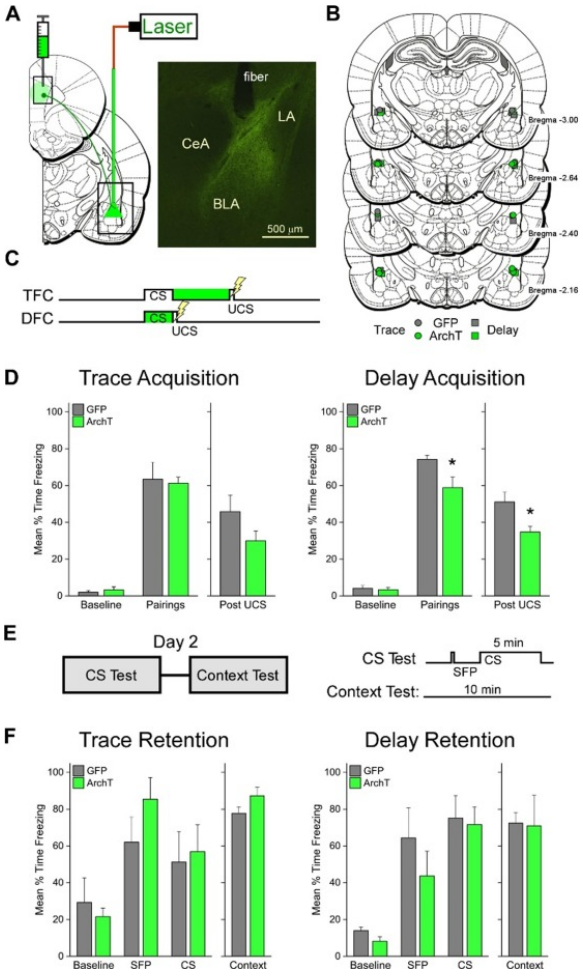

Fig. 2. Trial-limited silencing of PL input to BLA does not impair the formation of cued or contextual fear memory. (A) AAV9/CAG-ArchT-GFP or inactive virus was injected bilaterally into PL and optic fibers were positioned above the BLA. (B) Optic fiber placement is depicted for GFP and ArchT-expressing rats in the Trace (circles) and Delay (squares) groups. (C) Diagrams show the timing of laser light delivery on each trial of trace or

delay fear conditioning. (D) Bars show the average (±s.e.m.) time spent freezing by each group during the acquisition session. (E) Cued and Contextual fear retention tests occurred on the day after training in the absence of light delivery. The CS test consisted of a single brief 10-s CS followed by a 2-min stimulus-free interval (SFP) and a long 5-min CS presentation in a novel chamber. The context test consisted of a 10-min re-exposure to the training chamber. (F) Bars show the average (±s.e.m.) time spent freezing by each group during the CS and Contextual fear retention sessions. *p < 0.05 compared with GFP controls. Diagrams adapted from Paxinos & Watson, 2007, with permission from Elsevier.

