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Locus Coeruleus Contributions to Hippocampus-Dependent Memory Formation

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Locus Coeruleus Contributions to Hippocampus-Dependent Memory Formation

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Abstract

The locus coeruleus (LC) exerts its neuromodulatory effects via two distinct modes of activity: tonic and phasic. Tonic activity refers to extended periods of low to moderate firing (1-10Hz) that are thought to mediate relatively long-lasting changes in brain state by setting brain-wide noradrenergic tone. This type of activity is particularly important for regulating the overall level of arousal, which can, in turn, affect many psychological and physiological processes. Phasic activity refers to brief bouts of elevated firing rate (10-20Hz), usually in response to a specific environmental event. Phasic LC responses to task-related events are generally associated with improved performance across a variety of cognitive domains including perception, attention, and decision making. However, the role of precisely timed phasic LC responses in memory formation is relatively understudied. In particular, the role of phasic LC activity in hippocampus-dependent memory formation is unknown. In Part 1 of this dissertation, we characterize a trace fear conditioning task that requires mice to associate a tone and shock that are separated in time by 20 seconds. Using optogenetics, we show that this task requires intact hippocampal activity during both learning and memory retrieval. In Part 2, we characterize the phasic responses of the LC and its projections to the dorsal hippocampus during trace fear conditioning. We find learning related changes in these responses that are consistent with a role for LC in signaling the learned salience of environmental stimuli. We go on to show that amplifying these phasic responses can lead to enhancements in long-term memory. We also demonstrate that LC stimulation increases both norepinephrine and dopamine content in the dorsal hippocampus. Somewhat unexpectedly, we find that only the release of dopamine is needed to

enhance memory formation. The implications of these findings for LC function and dopamine release in the hippocampus during aversive learning are discussed.

Part 1

Acute disruption of the dorsal hippocampus impairs the encoding and retrieval of trace fear memories

1. Introduction

The hippocampus integrates spatial and temporal information to form complex memory representations. These include episodic memories in humans and contextual memories in animals (Eichenbaum, 2017). Simple associations, in contrast, can typically be learned without this structure. For example, rodents with damage to the hippocampus can acquire fear to an auditory cue that is immediately followed by shock (Chowdhury et al., 2005; Esclassan et al., 2009). However, if the shock is presented several seconds after the cue has ended, the same animals cannot form an association between them. This suggests that an important function of the hippocampus is to link discontiguous events – a property that allows it to encode sequences or form spatial maps, both of which involve associations between stimuli that are separated in time.

The ability to learn temporal associations can be studied in animals using trace conditioning. This is a Pavlovian procedure where a gap is included between the termination of the conditional stimulus (CS) and the onset of the unconditional stimulus (US). The majority of studies have found that the acquisition and retrieval of trace conditioning require the dorsal hippocampus (Chowdhury et al., 2005; Raybuck & Lattal, 2011, 2014) although there are exceptions (Cox et al., 2013; Czerniawski et al., 2009; Yoon & Otto, 2007). This variability could be attributed to the use of lesion and pharmacological techniques, both of which lack cell specificity and temporal precision. More recent studies have utilized optogenetic tools to directly manipulate hippocampal neurons or alter their activity indirectly by stimulating entorhinal inputs. When CA1 activity was decreased during learning, deficits in trace fear conditioning were observed

(Kitamura et al., 2014). In contrast, activation of CA1 neurons enhanced learning in young mice and ameliorated aging deficits in older animals(Sellami et al., 2017).

The goal of the current study was to directly compare the effects of CA1 stimulation on the acquisition and retrieval of trace fear memories. Based on previous work, we predicted that activation of dorsal CA1 pyramidal neurons would enhance learning while inhibition would impair both encoding and retrieval. The effect of CA1 activation on memory expression was less clear. Although it is possible to drive the retrieval of contextual fear memories by stimulating neurons in the dentate gyrus (Liu et al., 2012), the same procedure is far less effective in CA1 (Ramirez et al., 2013; Ryan et al., 2015). In addition, optogenetic activation of ventral CA1 has been shown to impair the retrieval of contextual fear (Jimenez et al., 2018). Accordingly, we predicted that direct stimulation of dorsal CA1 neurons would either impair or have no effect on the expression of trace fear.

2. Materials and Methods

2.1. Subjects:

Subjects in this study were 2-4 month old male and female C57BL/6J mice (Jackson Labs). Mice were maintained on a 12h light/12h dark cycle with *ad libitum* access to food and water. All experiments were performed during the light portion (7 a.m-7 p.m.) of the light/dark cycle. Mice were group housed until surgery, at which point they were single housed for the rest of the experiment. All experiments were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC).

