

Outcome-Locked Cholinergic Signaling Suppresses Prefrontal Encoding of Stimulus Associations

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Acetylcholine (ACh) is thought to control arousal, attention, and learning by slowly modulating cortical excitability and plasticity. Recent studies, however, discovered that cholinergic neurons emit precisely timed signals about the aversive outcome at millisecond precision. To investigate the functional relevance of such phasic cholinergic signaling, we manipulated and monitored cholinergic terminals in the mPFC while male mice associated a neutral conditioned stimulus (CS) with mildly aversive eyelid shock (US) over a short temporal gap. Optogenetic inhibition of cholinergic terminals during the US promoted the formation of the CS–US association. On the contrary, optogenetic excitation of cholinergic terminals during the US blocked the association formation. The bidirectional behavioral effects paralleled the corresponding change in the expression of an activity-regulated gene, *c-Fos* in the mPFC. In contrast, optogenetic inhibition of cholinergic terminals during the CS impaired associative learning, whereas their excitation had marginal effects. In parallel, photometric recording from cholinergic terminals in the mPFC revealed strong innate phasic responses to the US. With subsequent CS–US pairings, cholinergic terminals weakened the responses to the US while developing strong responses to the CS. The across-session changes in the CS- and US-evoked terminal responses were correlated with associative memory strength. These findings suggest that phasic cholinergic signaling in the mPFC exerts opposite effects on aversive associative learning depending on whether it is emitted by the outcome or the cue.

Key words: acetylcholine; associative learning; basal forebrain; mPFC; mice; photometry

Significance Statement

Drugs compensating for the decline of acetylcholine (ACh) are used for cognitive impairment, such as Alzheimer's disease. However, their beneficial effects are limited, demanding new strategies based on better understandings of how ACh modulates cognition. Here, we report that by manipulating ACh signals in the mPFC, we can control the strength of aversive associative learning in mice. Specifically, the suppression of ACh signals during an aversive outcome facilitated its association with a preceding cue. In contrast, the suppression of ACh signals during the cue impaired learning. Considering that this paradigm depends on the brain regions affected in Alzheimer's disease, our findings indicate that precisely timed control of ACh signals is essential to refine ACh-based strategies for cognitive enhancement.

Introduction

Acetylcholine (ACh) is implicated in diverse functions, including arousal, attention, and learning, through its modulation of cortical neural transmission and plasticity (Everitt and Robbins, 1997; Baxter and Chiba, 1999; McGaughy et al., 2000; Hasselmo and Sarter, 2011; Picciotto et al., 2012). The primary source of cortical ACh originates from the cholinergic neurons in the basal forebrain (BF), which consists of several subregions projecting to specific cortical and subcortical regions (Mesulam, 1995; Zaborszky et al., 1999; Woolf and Butcher, 2011). BF cholinergic neurons discharge in bursts during rapid eye movement sleep and waking but cease firings during slow-wave sleep (Lee et al., 2005; M. Xu et al., 2015). Atop the slow fluctuations, the activity of cholinergic neurons changes within milliseconds following the

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presentation of aversive and appetitive stimuli (Hangya et al., 2015; Harrison et al., 2016). When these salient outcomes are paired with a sensory stimulus, cholinergic neurons develop phasic responses to the outcome-predictive stimulus (Guo et al., 2019). These findings suggest that cholinergic neurons emit transient signals precisely time-locked to stimuli with innate and learned salience. Largely unknown is how such phasic cholinergic signaling modulates neural processes in the projection sites supporting cognitive function.

The mPFC is one of the major efferent projection targets of BF cholinergic neurons (Dalley et al., 2004; Bloem et al., 2014). Pharmacological manipulations of cholinergic receptors in the mPFC affect rodents' behavior in various cognitive tasks testing attentional processing (Hahn et al., 2003; Chudasama et al., 2004), aversive associative learning (Raybuck and Gould, 2010), olfactory associative learning (Boix-Trelis et al., 2007), contextual learning (Robinson-Drummer et al., 2017), and associative recognition memory (Sabec et al., 2018). In parallel, by using choline-sensitive microelectrodes, several recent studies detected transient ACh release in the mPFC in response to a cue predictive of reward (Parikh et al., 2007) and reward delivery area in a maze (Teles-Grilo Ruivo et al., 2017). Subsequent optogenetic experiments causally linked the cue-evoked activity with cue detection (Sarter et al., 2014; Gritton et al., 2016); however, the functional relevance of cholinergic signaling evoked by aversive outcome remains unknown.

To address this point, we manipulated and monitored cholinergic terminal activity in the mPFC while mice underwent an aversive associative learning task called trace eyeblink conditioning. This task required mice to associate a neutral stimulus with mild eyelid shock over a short temporal gap, a process critically dependent on the integrity of the prefrontal region (PrL) of the mPFC (Runyan et al., 2004; Takehara-Nishiuchi et al., 2005; Gilmartin and Helmstetter, 2010; Gilmartin et al., 2013; Volle et al., 2016). It offers precise control over stimulus presentations, making it an ideal assay to examine how outcome-evoked cholinergic signaling affects aversive associative learning. Here we show that phasic cholinergic signaling has opposing effects on aversive associative learning depending on whether it was evoked by the shock or shock-predictive stimulus.

Materials and Methods

Animals

Adult ChAT_(IRES)-Cre mice (RRID:IMSR_JAX:028861) and PV-Cre mice (RRID:IMSR_JAX:017320) were bred as homozygotes, where Cre recombinase is expressed under the ChAT and parvalbumin (PV) promoter without disrupting endogenous ChAT and PV expression. Eight- to 18-week-old male ChAT_(IRES)-Cre and PV-Cre mice were used in behavior and photometry experiments. Eight adult C57B6/J male mice (#000664; The Jackson Laboratory) were used for anatomic tracing experiments. Mice were housed under a 12 h light/dark cycle with *ad libitum* access to food and water. All surgeries and experiments were conducted under the regulation of the University of Toronto Animal Care Committee and the Canadian Council on Animal Care (AUP20012042).

Viral vectors

AAV8-CAG-FLEX-Arch-GFP and AAV8-CAG-FLEX-GFP were purchased from the vector core at the University of North Carolina at Chapel Hill. AAV9-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-HGHpA (RRID:Addgene_20298), AAV9-EF1a-DIO-EYFP (RRID:Addgene_27056), and AAV5-hSyn-FLEX-axon-GCaMPG6s (RRID:Addgene_112010) were purchased from Addgene.

Surgery

Viral infusion. Eight- to 12-week-old ChAT_(IRES)-Cre and PV-Cre mice were anesthetized (1%–2% isoflurane by volume in oxygen at a flow rate of 0.8 L/min; Fresenius Kabi) and administered with ketoprofen (5 mg/kg) subcutaneously. Mice were placed in a stereotaxic frame, and holes were made in the skull bilaterally above the BF (0.2 mm AP, $\pm$ 1.3 mm ML) (Paxinos and Watson, 2008). A 33 G microinfusion cannula was connected to a 10 μ l Hamilton syringe with Tygon tubing. One of the viral vectors (0.8–1 μ l/site) was infused to the BF (-5.4 mm DV) at a rate of 0.1 μ l/min. After infusion, the cannula was left in the brain for $\sim$ 15 min, allowing for the viral vectors to diffuse.

Optic fiber implantation. Three to 6 weeks after viral infusion, mice were placed in a stereotaxic frame as described above. Optic fibers (200- μ m-core diameter, 0.39 NA; Thorlabs) were implanted bilaterally at 30 degrees lateral to the medial angle above the PrL (1.9 mm AP, $\pm$ 1.4 mm ML, -1.8 mm DV). For fiber photometry experiments, a 400- μ m-core optic fiber (0.48 NA; Thorlabs) was implanted in the PrL (1.9 mm AP, -0.4 mm ML, -1.75 mm DV). The optic fibers were secured to the skull with resin cement (3M dental, Relyx unicem). To record EMG and deliver a periorbital shock, four Teflon-coated stainless-steel wires were implanted subcutaneously in the upper left orbicularis oculi and attached to a connector cap (Plastic One). The wires and the connector cap were secured to the skull with stainless-steel screws and dental acrylic resin. The mice were given 7–10 d to recover from the surgery before behavioral testing.

Retrograde tracing. Eight-week-old C57B6/J mice were placed on a stereotaxic frame as described above. The skin was retracted, and a hole was drilled in the skull at the targeted coordinates. A 200 nl volume of retrograde tracer, red RetroBeads (Lumafuor) was injected into the PrL (1.9 mm AP, 0.4 mm ML, -1.8 mm DV) at a rate of 40 nl/min. Two to 3 weeks after the injection, mice were transcardially perfused.

Behavioral experiments

Trace eyeblink conditioning. Mice were individually placed in a cylindrical Plexiglas container (20 cm in diameter and 25 cm high) in a sound- and light-attenuated chamber. Their behavior was monitored via an infrared web camera placed inside the chambers. Mice were connected to a cable and patch cords via zirconia sleeves (Thorlabs). For the first two sessions, mice were placed in the chamber for 50 min without any stimulus presentation to habituate them to the procedure and the surroundings. Conditioning began on the third session. The neutral conditioned stimulus (CS, 100 ms) was paired with the aversive unconditioned stimulus (US, 100 ms) with a stimulus-free period of 500 ms (trace interval) that separated the CS offset from the US onset. The CS was either a pure tone (85 dB; 2.5 kHz pure tone) delivered through a speaker or a blinking white light (50 Hz) delivered by an LED mounted on the sidewall of the chamber. The US was a mild electrical stimulation (0.2–3.0 mA; 100 Hz square pulse) generated by a stimulus isolator (ISO-Flex, AMPI) and was delivered near the left eyelid via a pair of wire electrodes. The intensity of electric stimulation was initially set as 0.2 mA. Subsequently, it was adjusted daily for each mouse to induce a consistent magnitude of head-turn responses. The timing of stimulus presentations was controlled by a microcontroller (Arduino Mega), and the corresponding timestamps were stored in a RZ-5 recording system (Tucker-Davis Technologies).