3.2. Experiment 3: Chemogenetic inhibition of prelimbic terminals in the basolateral amygdala

Our previous work showed that the trace interval is the time period in which PL activity is crucial for learning the CS-UCS association (Gilmartin, Miyawaki et al., 2013), and the results of Experiment 2 suggest that PL output to the BLA is not a driver of this effect. However, it is possible that communication between the PL and BLA beyond this brief window may be necessary for learning. To test whether direct PL input to the BLA is required at any point during fear acquisition, we used a chemogenetic approach to inhibit PL-BLA communication during the entire training session. We selectively infected PL cells projecting to the BLA with an inhibitory Gi-coupled DREADD using a combinatorial viral approach (see Methods). Fig. 3A shows a representative example of virus expression in BLA-projecting PL cells. There were no differences in freezing between the three control conditions (Saline-injected Gi rats (n = 5) and CNO-injected mCherry rats (1 mg/kg or 5 mg/kg, n = 5/group) during any session (Fs < 1.46; ps > 0.25). Therefore, these groups were collapsed into one Control group (n = 15). In Gi-DREADD expressing rats, pre-training systemic delivery of CNO at either a 1 mg/kg (n = 10) or 5 mg/kg (n = 5) dose did not affect the acquisition or retention of trace fear conditioning (Fig. 3C). A mixed model ANOVA on the acquisition session revealed a main effect of Trial ($F(6,162) = 71.62$; $p < 0.00001$), but no main effect of Group or Group × Trial interaction (Fs < 1.2; ps > 0.30). At drug-free testing the following day, all groups showed intact fear to the CS and context (Fig. 3D). In contrast with Experiment 2, this CS test consisted of eight 30-s CS presentations in order to assess fear to brief presentations of the CS. CS freezing was averaged into blocks of 4 CSs. A mixed model ANOVA on freezing during the CS test showed a Group × Period (Baseline, CS1-4, CS5-8) interaction ($F(4,54) = 3.78$, $p = 0.0088$; $\eta_p^2 = 0.219$). Planned comparisons on each period revealed that Gi rats injected with 5 mg/kg CNO showed elevated baseline freezing compared with Gi rats injected with 1 mg/kg CNO ($p = 0.0055$) and a trend towards elevated baseline freezing compared with Control rats that did not survive correction for multiple comparisons ($p = 0.045$, Holm-Sidak). Each group showed significantly greater freezing during the first four CSs compared with their own baseline (Control $p < 0.0001$; CNO 1 mg/kg $p < 0.0001$; CNO 5 mg/kg $p < 0.012$). No differences in freezing were observed during the CS. Similarly, no significant differences were observed in freezing during the context test ($F(2,27) < 1$).

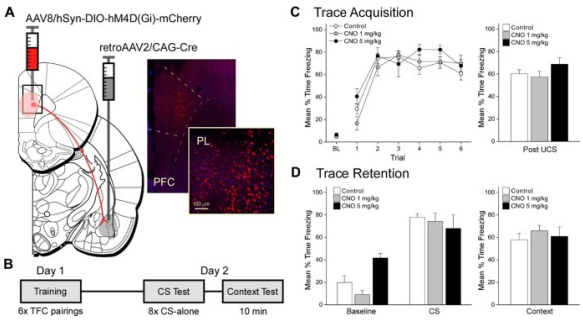

Fig. 3. Gi-DREADD-mediated suppression of PL input to BLA does not prevent the formation of cued or contextual fear memory. (A) AAV8/hSyn-DIO-hM4D(Gi)-mCherry or inactive mCherry control was injected bilaterally into PL and retroAAV2/CAG-Cre was injected bilaterally into the BLA. Micrographs show mCherry labeling of PL cells (inset 20×) projecting to the BLA. (B) Training and testing timeline. The CS test for all DREADD

experiments used discrete 30-s CS presentations. (C) Each point in the line graph shows the average (±s.e.m.) freezing during the CS and 20-s trace interval on each trial for each group. Each bar shows the average (±s.e.m.) time spent freezing during the 60-s post-shock period, averaged across trials. (D) Bars show the average (±s.e.m.) time spent freezing by each group during the CS (average of first 4 CSs) and Contextual fear retention sessions. Diagrams adapted from Paxinos & Watson, 2007, with permission from Elsevier.