2.2. Surgery:

Stereotaxic surgery was performed 2-3 weeks before behavioral experiments began. Mice were anesthetized with isoflurane (5% induction, 2% maintenance) and placed into a stereotaxic frame (Kopf Instruments). An incision was made in the scalp and the skull was adjusted to place bregma and lambda in the same horizontal plane. Small craniotomies were made above the desired injection site in each hemisphere. AAV was delivered at a rate of 2nl/s to dorsal CA1 (AP - 2.0mm and ML ± 1.5mm from bregma; DV -1.25mm from dura) through a glass pipette using a microsyringe pump (UMP3, World Precision Instruments). For stimulation experiments, the AAVs used were AAV9-CaMKIIa-hChR2(H134R)-eYFP (250nl/hemisphere, titer:8.96×10^{13}, Penn Vector Core) and AAV9-CaMKIIa-eGFP (250nl/hemisphere, titer:3.49×10^{13}, Penn Vector Core). For inhibition experiments, the constructs were AAV5-CaMKIIa-ArchT-GFP (350nl/hemisphere, titer:5.2×10^{12}, UNC Vector Core) and AAV5-CaMKIIa-GFP (350nl/hemisphere, titer:5.3×10^{12}, UNC Vector Core). After AAV infusions, an optical fiber (200um diameter, Thorlabs) was implanted above dorsal CA1 (dCA1) in each hemisphere (AP -2.0mm and ML ± 1.5mm from bregma; DV -1.0mm from dura). The fiber implants were secured to the skull using dental adhesive (C&B Metabond, Parkell) and dental acrylic (Bosworth Company).

2.3. Apparatus:

The behavioral apparatus has been described previously (Tayler et al., 2011). Briefly, fear conditioning occurred in a conditioning chamber (30.5 cm x 24.1 cm x 21.0 cm) within a sound-attenuating box (Med Associates). The chamber consisted of a

front-mounted scanning charge-coupled device video camera, stainless steel grid floor, a stainless steel drop pan, and overhead LED lighting capable of providing broad spectrum and infrared light. For context A, the conditioning chamber was lit with both broad spectrum and infrared light and scented with 95% ethanol. For context B, a smooth white plastic insert was placed over the grid floor and a curved white wall was inserted into the chamber. Additionally, the room lights were changed to red light, only infrared lighting was present in the conditioning chamber, and the chamber was cleaned and scented with disinfectant wipes (PDI Sani-Cloth Plus). In both contexts, background noise (65 dB) was generated with a fan in the chamber and HEPA filter in the room.

2.4. Trace Fear Conditioning Procedure

All behavioral testing occurred during the light portion of the light/dark cycle. Mice were habituated to handling and optical fiber connection for 5 minutes/day for 5 days before the beginning of behavior. Then, the mice were habituated to context B with one 5-minute session of free exploration each day for 2 days. Next, the mice underwent trace fear conditioning in context A. During training, mice were allowed to explore the conditioning chamber for 3 minutes before receiving six conditioning trials. Each trial consisted of a 20 second pure tone (85dB, 3000Hz) and a 2 second shock (0.9mA) separated by a 20 second stimulus-free trace interval. The intertrial interval (ITI) was 120 seconds. Mice were removed from the chamber 120 seconds after the last trial. Twenty-four hours later, the mice were placed in context B for a tone test consisting of a 3 minute baseline period followed by six 20-second tone presentations separated by a 140 second ITI. Freezing behavior was used to index fear and measured automatically

using VideoFreeze software (Med Associates). The next day, mice were placed back in the original conditioning chamber (context A) for either a 12- or 20-minute context test, depending on the experiment.

2.5. Experiment-Specific Methods

2.5.1. Experiment 1 - ChR2 stimulation during trace fear encoding

Blue light (465nm, 12mW measured at fiber tip) was delivered (20Hz, 15ms pulse width) to dCA1 in 42 second epochs during the training session. Light onset was simultaneous with onset of the tone and the light coterminated with the shock. No light was delivered during the tone or context tests. The context test was 20 minutes.

2.5.2. Experiment 2 - ArchT inhibition during trace fear encoding

Continuous green light (531nm, 12mW at fiber tip) illumination was delivered to dCA1 during training in the same 42 second epochs described for Experiment 1. Light was not present during testing and the context test was 20 minutes.

2.5.3. Experiment 3 - ChR2 stimulation during fear memory retrieval

Blue light was delivered to dCA1 as in Experiment 1, but during the tone test and the context test instead of training. In the tone test, light onset was simultaneous with tone onset and lasted 40 seconds. The context test consisted of four 3-minute epochs. The light was off for the first 3 minutes and on for the next 3 minutes; then, this sequence was repeated one time. Mice were sacrificed 90 mins following the end of the context test in order to quantify c-Fos expression.