During conditioning, the EMG activity was recorded from a pair of wire electrodes implanted in the left upper orbicularis oculi muscle as in previous studies (Kishimoto et al., 2001; Takehara et al., 2002). The EMG signal was filtered between 0.3 and 3.0 kHz, digitized at 6102 Hz, and stored using a RZ-5 recording system (Tucker-Davis Technologies).

Single-cue paradigm. This paradigm was used in optogenetic experiments (see Figs. 2, 4, and 5). Mice were placed in the chamber as described above. Daily conditioning was 50 min long and consisted of 100 trials of the tone CS presentations. The tone CS was followed by the US with the exception that the US was omitted in every 10th trial. These CS-alone trials are used to check the temporal pattern of EMG activity without the contamination of US artifacts and is a standard procedure in this paradigm (Kishimoto et al., 2001; Takehara et al., 2002). Trials were separated by a random intertrial interval between 20 and 40 s with a

mean of 30 s. In the last session (US off), the US was omitted, and the mice received 100 presentations of tone CS alone.

Differential paradigm. The differential paradigm was used for the photometric recording of cholinergic terminal activity (see Fig. 7) to compare the terminal responses to reinforced and nonreinforced stimuli in the same set of mice. Mice were placed in the chamber as described above. After 2 d of habituation, mice were placed in a square container (25 cm wide and 25 cm high, with rainbow patterns on three sides of the walls) to measure the basal cholinergic terminal responses to different stimuli. This session (Session 0) included 25 trials of light, tone, or eyelid shock presentations. The number of each trial type was set based on our preliminary observation that a minimum of 20 trials was required to obtain reliable stimulus-evoked terminal activity. They were separated in every 20–40 s (pseudo-randomized) over 38 min. Starting from the next day, each daily session included intermixed presentations of the tone or light stimulus alone (CS1-alone trials), the light or tone stimulus alone (CS2-alone trials), and pairings of the CS1 and mild eyelid shock (US; CS1-US trials). The stimulus assignment was counterbalanced across mice. During 10 days of conditioning (Sessions 1–10), each conditioning session included 25 CS1-alone, 100 CS1-US, and 25 CS2-alone trials. All trials were separated by a random intertrial interval between 20 and 40 s with a mean of 30 s. Three to 5 d after the last session, 7 mice underwent another session in which eyelid shock was presented by itself ~25 times. Across these presentations, the intensity of the shock was systematically changed across four levels: “Zero,” “Mild,” “Medium,” and “Strong” (5–7 presentations for each intensity level). In each mouse, the current that evoked minimum head-turn or scratching behavior was set as Mild intensity (0.271 ± 0.039 mA, $N = 7$). Medium and Strong intensities were set as the value 2 and 5 times as large as Mild intensity (Medium, 0.598 ± 0.083 mA, $N = 7$; Strong, 1.149 ± 0.125 mA, $N = 5$).

Behavioral analysis

As described previously (Volle et al., 2016; Jarovi et al., 2018), all EMG analyses were conducted using custom codes written in MATLAB (The MathWorks). For each session per mouse, the amplitude of EMG activity was computed by subtracting the minimum signal from the maximum signal in a series of 1 ms windows. EMG amplitude was averaged during a 300 ms window before CS presentation in each trial. The threshold was set as the median of averaged EMG amplitude plus 1 SD. EMG activity above the threshold was averaged together during a 300 ms pre-CS period (prevalue) and during a 200 ms window before US onset (CR value). The CR value was designed to capture the adaptive, anticipatory blinking responses that occur immediately before US onset. A trial was defined as a CR trial if the CR value was at least 5 times larger than the prevalue. In some trials, the prevalue exceeded 30% of the threshold because a mouse engaged in grooming, teeth grinding, or climbing behavior right before CS onset. These trials were defined as “hyperactive” and discarded. The percentage of the conditioned response (CR%) for each animal in a given session was the ratio of the CR trials relative to the total number of valid trials. To examine the effect of manipulations on the temporal patterns of CRs, we used the amplitude of EMG that was averaged across all trials in the last conditioning day. In each mouse, the averaged EMG amplitude was divided by the value during the pre-CS period. The normalized EMG amplitude was then averaged across all mice in each group (see Figs. 2E,I, 4D,H, and 5F). To quantify the speed of learning, we counted the number of sessions that each mouse took to show $\text{CR}\% > 38.7\%$ (learning criterion; see Figs. 2F, J, 4E,I, and 5G). This criterion was chosen based on the averaged CR% in the last three acquisition sessions of all control mice (38.7% , $N = 40$ mice). To quantify the degree to which mice acquired CRs selectively to the CS1, we calculated Diff-index (see Fig. 7E) by subtracting the across-session change in CR% in CS2-alone trials from that in CS1-alone trials.

Optogenetic manipulations

A blue (20 Hz, 2–5 mW, 40 ms pulse duration, 473 nm wavelength; Laserglow Technologies) or green (10–14 mW, 200 ms single pulse, 532 nm wavelength; Laserglow Technologies) laser was turned on for 200 ms starting from CS onset (ChR2-CS; Arch-CS) or 600 ms after CS onset (ChR2-US; Arch-US). The latter time window corresponds to the

onset of the US in CS–US paired trials and the omitted US in CS-alone trials. The laser was turned off on the first habituation session and Session 11.

Tissue collection and immunostaining

Three to 5 d after the last conditioning session, all mice (see Fig. 2) received an additional 20 min session in which they underwent 36 CS–US trials and 4 CS-alone trials with the same optogenetic manipulations during the US as those used during the acquisition sessions. Ninety minutes later, mice were subcutaneously administered with avertin (20 mg/kg) and transcardially perfused with 0.9% saline followed by chilled 4% PFA. The brains were immersed in 4% PFA for 48–72 h and were transferred to 30% sucrose/PBS and stored at 4°C. A series of 40 μm coronal sections were collected from the area containing the mPFC (AP: 1.3–2.4 mm) and BF (AP: 0.6 to -0.6 mm) using a cryostat (CM3050S, Leica Biosystems). The tissues were washed in PBS 3 times and incubated with 10% donkey (#D9663, Sigma-Aldrich, RRID:AB_2810235) or goat serum (#ab156046, Abcam) for 2 h at room temperature followed by 3 times wash in PBS.

1. To check the transgene expression and specificity, the BF sections were incubated with primary antibodies against ChAT (goat, #AB144P, 1:200, Millipore, RRID:AB_90661) or PV (mouse, #MAB1572, 1:800, Sigma-Aldrich, RRID:AB_2174013) for 24–36 h at 4°C. Following 3 times wash in PBS, the brain sections were then incubated with secondary antibody (rhodamine-conjugated donkey-anti-goat, #705-025-147, 1:200, Jackson ImmunoResearch Laboratories, RRID:AB_2340389; AlexaFluor-594-conjugated goat-anti-mouse, #115-585-003, 1:200, Jackson ImmunoResearch Laboratories, RRID:AB_2338871) for 2 h at room temperature. Finally, the BF sections were mounted using an antifade mountant with DAPI (#P36935, Thermo Fisher Scientific).
2. To examine the effect of BF cholinergic terminal manipulations on prefrontal vasoactive intestinal polypeptide- (VIP), somatostatin- (SST), and PV-positive interneuronal activation, the sections were triple-stained with primary antibodies against VIP (rabbit, #20077, 1:600, Immunostar, RRID:AB_572270), PV (mouse, #P3088, 1:800, Sigma-Aldrich, RRID:AB_477329), SST (rat, #MA5-16987, 1:400, Thermo Fisher Scientific, RRID:AB_2538460), and c-Fos (rabbit, #2250, 1:600, Cell Signaling Technology, RRID:AB_2247211; or mouse, #ab208942, 1:600, Abcam, RRID:AB_2747772) for 36–48 h at 4°C. After washing 3 times, the tissues were incubated with a cocktail of secondary antibodies (DyLight 405-conjugated donkey-anti-mouse, #715-475-150, 1:200, RRID:AB_2340839; AlexaFluor-488-conjugated donkey-anti-rat, #712-545-153, 1:200, RRID:AB_2340684; rhodamine-conjugated donkey-anti-rabbit, #711-025-152, 1:200, RRID:AB_2340588; AlexaFluor-488-conjugated donkey-anti-mouse, 1:200, #715-545-150, all from Jackson ImmunoResearch Laboratories, RRID:AB_2340846) for 2 h at room temperature. Subsequently, the sections were washed 3 times in PBS and were mounted with an antifade mountant (#P36961, Thermo Fisher Scientific).

Image acquisition

Images used for cell counting were obtained using a confocal microscope (Carl Zeiss) under a 20 $\times$ objective. Images used for axon colocalization were obtained using the confocal microscope under a 60 $\times$ objective. Images used for viral spread and optic fiber track identification were collected using an upright fluorescence microscope with a 10 $\times$ objective.

Cell counting

Cell counting was conducted manually using FIJI (ImageJ) software (National Institutes of Health). Transgene expression specificity and efficiency were calculated as a percentage of the total number of ChAT⁺ or PV⁺ cells relative to the total number of fluorophore-labeled cells and a percentage of the total number of fluorophore-labeled cells relative to the total number of ChAT⁺ or PV⁺ cells. In each image, the number of c-Fos⁺ cells was quantified and divided by the area to obtain a density measure. To assess prefrontal interneuronal activation following optogenetic manipulation of cholinergic terminal activity, the c-Fos expression in different interneurons (a proportion of the total number of c-Fos and

SST⁺ or PV⁺ or VIP⁺ colocalized cells relative to the total number of SST⁺ or PV⁺ or VIP⁺ cells) was calculated. To compare the manipulation effects on task-induced activity across three interneuron types, the proportion of double-positive cells in the Chr2 or Arch group was divided by that of the Control group in each interneuron type.