To confirm the functional expression of Gi-DREADDs using this combinatorial viral approach, we recorded BLA neuronal activity in one DREADD-expressing rat from the 1 mg/kg CNO group. Four days after the retention tests, the rat was implanted with a 16-channel silicon probe into the BLA for subsequent recording of baseline and CS-evoked activity in the absence and presence of CNO (Fig. 4A). On the day of recording, the rat was injected with saline (1 ml/kg, i.p.) and presented with 6 CS-alone presentations followed by a single trace conditioning trial and an additional 6 CS-alone presentations. This served as a baseline for CS-evoked activity with which to compare the effects of CNO. The rat was then removed from the chamber, injected with CNO (5 mg/kg, i.p.), and placed back in the chamber. Baseline activity was monitored for 20 min, after which the same CS procedure was repeated (Fig. 4B). The 20-min period immediately after CNO-injection was analyzed separately. Eighteen units were isolated during this 20-min period, of which 11 were individual neurons and 7 were multi-unit activity. Using the first 4 min of the session (<5 min after CNO injection) as baseline activity, firing rates were standardized to z-scores in each minute. The average z-score in the last 10 min of the 20-min session was used to classify units as CNO-responsive (>2 or < -2 z). The majority of units (10/18) showed a decrease in firing across the session, while only 1 unit showed an increase in firing. The rest show no change or a modest decrease. Fig. 4C shows the average normalized firing for all units classified as decreasing.

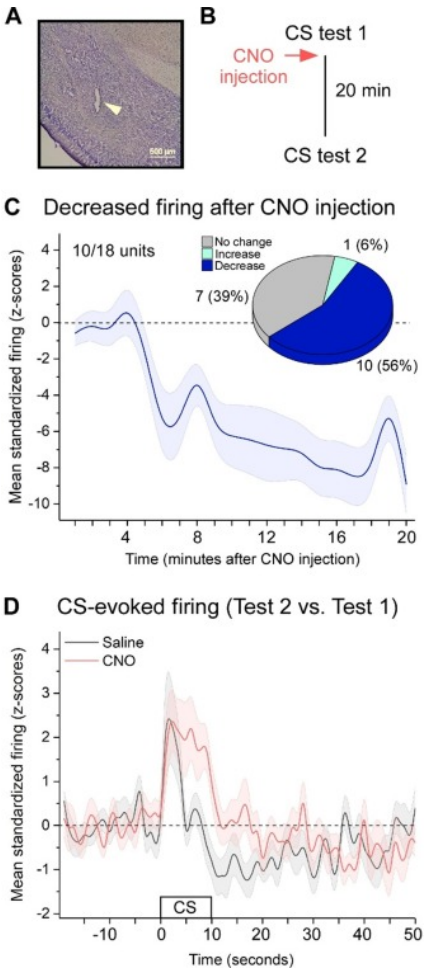

Fig. 4. CNO decreased the firing rates of the majority of BLA units recorded in vivo. (A) Electrode placement in the BLA from a Gi-DREADD-expressing rat from Experiment 3. (B) Recording procedure for verifying functional hM4D(Gi) function. (C) The line graph shows the average (±s.e.m.) normalized firing rate of the population of units recorded during the CNO injection session that showed significantly decreased firing in the second half of the 20-min session (10/18 units). This population is represented by dark blue in the pie chart. Z-scores were calculated based on the first 4 min of the session, immediately after CNO injection. (D) Line graphs show the average (±s.e.m.) normalized firing to the CS-alone trials of Test 1 (Saline) and Test 2 (CNO). The neuronal response to the CS was broadened in the presence of CNO.

Thirteen units were isolated during the Saline CS test session and 14 units were isolated during the CNO CS test session. While most of these units were likely the same from one session to the next, they were treated as independent populations. Fig. 4D shows the population response to the CS (averaged over all 12 CS-alone presentations) during the first test session after saline injection and during the second test session after CNO injection. CNO-activation of Gi-DREADD in PL-BLA did not prevent CS-evoked firing, but it altered the duration of firing to the CS. This shift may reflect a change in the local balance of excitation and inhibition as a consequence of reduced PL input. Thus, the Gi-DREADD, expressed selectively in PL afferents alters BLA activity when activated by CNO. Together, the results of Experiments 2 and 3 show that the formation of trace cued and contextual fear memories is intact despite the loss of prefrontal cortical input to the BLA at the time of training.

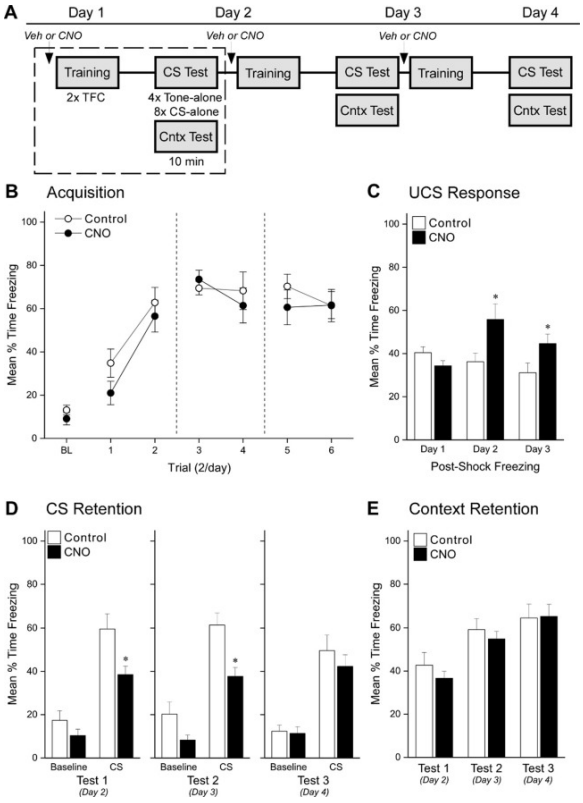

Fig. 5. Inhibiting PL input to BLA impairs learning using a weak-training procedure. (A) Rats were injected with vehicle or CNO prior to a weak 2-trial training session and were tested the following day (boxed procedure). This train-test procedure was repeated for 2 more days. (B) Each point shows the average (±s.e.m.) freezing during the CS and trace interval (30 s total) on each trial of acquisition across 3 days. (C) Each bar shows the average (±s.e.m.) freezing during the first minute after the UCS, averaged across 2 trials each day. (D) Graphs show the average (±s.e.m.) freezing during the first four CS presentations at each test. (E) Bars show the average (±s.e.m.) freezing during the 10-minute context test on each day. * $p < 0.05$ compared with vehicle controls.

3.3. Experiment 4: Chemogenetic inhibition of PL-BLA impairs learning in a weak training protocol.

The results of the previous experiments showed that direct communication between the PL and BLA is not crucial for the formation of trace fear memory. This suggests that direct prefrontal input to the amygdala does not participate in memory formation. An alternative interpretation is that PL-BLA input does participate in learning, but other circuits can compensate for its absence over the course of training. If this were the case, the loss of PL input during a weaker training procedure with only two trials may not be sufficient to recruit a compensatory circuit and may thus reveal a role for PL-BLA communication. Trace conditioning using anywhere from one to ten trials requires the PL and BLA (Gilmartin and Helmstetter, 2010, Gilmartin et al., 2012, Gilmartin et al., 2013b, Gilmartin et al., 2013a, Guimarais et al., 2011, Kochli et al., 2015, Runyan and Dash, 2004, Runyan et al., 2004). In the present experiment, rats were injected with vehicle or CNO prior to a weak training session. Rats received 2 pairings of the CS and a weak 0.5-mA UCS. Fear to the CS, neutral tones, and context were tested the following day (Test 1, day 2). This train-test procedure (Fig. 5A) was repeated for two more days and the final CS and Context tests were administered on Day 4. This resulted in a total of 6 CS-UCS pairings, intermixed with non-reinforced CS trials (i.e., test trials), which made the CS-UCS association more difficult compared with the single-day 6-trial procedure. Thus, we could assess the contribution of PL-BLA to trace conditioning under weak conditions.