Fiber photometry

Recording. To measure the bulk fluorescence from a genetically encoded calcium sensor, GCaMP6s was expressed in BF cholinergic terminals in the PrL. We used a fiber photometry system with two excitation wavelengths. The two LEDs were modulated at distinct frequencies (Tucker-Davis Technologies, RZ-5 recording system; Doric LED driver): 381 Hz for 465 nm (calcium-dependent excitation wavelength) and 221 Hz for 405 nm (calcium-independent excitation wavelength). The excitation light was delivered through a fiber cable (400 μ m; Thorlabs) to a 400- μ m-core optic fiber (0.48 NA; Thorlabs) connected via a zirconia sleeve (Thorlabs). The intensity of each excitation LED was set so that the output from the implanted optic fiber tip was ~ 30 μ W (PM100D; Thorlabs). The fluorescence emission signal of GCaMP6s was converted into a continuous electric signal by a femtowatt photoreceiver (Newport; Doric). It was then demodulated and sampled at 1 kHz and stored in a RZ-5 recording system (Tucker-Davis Technologies). To avoid potential photobleaching by continuous excitation during the entire session (~ 75 min), the LEDs were turned on for 9 s starting from 4 s before each stimulus onset. Because in our system, the emission signal became stabilized within 1.5 s after LED onset, we used the signals starting from 1.5 s after LED onset for subsequent analyses.

Data analysis. Photometry signals were analyzed with custom codes written in MATLAB (The MathWorks). To isolate calcium-dependent signals from general fluorescent changes, we first aligned the 405 signal to the 465 signal in each trial by using their values in the time window outside the stimulus presentation periods (a 2 s window immediately before stimulus onset and a 0.9 s window 6 s after stimulus onset). The two sets of values were used to fit a linear regression line, and the 405 signal was corrected with the slope and offset of the regression model. This step made the 405 signal comparable to the 465 signal unless the 465 signal fluctuated in response to the change in calcium in cholinergic terminals. The $\Delta F/F$ was calculated as (465 nm signal – corrected 405 nm signal)/corrected 405 nm signal. For each session per mouse, US-evoked cholinergic response was calculated by subtracting the averaged $\Delta F/F$ trace in CS1-alone trials from averaged $\Delta F/F$ trace in CS1-US paired trials. The area under the curve (AUC) was calculated as the sum of $\Delta F/F$ during a 2.5 s window after stimulus onset (0–2.5 s after CS onset for CS response and 0.6–3.1 s after CS onset for US response). The baseline activity was calculated as the sum of $\Delta F/F$ during a 2.5 s window starting from 2.5 s before stimulus onset. To examine the relationship between cholinergic responses to tone VS light in naive mice in relation to the optic fiber location (see Fig. 6F), an increase in response was considered if stimulus-evoked response (AUC) was larger than 100 and also greater than its baseline AUC by 100. To compare the change in the terminal responses to the CS1, CS2, and US across sessions, the AUC in Session 1 was subtracted from the AUC in all sessions (Δ AUC) (see Fig. 7D).

Statistical analysis

The sample size was chosen based on our previous studies (Takehara et al., 2002; Volle et al., 2016; Jarovi et al., 2018). The data were presented as the group mean $\pm$ SEM. Statistical analyses were performed with GraphPad Prism. To determine the statistical significance, we used one-way (repeated) ANOVA, two-way mixed/repeated-measures ANOVA, unpaired *t* test, and Pearson correlation. Degrees of freedom were corrected with Greenhouse–Geisser correction when sphericity was violated. For *post hoc* analyses comparing c-Fos expression among groups, Dunnett's T3 multiple comparisons test was used following one-way ANOVA because of different SDs across groups. For *post hoc* analyses comparing cholinergic responses to stimulus, Tukey's multiple comparison test was used following one-way repeated-measures ANOVA.

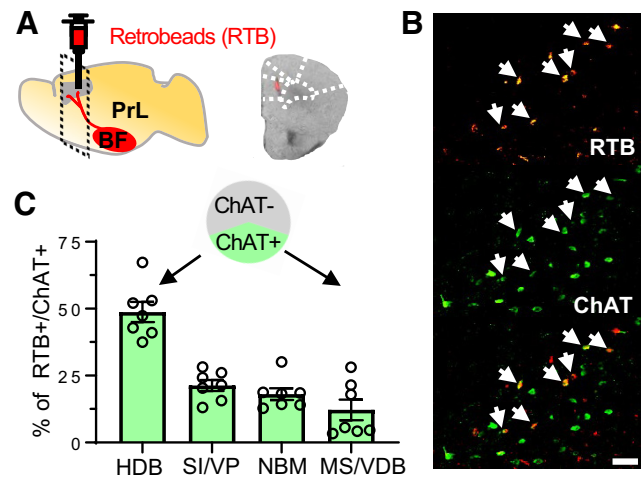


Figure 1. The horizontal diagonal band of Broca provides the primary cholinergic input into the prelimbic cortex. **A**, Experimental design and a representative image showing Red retrobeads (RTB) injected into the PrL of the mPFC. **B**, Representative images of RTB-labeled cells (top), cholinergic neurons immunolabeled with ChAT (middle), and their overlap (bottom) in the BF. Scale bar, 50 μ m. **C**, Top, The proportion of cells immunoreactive for ChAT among RTB-labeled cells. Bottom, The proportion of PrL-projecting cholinergic neurons in various BF subregions (mean $\pm$ SEM, $N = 7$), including the HDB, SI/VP, NBM, and MS/VDB.

Results

Suppression of shock-evoked cholinergic terminal activity facilitates the formation of stimulus–shock associations

Because the BF contains several subregions and extends across the anterior–posterior axis, we first located the source of the densest BF projections to the PrL. The injection of a retrograde tracer (Retrobeads) into the PrL labeled cells in multiple BF subregions (Fig. 1A,B). Among them, $\sim 38\%$ were immunoreactive for choline acetyltransferase (ChAT, the enzyme responsible for the synthesis of ACh), suggesting that they were cholinergic neurons (Fig. 1C). The most robust labeling was found in the horizontal diagonal band of Broca (HDB) of the BF along with weaker labeling in the substantia innominata/ventral pallidum (SI/VP), medial septum/ventral diagonal band of Broca (MS/VDB), and nucleus basalis of Meynert (NBM; Fig. 1C; $N = 7$). Based on these findings, all subsequent experiments targeted the projection from the HDB to the PrL.

We first manipulated the activity of cholinergic terminals during aversive outcomes and examined the impact on memory acquisition in trace eyeblink conditioning (Fig. 2A,B). In this paradigm, mice received pairings of a tone (CS) with a mild eyelid shock (US) over a short temporal gap (500 ms; Fig. 2B). With learning, they developed anticipatory blinking responses (CRs), which were detected by recording EMG activity from the eyelid (Fig. 2B). ChAT^(IRES)-Cre knock-in mice received the infusion of adeno-associated viral vectors (AAVs) carrying cre-dependent forms of transgenes into the HDB (Fig. 2A). Archaelhodopsin-3 (Arch) with GFP or GFP alone was expressed for terminal silencing, while channelrhodopsin-2 (ChR2) with EYFP or EYFP alone was expressed for terminal stimulation. These transgenes were expressed in $\sim 80\%$ of cholinergic neurons in the HDB (Fig. 2C, G; GFP, $81.3 \pm 2.7\%$, $N = 10$; EYFP, $76.2 \pm 6.0\%$, $N = 8$) and were expressed almost exclusively in cholinergic neurons (GFP, $97.1 \pm 1.4\%$; EYFP, $91.6 \pm 1.6\%$).

During daily conditioning sessions, mice received laser pulses through implanted optic fibers targeting the PrL (Fig. 2C,G). When the green laser (532 nm) was applied for 200 ms during the US, both GFP- and Arch-expressing mice increased the