All rats were transfected with AAV8/hSyn-DIO-HM4iD-mCherry in BLA-projecting PL cells as in Experiment 3. Twenty to thirty minutes prior to conditioning on each day, rats were injected with saline vehicle or CNO (5 mg/kg, i.p.). One rat in the CNO group did not show mCherry expression in PL and was excluded from all analyses. This resulted in a final group size of 10 rats/drug. As we observed in Experiment 3, CNO administered prior to training did not affect the within-session performance of freezing across days (Fig. 5B). In the mixed model ANOVA, there was no main effect of Group ($F(1,18) < 1$; $p = 0.352$) or Group × Period interaction ($F < 1$). There was a main effect of Period ($F(6,108) = 36.77$; $p < 0.001$; $\eta_p^2 = 0.67$), which reflected the increased freezing across trials. Freezing in reaction to the UCS was unaffected by CNO on Day 1 but was significantly elevated on subsequent days of training in the CNO group compared with controls (Fig. 5C). The mixed model ANOVA revealed a Group × Day interaction ($F(2,36) = 5.18$; $p < 0.011$; $\eta_p^2 = 0.22$), and planned comparisons showed a group difference in post-shock freezing on days 2 and 3 (ps < 0.05). Inhibiting the PL-BLA connection with CNO prior to 2 CS-trace-UCS pairings led to impaired cued fear recall when tested drug-free (Fig. 5D). Because we hypothesized a deficit under weak training conditions, we first analyzed cued and contextual retention at test 1, one day after having received only two CS-trace-UCS pairings. A mixed model ANOVA on the first CS retention test showed a main effect of Group ($F(1,18) = 6.33$; $p < 0.022$; $\eta_p^2 = 0.26$) and a main effect of Period (Baseline vs. CS, $F(1,18) = 83.36$; $p < 0.0001$; $\eta_p^2 = 0.82$), but no Group × Period interaction ($F(1,18) = 3.32$; $p = 0.085$; $\eta_p^2 = 0.16$). There were no differences in contextual freezing during the context test (Fig. 5E; $F < 1$; $p = 0.385$). This suggests that the lack of PL input to BLA during a brief training session impairs the formation of cued but not contextual fear memory. Additional training (2-trials/day for two more days) and testing did not change this overall pattern. Contextual fear increased across days and did not differ between groups. This was reflected in the main effect of Day ($F(2,36) = 35.88$; $p < 0.001$; $\eta_p^2 = 0.67$), but no main effect of Group or Group × Day interaction (Fs < 1; ps > 0.52). An analysis of CS freezing across all three CS tests showed that freezing to the CS was reduced in CNO rats compared with controls following 2 or 4 training trials, and no different from controls by the final test, after a total of 6 pairings (Fig. 5D). This observation was supported by a Group × Day interaction ($F(2,36) = 3.40$; $p < 0.044$; $\eta_p^2 = 0.15$). Planned comparisons showed a significant group difference on Tests 1 and 2 (ps < 0.015, Holm-Sidak). While this observation is consistent with additional training overcoming the CNO-induced deficit, it is important to note that behavior after 6 pairings in this design could be influenced by variables not present in the single-day protocol, such as non-reinforced test trials between spaced training sessions with a weak shock. Overall, the results of this experiment suggest that when rats are given only two

pairings of the CS with a weak UCS, a role for direct communication between the PL and BLA in fear learning is revealed. Specifically, PL-BLA input is important for cued, but not contextual, fear learning during trace fear conditioning.

4. Discussion

The present study suggests that direct communication between the PL and the BLA contributes to the acquisition of trace fear conditioning under weak, but not strong, training conditions. Chemogenetic silencing of PL input to the BLA during training impaired the formation of trace cued fear memory acquired after two CS-UCS pairings, but neither chemogenetic nor optogenetic silencing of this connection impaired trace fear learning after six trials. Moreover, the contextual fear memory was intact under all training conditions. These findings reveal that direct PL input to the BLA facilitates the acquisition of cued fear early in training, but other circuits support fear learning with additional trials.

The selective role for direct PL-BLA communication early in training suggests that this connection may be part of a circuit that supports memory formation under ambiguous CS-UCS relationships. Ambiguity about the predictive relationship among available discrete and contextual cues and the shock is present early in training until consistent contingencies are established. The acquisition of contingency is quite rapid in standard delay fear conditioning, but the addition of a temporal gap in trace fear conditioning prevents contiguity from quickly resolving the ambiguity, making trace conditioning tasks more difficult (Beylin et al., 2001) and dependent on prefrontal and hippocampal circuitry (Czerniawski et al., 2009, Gilmartin and Helmstetter, 2010, Gilmartin et al., 2013b, Gilmartin et al., 2014, Guimarais et al., 2011, McEchron et al., 1998, Yoon and Otto, 2007). Communication between these regions and the amygdala early in training may therefore facilitate learning about the predictive strength of the CS until the associative nature is established by the broader network over additional training. Paz and colleagues have demonstrated functional shifts from early to late training in the correlated activity between the amygdala and prefrontal cortex or rhinal cortices during the acquisition of aversive or appetitive associative memories (Livneh and Paz, 2012, Paz et al., 2006). For example, in an aversive trace conditioning task, activity in the primate dorsal anterior cingulate cortex (dACC), which, along with primate dlPFC, shares functional and anatomical homology with the rodent PL, was correlated with the BLA during early tone-odor pairings (Livneh & Paz, 2012). Correlated activity declined when the predictive value of the CS became consistent with additional training, but remained high throughout training to a different CS under partial reinforcement conditions (Livneh & Paz, 2012). Thus, under conditions of ambiguity, such as partial reinforcement or early in continuous reinforcement training, primate dACC-BLA correlated activity is associated with memory formation. How might this connection facilitate learning under these conditions? Recently, Madarasz et al hypothesized that the nature of fear learning under ambiguous conditions may best be explained by a structure model, in which several possible relationships among cues within the environment are weighed (Madarasz et al., 2016). With additional trials, learning then transitions to a parameter model to learn the parameters of the best predictive relationship(s) obtained via structure learning. This group showed that the amygdala is capable of structure learning and postulated that cortical input may influence the selection of learning model (Madarasz et al., 2016). The PL is typically recruited into associations with a degree of ambiguity, such as trace conditioning and even delay eyeblink conditioning when the UCS is relatively weak (Gilmartin et al., 2014, Guimarais et al., 2011, Oswald et al., 2006). The PL is also recruited for associative learning or behavioral selection in situations of predictive uncertainty, such as cue competition, blocking, or context-guided learning and behavior (Furlong et al., 2010, Sharpe and Killcross, 2014, Sharpe and Killcross, 2015). The diverse connectivity of the PL likely explains its role in these situations. The PL contributes to latent contextual processing and contextual fear learning (Gilmartin and Helmstetter, 2010, Heroux et al., 2017, Zelikowsky et al., 2013, Zelikowsky et al., 2014, Zhao et al., 2005), and input from the hippocampus to PL or IL mediates the use of contextual information in aversive learning or fear relapse, respectively (Marek et al., 2018, Santos et al.,

2020, Twining, Lepak, Kirry, & Gilmartin, 2020). Likewise, prefrontal communication with the amygdala may mediate the use of cue-related encoding to facilitate structure-learning by the amygdala early in learning when the CS-UCS relationship is most ambiguous, especially in a trace design. The pattern of results in our study is not unlike that observed after lesions of the dorsal hippocampus during delay fear conditioning. Under typical training conditions, the acquisition of delay fear conditioning is independent of the dorsal hippocampus. However, Fanselow and colleagues found that pre- or post-training hippocampal lesions impaired cued fear learning in the delay fear conditioning paradigm under weak training conditions, such as weak shock reinforcement and fewer training trials (Quinn, Wied, Ma, Tinsley, & Fanselow, 2008). They interpreted this observation as reflecting the reliance on rapid episodic binding by the hippocampus early in training, whereas strong associative strength acquired under stronger training parameters would support behavior in the absence of DH input. Thus, prefrontal cortex and hippocampus may promote associative learning with limited stimulus experience or weak reinforcers through region-specific functions. In the case of trace fear conditioning, the PL-BLA pathway contributes to this early learning. Whether it does so by promoting structure learning mechanisms in the amygdala or by modifying stimulus processing in the amygdala remains to be determined.