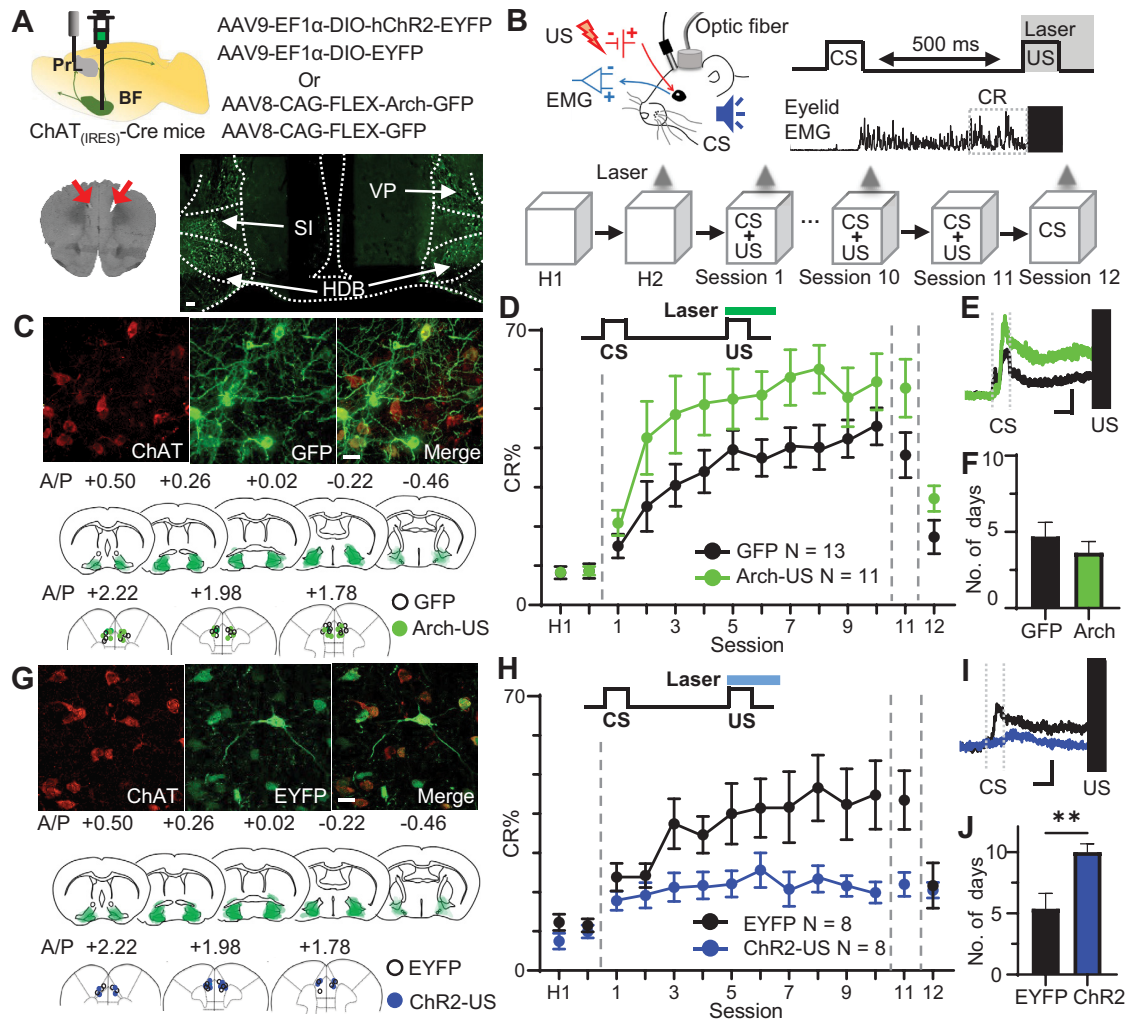


Figure 2. Suppression of shock-evoked cholinergic terminal activity facilitates aversive associative learning. **A**, Top, The site of viral vector infusion and optic fiber implantation. Bottom right, Representative image showing optic fibers placement in the PrL. Bottom left, Representative image showing green fluorescent signals from virally infected cells in the BF. Scale bar, 100 μ m. **B**, Top, Trace eyeblink conditioning and EMG activity from a representative trial with CR expression. Bottom, Experimental schedule. Triangle represents laser delivery. **C**, Top, Representative image showing the colocalization of ChAT and virally infected cells expressing GFP. Scale bar, 10 μ m. Bottom, Schematic representations of virus spread in the BF and optic fiber placements in the PrL. **D**, The percentage of trials containing CRs (CR%) of mice expressing Arch ($N = 13$) or GFP ($N = 11$). Top, Diagram represents the timing of laser delivery. **E**, The averaged normalized EMG amplitude in Arch-expressing (green, $N = 13$) and GFP-expressing mice (black, $N = 11$) on Session 10. Scale bar, $0.1 \text{ s} \times 100\%$ change from baseline. **F**, The number of sessions required to reach the learning criterion in Arch- and GFP-expressing mice. **G**, The same as in **C**, but for mice expressing ChR2 or EYFP. **H–J**, The same as **D–F**, but for ChR2-expressing (blue, $N = 8$) and EYFP-expressing (black, $N = 8$) mice. Data are mean $\pm$ SEM. $**p < 0.01$.

frequency of CR expression across sessions; however, Arch-expressing mice showed CRs more frequently than GFP-expressing mice (two-way mixed ANOVA, Session $\times$ Group interaction, $F_{(9,198)} = 0.546$, $p = 0.840$; a main effect of session, $F_{(2,045, 45)} = 11.56$, $p < 0.001$; a main effect of group, $F_{(1,22)} = 4.834$, $p = 0.039$; after Greenhouse–Geisser correction; Fig. 2D). In both groups, the amplitude of eyelid EMG was increased shortly after CS onset and maintained the elevated level toward US onset (Fig. 2E). Also, two groups took a comparable number of sessions to reach the typical asymptomatic performance in this task (i.e., expressing CRs in 38.7% of CS presentations, “learning criterion”; t test, $t_{(22)} = 0.861$, $p = 0.399$; Fig. 2F). When the laser was omitted, the Arch-expressing mice still showed CRs frequently (Session 11; t test, $t_{(22)} = 1.848$, $p = 0.078$; Fig. 2D), suggesting that the manipulation enhanced the formation of CS–US associations but not the movement of the eyelid muscle. Furthermore, both groups reduced the frequency of CR expression when the US was

omitted (Session 12; t test, $t_{(22)} = 1.769$, $p = 0.091$; Fig. 2D), confirming that the manipulation facilitated CS–US association formation rather than nonassociative sensitization.

In stark contrast to the enhanced learning with the terminal inhibition, the terminal excitation severely impaired learning. When the blue laser (473 nm; 20 Hz) was applied during the US, ChR2-expressing mice expressed CRs less frequently than EYFP-expressing mice (two-way mixed ANOVA, Session $\times$ Group interaction, $F_{(9,126)} = 1.531$, $p = 0.144$; a main effect of session, $F_{(1,946, 27.25)} = 3.045$, $p = 0.065$; a main effect of group, $F_{(1,14)} = 7.448$, $p = 0.016$; after Greenhouse–Geisser correction; Fig. 2H). Although the ChR2 group mice exhibited some eyelid movement after the CS, they failed to develop the adaptive blinking responses that normally occurred near the expected US onset (Fig. 2I). Also, ChR2-expressing mice took longer days to reach the learning criterion than EYFP-expressing mice (t test, $t_{(14)} = 3.243$, $p = 0.006$; Fig. 2J). The impaired CR expression in ChR2-expressing mice persisted in the session without the laser

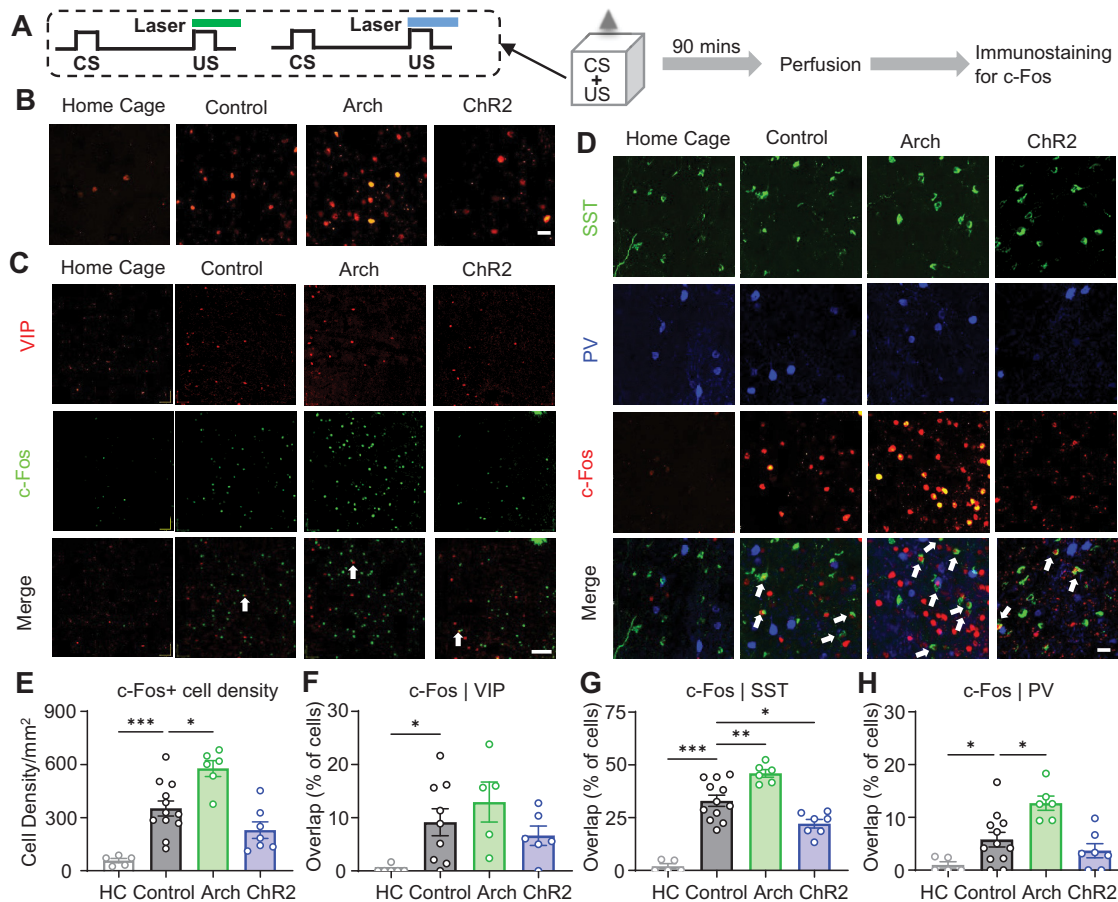


Figure 3. Shock-evoked cholinergic terminal activity affects the task-related activation of prefrontal cells. **A**, Experimental design. Green and blue lines indicate the timing of laser delivery. **B**, Representative images showing cells expressing c-Fos (red) in the PrL. Scale bar, 20 μ m. **C**, Representative images showing cells expressing VIP (red) or c-Fos (green). Scale bar, 100 μ m. **D**, Representative images showing cells expressing SST (green), PV (blue), or c-Fos (red). Scale bar, 20 μ m. **E**, The densities of c-Fos-expressing cells in the deep layers of the PrL in HC control ($N = 5$), GFP/EYFP-expressing (CTL, $N = 12$), Arch-expressing ($N = 6$), and ChR2-expressing ($N = 7$) mice. **F–H**, The proportion of c-Fos-expressing cells among cells immunoreactive for VIP, SST, or PV. Data are mean $\pm$ SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

stimulation (Session 11, t test, $t_{(14)} = 1.761$, $p = 0.019$; Fig. 2H), suggesting that cholinergic terminal stimulation impaired the acquisition, but not the expression of CRs. These findings also confirm that the enhanced learning brought about by the suppression of cholinergic terminals (Fig. 2D) was not because of rebound potentials that might occur when using Arch (Mahn et al., 2016).

Shock-evoked cholinergic signal modulates the learning-induced activation of PrL neurons

Next, we investigated how shock-locked phasic cholinergic signals affected local neural processing. To this end, we monitored the expression of an activity-regulated gene, c-Fos, as a measure of task-induced neural activation in the PrL. Three to 5 d after completing the 14 daily sessions (Fig. 2), mice underwent the conditioning for 20 min with optogenetic manipulation (Fig. 3A). The brains were sampled 90 min later for immunohistochemistry against c-Fos. After the conditioning, the proportion of neurons expressing c-Fos was elevated in control mice expressing GFP or EYFP (CTL) compared with naive mice that stayed in the home cage (HC; one-way ANOVA, $F_{(3,27)} = 20.052$, $p < 0.001$; *post hoc* Dunnett's T3, HC vs CTL, $p < 0.001$; Fig. 3B, E). Photo-inhibition of cholinergic terminals further increased the density of c-Fos-expressing cells (CTL vs Arch, $p = 0.016$), whereas their photo-excitation did not significantly change

expression (CTL vs ChR2, $p = 0.330$). The latter finding alludes that shock-evoked cholinergic signaling might not directly affect the most abundant cell type, pyramidal neurons.

We thus consider a possible involvement of interneurons and conducted double immunostaining of c-Fos and markers for interneurons expressing SST, PV, or VIP. Conditioning increased c-Fos expression in interneurons expressing VIP ($F_{(3,22)} = 4.223$, $p = 0.017$; HC vs CTL, $p = 0.041$; Fig. 3C,F), SST ($F_{(3,26)} = 40.905$, $p < 0.001$; HC vs CTL, $p < 0.001$; Fig. 3D,G), and PV ($F_{(3, 23.41)} = 12.73$, $p < 0.001$; HC vs CTL, $p = 0.039$; Fig. 3D,H). The cholinergic terminal inhibition increased the task-induced activation of SST⁺ (CTL vs Arch, $p = 0.006$) and PV⁺ (CTL vs Arch, $p = 0.018$), but not VIP⁺ interneurons (CTL vs Arch, $p = 0.114$). The cholinergic terminal excitation suppressed the task-induced activation of SST⁺ interneurons (CTL vs ChR2, $p = 0.026$), but not PV⁺ (CTL vs ChR2, $p = 0.849$) or VIP⁺ interneurons (CTL vs ChR2, $p = 0.953$). To directly compare the manipulation effects on task-induced activity of the three interneuron types, the c-Fos expression in ChR2- and Arch-expressing mice were divided by that in control mice in each interneuron type ("vs Control" in Table 1). We found that c-Fos expression in VIP⁺, SST⁺, and PV⁺ neurons was greater in Arch-expressing mice than ChR2-expressing mice (two-way mixed ANOVA, Manipulation $\times$ Interneuron type interaction, $F_{(2,31)} = 2.710$,

Table 1. Raw and normalized count of task-activated interneurons in each interneuron type, the counts of cells immunoreactive for each interneuron marker, and those for both the interneuron marker and c-Fos^a

Interneuron type		HC	Control	Arch	ChR2
VIP	cFos ⁺ and VIP ⁺	0.2 ± 0.2	2.8 ± 0.6	4.0 ± 1.1	2.6 ± 0.9
	VIP ⁺	20.3 ± 5.8	40.2 ± 3.3	31.8 ± 4.6	34.8 ± 4.2
	vs Control			1.4 ± 0.4	0.7 ± 0.2
SST	cFos ⁺ and SST ⁺	0.4 ± 0.2	12.1 ± 1.5	15.2 ± 2.0	7.4 ± 4.1
	SST ⁺	18.0 ± 1.0	34.5 ± 3.0	33.3 ± 3.7	31.9 ± 5.1
	vs Control			1.4 ± 0.1	0.7 ± 0.1
PV	cFos ⁺ and PV ⁺	0.3 ± 0.2	2.0 ± 0.4	3.8 ± 1.0	1.3 ± 0.4
	PV ⁺	14.2 ± 2.3	33.3 ± 3.2	29.5 ± 5.6	29.3 ± 2.2
	vs Control			2.2 ± 0.2	0.6 ± 0.2

^avs Control^a shows the proportion of the latter double-labeled cells in Arch and ChR2 groups divided by that in control group.

$p = 0.082$; a main effect of manipulation, $F_{(1,31)} = 31.93$, $p < 0.001$; a main effect of interneuron type, $F_{(2,31)} = 2.030$, $p = 0.148$; Table 1). These patterns suggest that shock-evoked cholinergic activity modulated all three interneuron types in a comparable manner.

Suppression of stimulus-evoked cholinergic terminal activity impairs the formation of stimulus–shock associations

In addition to strong firing responses to aversive stimuli (Hangya et al., 2015), BF cholinergic neurons become excited by sensory stimuli as they become associated with aversive outcomes (Guo et al., 2019). To investigate how the stimulus-evoked cholinergic signal affects associative learning, we next manipulated cholinergic terminal activity in the PrL during the CS. As in the experiment of the terminal manipulation during the US (Fig. 2A), Arch and ChR2 were expressed in cholinergic neurons in the HDB, and mice underwent the same 14 daily conditioning sessions (Fig. 4A). The laser was delivered through optic fibers in the PrL for 200 ms starting from CS onset (Fig. 4A,B,F). Throughout 10 acquisition sessions, Arch-expressing mice showed CRs less frequently than GFP-expressing mice (two-way mixed-measures ANOVA, Session × Group interaction, $F_{(9,189)} = 0.999$, $p = 0.443$; a main effect of session, $F_{(2,262, 47.49)} = 6.629$, $p = 0.002$; a main effect of group, $F_{(1,21)} = 7.202$, $p = 0.014$; after Greenhouse–Geisser correction; Fig. 4C). The temporal patterns of EMG activity were comparable between the two groups (Fig. 4D); however, Arch-expressing mice took longer sessions to reach the learning criterion than GFP-expressing mice (t test, $t_{(21)} = 3.06$, $p = 0.006$; Fig. 4E). When the laser was turned off, they still expressed the CRs infrequently (Session 11, t test, $t_{(20)} = 1.725$, $p = 0.013$; Fig. 4C), suggesting impairments in CR acquisition but not CR expression.

In contrast, ChR2-expressing mice showed comparable CR acquisition to EYFP-expressing mice (Session × Group interaction, $F_{(9,126)} = 0.767$, $p = 0.647$; a main effect of session, $F_{(2,203, 30.84)} = 4.727$, $p = 0.014$; a main effect of group, $F_{(1,14)} = 1.923$, $p = 0.187$; after Greenhouse–Geisser correction; Fig. 4G). Both groups showed comparable temporal patterns of CRs (Fig. 4H) and reached the learning criterion at a similar speed (t test, $t_{(14)} = 1.229$, $p = 0.234$; Fig. 4I). These findings suggest that stimulus-evoked phasic cholinergic activity promotes aversive associative learning, which was opposite from the inhibitory effects of shock-evoked phasic cholinergic activity (Fig. 2).

mPFC-projecting BF PV-positive neurons are not involved in the formation of stimulus–shock associations

Consistent with previous anatomic studies (Henny and Jones, 2008; Ährlund-Richter et al., 2019), our retrograde tracing data

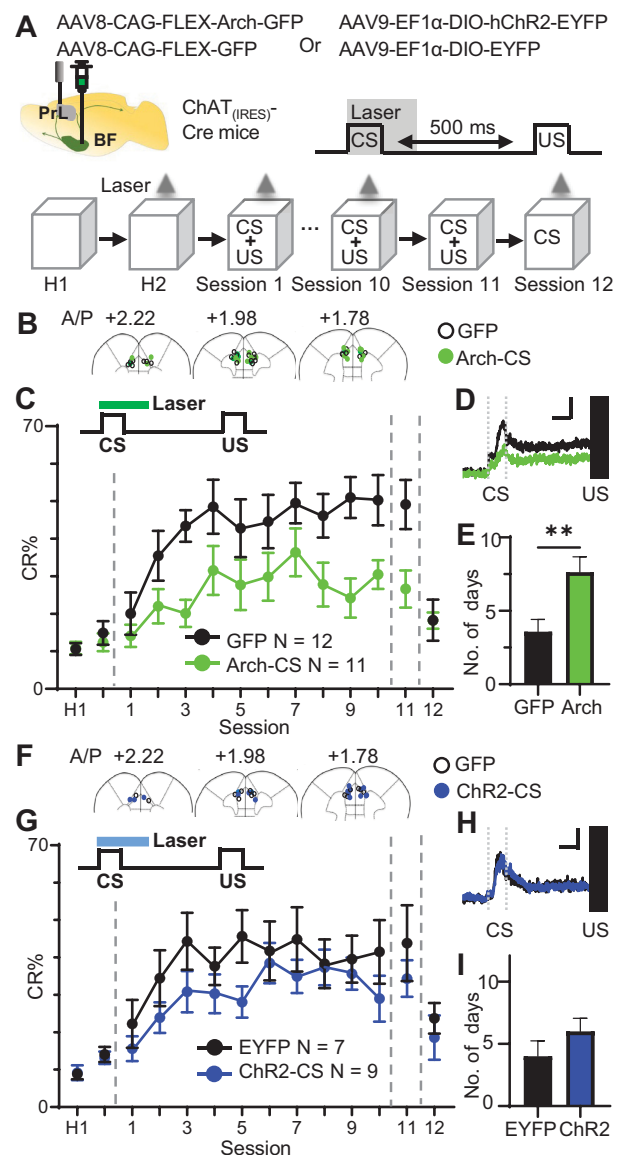


Figure 4. Suppression of stimulus-evoked cholinergic terminal activity impairs aversive associative learning. **A**, Experimental design and schedule. **B**, Optic fiber placements in the PrL. **C**, The percentage of trials containing CRs (CR%) of Arch-expressing ($N = 11$) or GFP-expressing ($N = 12$) mice. Top, Diagram represents the timing of laser delivery. **D**, The averaged normalized EMG amplitude in Arch-expressing (green, $N = 12$) and GFP-expressing mice (black, $N = 11$). Scale bar, $0.1 \text{ s} \times 100\%$ change from baseline. **E**, The number of sessions required to reach the learning criterion in Arch- and GFP-expressing mice. **F–I**, The same as in **B–E**, but for mice expressing ChR2 (blue, $N = 9$) or EYFP (blue, $N = 7$). Data are mean $\pm$ SEM. $^{**}p < 0.01$.