Glutamatergic input from the prefrontal cortex or hippocampus can regulate amygdala activity through modulation of local excitatory and inhibitory circuits (Arruda-Carvalho and Clem, 2014, Bazelot et al., 2015, Hubner et al., 2014). Loss of one of these inputs could alter encoding in a way that is disruptive to learning under weak conditions, but which can be overcome with additional training and other input. Previous work has shown for instance that prefrontal lesions increase the neuronal response to footshock in the central nucleus of the amygdala (Correll, Rosenkranz, & Grace, 2005). This area is inhibited by the BLA and even brief inhibition of BLA footshock-UCS firing in a discriminative cued fear design slowed acquisition of conditioned suppression of lever pressing, which was attributed to altered cue salience (Sengupta et al., 2018). In the rat (delay conditioning) and primate (trace conditioning) amygdala, neuronal firing to the reinforcer during training negatively correlated with CS-elicited conditional responses (Belova et al., 2007, Johansen et al., 2010). If loss of PL input were to alter UCS-elicited activity in amygdala circuits, this could impair the acquisition of trace conditioning. Interestingly, in our study, while CNO treatment in Gi-DREADD rats had no effect on within-session behavior on day 1 of the weak training protocol, it did contribute to elevated post-shock freezing relative to controls on days 2 and 3 of training. This pattern may reflect a change in UCS processing that elevates freezing to the shock and reduces cue salience. It could also reflect a change in CS encoding, specifically the ability of the CS to drive preparatory modulation of ascending pain signals that occur during conditioning and contribute to asymptotic fear (Fanselow and Bolles, 1979, Fanselow, 1998). This modulation is linked to expectation-driven top-down suppression of pain- and learning-related circuits by the prefrontal cortex and amygdala (Furlong et al., 2010, Johansen et al., 2010, McNally et al., 2011). Therefore, loss of PL input to BLA during the first training session may have reduced the predictive value of the CS such that during subsequent training sessions the UCS was less expected compared with controls. A less expected UCS would elicit a greater neural response and more post-shock freezing. However, post-shock freezing was unaffected or even decreased when PL-BLA communication was silenced in the 6-trial paradigm with no effect on cued memory the following day, which suggests that this explanation does not fully explain the impaired learning after two trials. The altered neuronal firing response to the CS during retrieval in our verification recording suggests that Gi-DREADD inhibition of glutamatergic input from PL may alter CS-related activity in BLA units. A future study examining the consequence of PL-BLA silencing on CS- and UCS-encoding during acquisition may reveal the underlying mechanism by which PL input facilitates learning after limited or weak training.

In conclusion, silencing PL cell bodies during the trace interval impairs memory formation (Gilmartin, Miyawaki et al., 2013) and reduces BLA Arc expression (present study). The direct connection between the two areas is important only early in training under weak conditions when the CS-trace-UCS relationship is most ambiguous. This suggests a role for PL-BLA in the establishment of fear memories after limited experience, which has

implications for understanding individual variability in disorders such as PTSD. Genetic or environmental factors that negatively impact the development of this connection, such as severe stress in childhood (Guadagno et al., 2018), may alter how fear to threat-predictive cues is organized in broader memory circuits, contributing to pathological fear.

CRediT authorship contribution statement

Adam J. Kirry: Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Robert C. Twining:** Methodology, Investigation, Formal analysis, Visualization, Writing - review & editing. **Marieke R. Gilmartin:** Conceptualization, Methodology, Formal analysis, Visualization, Writing - original draft, Writing - review & editing, Resources, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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