showed that $\sim 62\%$ of PrL-projecting cells in the BF were not immunoreactive for ChAT (Fig. 1C), suggesting that they were GABAergic or glutamatergic neurons (Brashear et al., 1986; Zaborszky et al., 1999; Hur and Zaborszky, 2005). Unlike cholinergic neurons, noncholinergic neurons do not show phasic firings that are precisely time-locked to aversive stimuli (Hangya et al., 2015; Harrison et al., 2016). Thus, the manipulation of noncholinergic terminals during the US should not affect CR acquisition if the enhanced learning (Fig. 2D) was because of the suppression of shock-locked cholinergic signals. In addition, among GABAergic neurons in BF, many express PV (Gritti et al., 2003; Henny and Jones, 2008; McKenna et al., 2013). Because

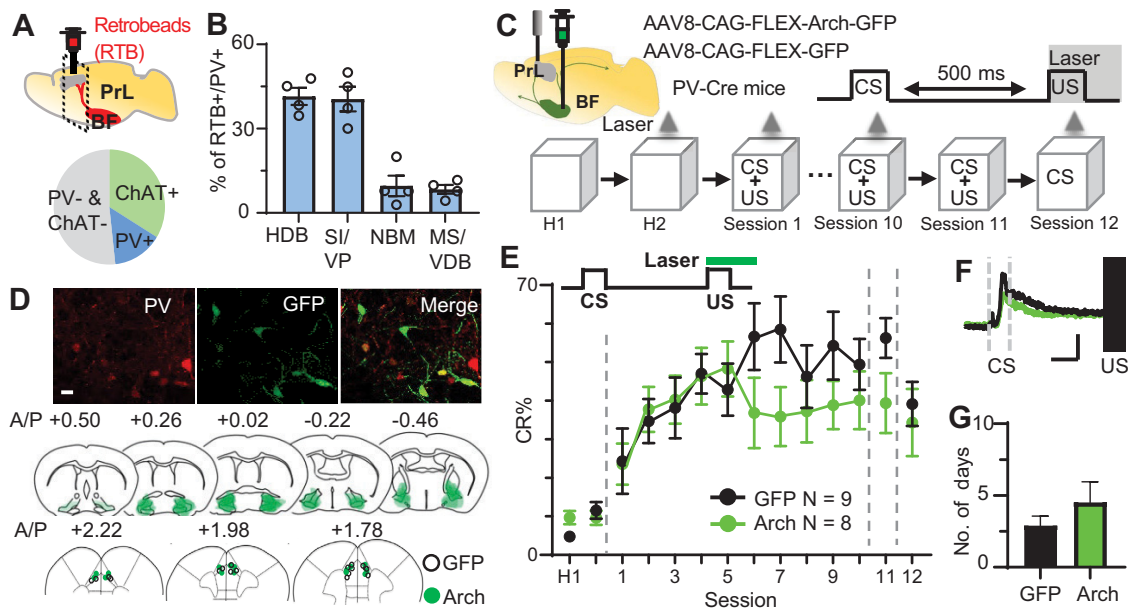


Figure 5. Prefrontal-projecting BF PV⁺ cells are not involved in aversive associative learning. **A**, Top, Experimental design. Bottom, The proportion of BF cells immunoreactive for ChAT or PV among cells labeled with Retrobeads (RTB) infused into the PrL. **B**, The proportion of RTB-labeled PV⁺ cells in BF subregions ($N=4$), as in Figure 1C. **C**, Experimental design and schedule. **D**, Top, Representative images showing colocalization of GFP with PV⁺ cells in the BF. Scale bar, 10 μ m. Bottom, Schematic representations of virus spread in the BF and optic fiber placements in the PrL. **E**, The percentage of trials containing CRs (CR%) of Arch-expressing ($N=8$) and GFP-expressing ($N=9$) mice. Top, Diagram represents the timing of laser delivery. **F**, The averaged normalized EMG amplitude in Arch-expressing (green, $N=9$) and GFP-expressing mice (black, $N=8$). Scale bar, 0.1 s $\times$ 100% change from baseline. **G**, The number of sessions to reach the learning criterion in Arch- and GFP-expressing mice. Data are mean $\pm$ SEM.

these PV⁺ neurons are excited by neighboring cholinergic neurons (Yang et al., 2014), shock-evoked excitation of cholinergic neurons might activate PV⁺ neurons and, in turn, indirectly modulate the PrL.

To address these points, we began by confirming the projection of BF PV⁺ neurons to the PrL with retrograde tracing. Among BF noncholinergic neurons projecting to the PrL, ~22% were PV⁺ ($N=4$; Fig. 5A), and most of them were found in the HDB and SI/VP (Fig. 5B). To manipulate the activity of these PrL-projecting PV⁺ neurons in the BF, AAV encoding a Cre-dependent Arch or GFP gene was infused into the HDB of PV-Cre mice expressing Cre recombinase in PV⁺ neurons (Fig. 5C). These opsins were expressed in about half of PV⁺ neurons in the BF ($53.4 \pm 9.5\%$), and most of the infected neurons were immunoreactive for PV ($71.2 \pm 11.7\%$; Fig. 5D). Although the specificity of transgene expression was lower than that in cholinergic neurons (Fig. 2C,G), it was comparable to previous work using the PV-Cre transgenic mouse line to target BF PV⁺ neurons (T. Kim et al., 2015). When green laser was delivered during the US through optic fibers implanted in the PrL (Fig. 5C), Arch-expressing mice acquired CRs comparably to GFP-expressing mice (two-way mixed ANOVA, Session $\times$ Group interaction, $F_{(9, 135)} = 1.397$, $p = 0.195$; a main effect of session, $F_{(2, 797, 41.96)} = 2.962$, $p = 0.046$; a main effect of group, $F_{(1, 15)} = 0.993$, $p = 0.335$; after Greenhouse–Geisser correction; Fig. 5E). Their CR% was comparable in sessions without the laser (Session 11; t test, $t_{(15)} = 1.858$, $p = 0.083$; Fig. 5E) or without the US (Session 12; t test, $t_{(15)} = 0.473$, $p = 0.643$; Fig. 5E). The two groups also showed similar temporal patterns of CRs (Fig. 5F). Also, the number of sessions to reach the learning criterion did not differ between the two groups (t test, $t_{(15)} = 1.045$, $p = 0.312$; Fig. 5G). These findings support a view that memory enhancement was induced uniquely by the manipulation of outcome-locked phasic cholinergic signals and that this effect was not supported indirectly through cortically projecting PV⁺ cells in the BF. They, however, leave a

possibility open that the lack of behavioral effects might be because of insufficient targeting or inhibition of projections of BF PV⁺ cells.

Cholinergic terminal activity in the mPFC tracks the development of stimulus–shock associations

Last, to confirm the presence of stimulus- and shock-evoked phasic cholinergic signaling during our task, we conducted photometric recording from cholinergic terminals in the PrL while mice formed CS–US associations. A Cre-dependent AAV that expresses an axon-targeted form of the genetically encoded calcium indicator, GCaMP6s (Broussard et al., 2018) was infused into the HDB in ChAT_(IRES)-Cre knock-in mice (Fig. 6A). The terminal fields expressing GCaMP6s were distributed across all layers in the PrL and were also immunoreactive for ChAT (Fig. 6B). Through a fiber implanted in the PrL, we measured the calcium-dependent excitation wavelength (465 nm) and the calcium-independent isosbestic wavelength (405 nm; Fig. 6C). This approach allows for ratiometric measurements of GCaMP6s activity, thereby correcting motion-related artifacts (Lerner et al., 2015). In naive mice, cholinergic terminals were excited strongly by light and eyelid shock but only marginally by tone (Fig. 6D). To quantify the magnitude of stimulus-evoked terminal responses, we integrated the signal over a 2.5 s window before and after the stimulus presentations (the AUC). Compared with the AUC at baseline, the AUC became significantly larger after the light and shock, but not tone presentations (Fig. 6E; two-way repeated-measures ANOVA, Period $\times$ Stimulus type interaction, $F_{(1, 933, 46.39)} = 16.51$, $p < 0.001$; main effect of Stimulus Type, $F_{(1, 911, 45.85)} = 9.660$, $p < 0.001$; main effect of Period, $F_{(1, 2400)} = 21.01$, $p < 0.001$; *post hoc* Tukey: baseline VS tone, $p = 0.991$; baseline VS light, $p = 0.019$; baseline VS eyelid shock, $p < 0.001$). The light- and shock-evoked responses were comparable to one another but larger than the tone-evoked responses (*post hoc* Tukey, tone vs eyelid shock, $p < 0.001$; tone vs light, $p = 0.005$;

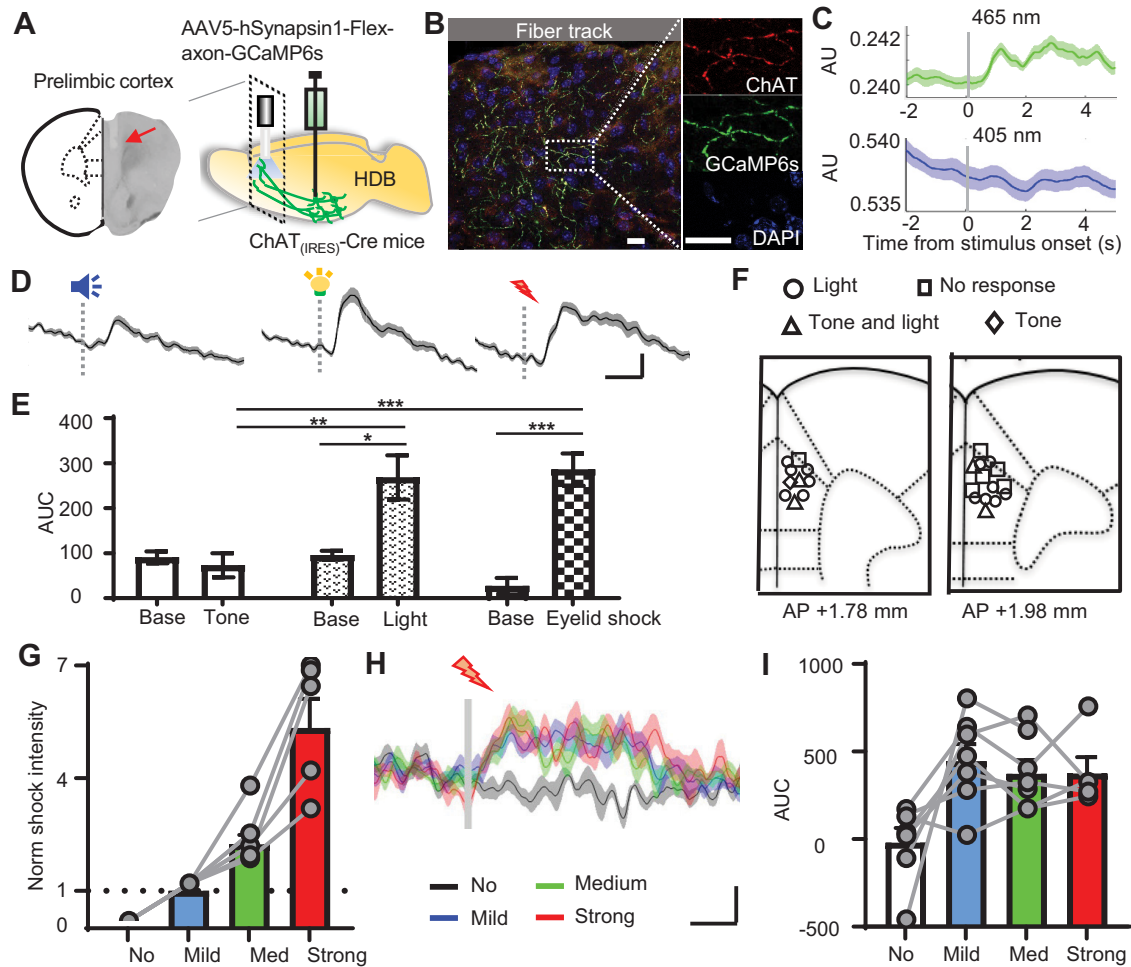


Figure 6. Cholinergic terminals are activated by eyelid shock and light in naive mice. **A**, Experimental design and a representative image of an optic fiber track in the PrL. **B**, Higher-magnification images showing colocalization of GCaMP6s-expressing axons (green) and ChAT-positive axons (red) in the PrL. Scale bars, 15 μ m. **C**, Averaged traces of calcium-dependent (465 nm, top) and calcium-independent signals (405 nm, bottom) on a presentation of eyelid shock from 1 mouse. Vertical lines indicate stimulus onset. Shade represents SEM across 29 shock presentations. **D**, Averaged traces of motion-corrected calcium signals ($N = 25$) during the presentations of tone (left), light (middle), and eyelid shock (right). Shade represents SEM across mice. Dotted lines indicate the stimulus onset. Scale bars, 1 s $\times$ 0.1% $\Delta F/F$. **E**, The magnitude of calcium activity before (Base) and after stimulus presentations, quantified as the AUC. **F**, Diagrams of the placement of optic fibers. Each marker represents the location of fiber tips in a mouse. Marker types indicate the presence/absence of tone- or light-evoked responses greater than the maximum value in the baseline. **G**, Three levels of shock intensity used to measure shock-evoked terminal responses (Mild, $N = 7$; Medium, $N = 7$; and Strong, $N = 5$). **H**, Averaged cholinergic terminal responses to eyelid shock. Shade represents SEM across mice. Scale bar, 1 s $\times$ 0.1% $\Delta F/F$. **I**, The magnitude of shock-evoked responses. Data are mean $\pm$ SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

eyelid shock vs light, $p = 0.998$). The magnitude of tone-evoked responses varied greatly across mice, some with an apparent increase in the signal from baseline and others with no change (data not shown). We suspected that this variation might be because of the location of optic fiber within the PrL; however, we did not find any relation between the stimulus-evoked responses and the placements of the fibers in the PrL (Fig. 6F).

When the intensity of eyelid shock was systematically changed (Fig. 6G), the magnitude of shock-evoked responses was maintained stably across the different intensity levels (Fig. 6H,I; one-way repeated-measures ANOVA, $F_{(2,16)} = 0.215$, $p = 0.809$). This observation indicates that cholinergic terminal activity was not sensitive to the pain level.

We then examined how cholinergic terminal responses changed with aversive associative learning. To isolate learning-related changes from unrelated changes, we used a differential paradigm (Fig. 7A) that included two CSs. One of them (CS1; tone or light, counterbalanced) was paired with the eyelid shock, while the other stimulus (CS2; light or tone) was presented alone. The CS2 served as an internal control to test whether the changes

in the terminal activity were related to habituation to repeatedly presented stimuli. To isolate stimulus-evoked terminal activity, the CS1 was presented alone $\sim 20\%$ of the time. These three types of trials, CS1-US (100 trials), CS1-alone (25 trials), and CS2-alone (25 trials), were intermixed and presented every 20–40 s. Across 10 daily sessions, mice developed anticipatory blinking responses to the CS1 regardless of CS1 sensory modality (two-way repeated-measures ANOVA, Sessions $\times$ Trial types interaction, $F_{(8,128, 197.2)} = 8.695$, $p < 0.001$; follow-up one-way repeated-measures ANOVA, CS1-alone trials, $F_{(4,341, 104.2)} = 4.857$, $p < 0.001$; CS1-US trials, $F_{(4,295, 103.1)} = 6.379$, $p < 0.001$; after Greenhouse–Geisser correction; Fig. 7B). Unexpectedly, mice initially blinked more frequently to the CS2 than the CS1; however, they learned to withhold the responses to the CS2 (CS2-alone trials, $F_{(5,862, 140.7)} = 4.001$, $p = 0.001$; Fig. 7B).

In parallel, the cholinergic terminals were strongly excited by the US and CS2 in the first session; however, these responses were gradually weakened over subsequent sessions (Fig. 7C). In contrast, the terminal responses to the CS1 were initially weak but gradually strengthened as mice formed CS–US associations.

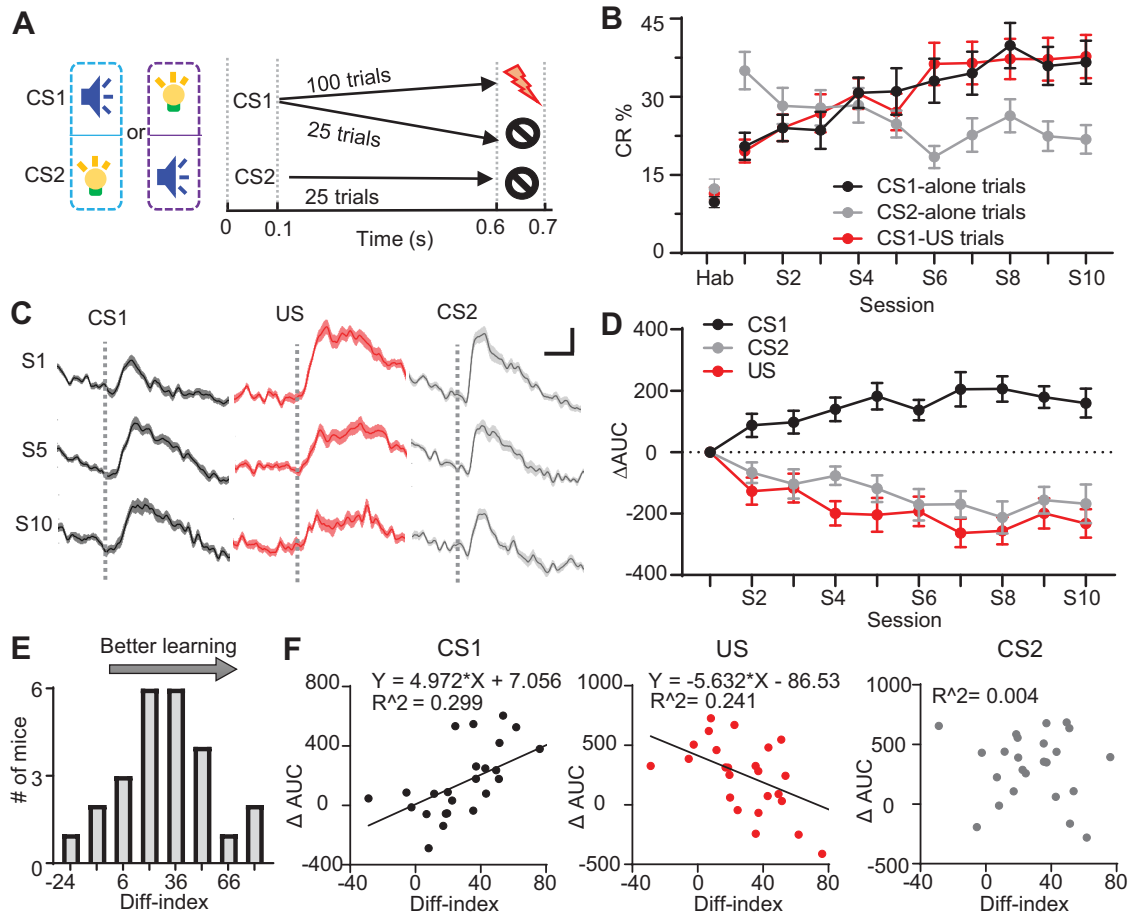


Figure 7. Cholinergic terminals weaken innate shock-evoked responses but strengthen learned responses to shock-predictive stimuli. **A**, Differential paradigm. **B**, Frequency of CR expression (CR%) in three trial types. **C**, Photometric traces of motion-corrected calcium responses ($N = 25$) to the CS1 (left), US (middle), and CS2 during the conditioning (S1, 5, 10). Shade indicates SEM across mice. Dotted lines indicate the onset of the stimulus. Scale bars represent $1 \text{ s} \times 0.1\% \Delta F/F$. **D**, The change in the magnitude of calcium activity evoked by the CS1, US, and CS2 across sessions (normalized to Session 1; ΔAUC). **E**, Distribution of the across-session improvement in behavioral differentiation between the CS1 and CS2 (Diff-index, $N = 25$). **F**, The correlation between Diff-index and the across-session change in the evoked terminal responses (ΔAUC). Data are mean $\pm$ SEM.

To confirm these visual impressions statistically, we compared the change in the AUC from session 1 (ΔAUC) among three stimuli. The magnitude of the CS1-evoked responses was increased across sessions (two-way repeated-measures ANOVA on ΔAUC , Session $\times$ Stimulus interaction, $F_{(6,101, 146.4)} = 3.438$, $p = 0.003$; follow-up one-way repeated-measures ANOVA, $F_{(5.851, 140.4)} = 5.215$, $p < 0.001$; Fig. 7D), while that of the CS2- ($F_{(4.948, 118.8)} = 4.524$, $p < 0.001$) and US-evoked responses was decreased ($F_{(6.466, 155.2)} = 5.754$, $p < 0.001$; Fig. 7D). These across-session changes were observed regardless of which stimulus was used as the CS1 (tone or light; data not shown). We then investigated the correlation between the changes in stimulus-evoked terminal responses with the degree of selective CR acquisition to the CS1 (Diff-index; see Materials and Methods; Fig. 7E). The Diff-index was positively correlated with the change in CS1-evoked responses ($R^2 = 0.299$, $p = 0.005$) and negatively correlated with the change in the US-evoked responses ($R^2 = 0.241$, $p = 0.012$). In contrast, the Diff-index was not correlated with the change in CS2-evoked responses ($R^2 = 0.004$, $p = 0.769$). Collectively, these findings suggest that the formation of stimulus–shock association parallels the weakening of innate responses to the shock and the strengthening of learned responses to the shock-predictive stimulus.

Discussion

BF cholinergic neurons emit phasic signals time-locked to aversive outcome (Hangya et al., 2015; Harrison et al., 2016) and environmental stimuli predictive of the outcome (Guo et al., 2019). By manipulating the activity of cholinergic terminals in the PrL, we showed that the stimulus- and outcome-evoked cholinergic signals induced opposite effects on aversive associative learning by modulating the task-induced activation of the mPFC network.

By monitoring calcium dynamics of cholinergic terminal activity in the PrL of the mPFC, we found that cholinergic terminals initially responded strongly to eyelid shock (Fig. 6D,E). As mice associated the shock with a preceding stimulus, however, the shock-evoked response was gradually weakened (Fig. 7C,D). Moreover, the greater the decrease in shock-evoked responses, the stronger the stimulus–shock association the mice formed (i.e., more frequent CR expression; Fig. 7E,F), indicating that the weakened shock-evoked cholinergic signaling reflects associative learning, but not habituation to the repeatedly presented shock. The innate, strong shock-evoked responses are consistent with previous electrophysiological (Hangya et al., 2015; Guo et al., 2019) and optical imaging (Harrison et al., 2016) data showing that BF cholinergic neurons respond to primary reinforcers with millisecond precision. In the sensory cortex (Chubykin et al., 2013;

Pinto et al., 2013; Liu et al., 2015; Guo et al., 2019) and amygdala (Jiang et al., 2016), optogenetic activation of this reinforcer-locked cholinergic terminal activity facilitates the encoding of stimulus associations, supporting a view that the phasic cholinergic signal serves as an error-correcting teaching signal (Hangya et al., 2015; Sturgill et al., 2020). In contrast, in the PrL, the encoding was facilitated by the inhibition, but not the activation, of cholinergic terminals during the shock delivery (Fig. 2D,H). These behavioral effects suggest that, at least in the PrL, outcome-locked cholinergic signaling does not encode prediction errors that drive learning (Rescorla and Wagner, 1972; J. J. Kim et al., 1998). Alternatively, although unconventional, cholinergic signaling might encode prediction errors that inhibit the formation of stimulus–shock associations.

In parallel to prediction errors, uncertainty is another critical parameter that affects learning. Theories posit that even in a simple case, such as classical conditioning, animals learn to predict an outcome from the occurrence of other environmental cues by estimating not only the strength of the cue–outcome association but also their uncertainty (Courville et al., 2006; Kruschke, 2008; Bach et al., 2011; Gershman and Niv, 2015). In particular, ACh is presumed to track the known unreliability of predictive relationships between a cue and outcome within a familiar environment (Yu and Dayan, 2002, 2005; Vossel et al., 2014; Marshall et al., 2016). In our paradigm, the CS was predictive of the US in most trials, but sometimes it was not (i.e., CS-alone trials). Also, despite our effort into adjusting the shock intensity, the degree of pain sensation inevitably varied across trials. These factors could induce uncertainty about CS–US contingency, highlighting the need to search for other cues that might predict the outcome more reliably. Thus, optogenetic excitation of cholinergic terminals almost completely blocked association formation (Fig. 2H) because it might have inflated the estimation of uncertainty regarding the CS–US contingency. On the contrary, their inhibition lowered the uncertainty estimate, leading to faster, more robust learning (Fig. 2D). A critical next step is to investigate the manipulation effects under various levels of the US probability. In particular, the change of cholinergic signal should affect learning greatly with 50% US probability (highest uncertainty) but marginally with 100% US probability (no uncertainty).

By using c-Fos expression as a measure of task-induced neural activation, we previously showed that better learning in trace eyeblink conditioning paralleled the greater activation of the mPFC network in rats (Volle et al., 2016; Jarovi et al., 2018). Consistent with this finding, the photo-inhibition of shock-evoked cholinergic terminal activity, which enhanced learning (Fig. 2D), resulted in the greater c-Fos expression in the mPFC (Fig. 3B,E). This finding confirms that the cholinergic terminal manipulations affected aversive associative learning by inducing the mPFC network state instrumental to associative learning. Notably, the manipulation effects on task-induced c-Fos expression were generally in the same direction in both overall c-Fos expression (Fig. 3E) and all three types of interneurons (Fig. 3F–H; Table 1). Although these findings echo anatomic findings that both pyramidal and all these interneuron types receive cholinergic inputs (Sun et al., 2019), task-induced c-Fos expression might not be sensitive enough to differentiate specific contributions of these cell types to microcircuit operations supporting aversive associative learning (H. Xu et al., 2019; Cummings and Clem, 2020). Future studies with more sensitive measures, such as fiber photometry and calcium imaging, are warranted to identify the cellular targets of the outcome-evoked cholinergic signal.

In addition to the strong innate responses to eyelid shock (Fig. 6E,F), PrL cholinergic terminals were also excited by

the auditory and visual stimuli during the conditioning (Fig. 7C). Initially, the nonreinforced CS2 evoked stronger terminal responses than the reinforced CS1 (Fig. 7C). Because this difference was observed regardless of which stimuli were used as the CS2 (data not shown), it is likely because of the higher salience of rare CS2 (25 of 150 trials) than common CS1. As mice associated the US with the CS1, the cholinergic terminals strengthened their responses to the CS1 while weakening the responses to the CS2 (Fig. 7D). Moreover, the suppression of the CS1-evoked cholinergic terminal activity mildly impaired associative learning (Fig. 4C). These findings align with the well-established role of cholinergic signaling in detecting salient environmental stimuli that guide well-learned responses (Posner et al., 1980; Chiba et al., 1999; Dalley et al., 2004; Maddux et al., 2007; Parikh et al., 2007). The present results suggest that this role is dissociable from the role played by outcome-evoked cholinergic signaling. At the beginning of learning, shock-evoked cholinergic signal constrains prefrontal activity to evaluate the validity of all possible cue–outcome associations in the environment. Once a stimulus was identified as a valid predictor of the shock, the cholinergic signal is strengthened to the stimulus and then facilitates the encoding of their association. The manipulation of either of these processes affects associative learning, but in the opposite direction.

A question remains open as to how the cue- and outcome-evoked phasic cholinergic signaling induces opposite effects on aversive associative learning. One possibility is that the CS and US information may arrive at distinct sets of neurons or synaptic sites that are modulated differently by phasic cholinergic signaling. In the PrL, both excitatory and inhibitory neurons express nicotinic and muscarinic ACh receptors (Levey et al., 1991; van der Zee et al., 1992; Poorthuis et al., 2013; Oda et al., 2018), each of which is further divided into several subtypes. Some of these subtypes are excitatory (e.g., $\alpha 4\beta 2$), while others are inhibitory (e.g., M2, M4) (Gulledge et al., 2007; Hedrick and Waters, 2015). Also, cholinergic receptors are expressed in both presynaptic and postsynaptic sites (Levey et al., 1991; Mrzljak et al., 1993, 1995). In addition, cholinergic neurons are known to corelease GABA (Saunders et al., 2015a, b; Granger et al., 2016). Therefore, phasic cholinergic signaling can depolarize or hyperpolarize local neurons, which would fine-tune how these neurons respond to synaptic inputs carrying the CS or US information. Although the exact pathway has not been mapped out, the PrL likely receives the CS information from the sensory cortices (Concina et al., 2018) and the US information from subcortical regions, such as the amygdala (Senn et al., 2014; Karalis et al., 2016). Comparing cholinergic modulations among different types of long-range synaptic connections will open a window into the precise mechanism underlying the complex cholinergic modulation of PrL processing.

In conclusion, we have shown that cholinergic projections to the mPFC constrain aversive associative learning by modulating task-induced neuronal activation in the mPFC. Future studies need to specify which cholinergic receptors this modulation depends on and how it affects the development of neuronal ensembles coding for stimulus associations. It is also essential to investigate whether these findings hold in female mice. Thus, our findings mark a first step toward uncovering intricate modulation that ACh exerts on cortical processing supporting aversive associative learning.

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