2.5.4. Experiment 4 - ArchT inhibition during fear memory retrieval

Green light was delivered continuously to dCA1 as in Experiment 2, but during the testing periods rather than training. In the tone test, light onset was simultaneous with tone onset and lasted 40 seconds. The context test was 20 minutes and green light was delivered throughout the test in order to ensure c-Fos expression would be representative of neural activity that occurred while the laser was on.

2.6. Immunohistochemistry

Ninety minutes after behavioral testing, mice were transcardially perfused with 4% PFA. Following 24 hours of post-fixation, 40um coronal sections were cut and stained for c-Fos. Slices were washed three times in 1X phosphate buffered saline (PBS) at the beginning of the procedure and after all antibody and counterstaining steps. All antibodies and counterstains were diluted in a blocking solution containing .2% Triton-X and 2% normal donkey serum in 1X PBS, unless otherwise indicated. First, sections were incubated for 15 minutes in the blocking solution. Then, slices were incubated for 24 hours at four degrees in anti-c-Fos rabbit primary antibody (1:5000, ABE457, Millipore). Next, slices were placed in biotinylated donkey anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch) for 60 minutes at room temperature, followed by Streptavidin-Cy3 (1:500, Jackson ImmunoResearch) for 45 minutes. Finally, sections were stained with DAPI (1:10,000 in PBS, Life Technologies) for 10 minutes, mounted on slides, and coverslipped with Vectashield anti-fade mounting media (Vector Labs).

2.7. Image Acquisition and Cell Quantification:

Images were acquired at 20X magnification using a fluorescence slide scanner (BX61VS, Olympus). After acquisition, images were cropped to contain approximately 30,000-40,000 μm^2 of dorsal CA1. A blinded experimenter performed cell counts on 3-4 sections from each animal (6-8 hemispheres). c-Fos+ cells were counted using the multi-point tool in Image-J. Cell counts were averaged across slices to obtain one value per animal.

2.8. Statistical analysis

For analysis of behavioral data from training and tone test sessions, freezing scores in each phase type (baseline, tone, trace) were averaged for each animal. All behavioral data were analyzed using two-way repeated-measures ANOVA followed by Bonferroni-corrected *post hoc* comparisons when necessary. Cell count data were analyzed using unpaired t-tests. A threshold of $p < .05$ was used to determine statistical significance. All data are shown as mean ± SEM. All data were analyzed with GraphPad Prism (v8) and all figures were generated using Prism and BioRender.

3. Results

3.1. Inhibition of dCA1 impairs trace fear memory retrieval

To silence dCA1 during retrieval, we expressed the inhibitory opsin ArchT in pyramidal neurons using the αCaMKII promoter. Animals then received 6 trace fear conditioning trials in the absence of laser stimulation (Figure 1A). Each trial consisted of a 20-second auditory CS followed by a 20-second trace interval and then a 2s footshock. The intertrial interval (ITI) was 120s. As expected, freezing increased during the tone and trace interval relative to the baseline period and there were no differences

between ArchT mice and eGFP control animals (Main effect of stimulus period $F(2, 20) = 122$, $p < .05$; No effect of group, $F(1, 10) = 0.48$, $p > .05$, No stimulus period x group interaction $F(2, 20) = 0.49$, $p > .05$) (Figure 1B).

The next day, animals received a tone test in a novel environment. The test was identical to training except that no shocks were presented and continuous green light was delivered to dCA1 during the tone and trace intervals (Figure 1A). Group differences were not observed at baseline (BL); however, ArchT stimulation significantly reduced freezing during the tone and trace intervals (Group x stimulus period interaction $F(2, 20) = 10.9$, $p < .05$; Bonferroni post-hoc tests, BL ($p > .05$), tone and trace ($p < .05$) (Figure 1C). The following day, mice were placed back in the original training environment for 20-minutes to assess context fear. Continuous green light was delivered to dCA1 during the entire test (Figure 1A). Similar to the trace fear data, stimulation of ArchT significantly reduced freezing to the context (Main effect of group $F(1, 10) = 23.81$, $p < .05$; Main effect of time $F(3, 30) = 10.48$, $p < .05$; No group x time interaction $F(3, 30) = 1.73$, $p > .05$) (Figure 1D).

To confirm that ArchT was expressed in dCA1 and that laser stimulation reduced neural activity, mice were sacrificed 90-min after the context test. We observed strong bilateral expression of ArchT and eGFP throughout the dCA1 (Figure 1E). We also found reduced expression of the immediate early gene c-Fos in ArchT mice relative to eGFP controls, indicating that our manipulation successfully reduced neural activity $t(10) = 4.83$, $p < .05$) (Figure 1F). Together, these data demonstrate that reduced activity in dCA1 impairs the retrieval of both trace and context fear memories.

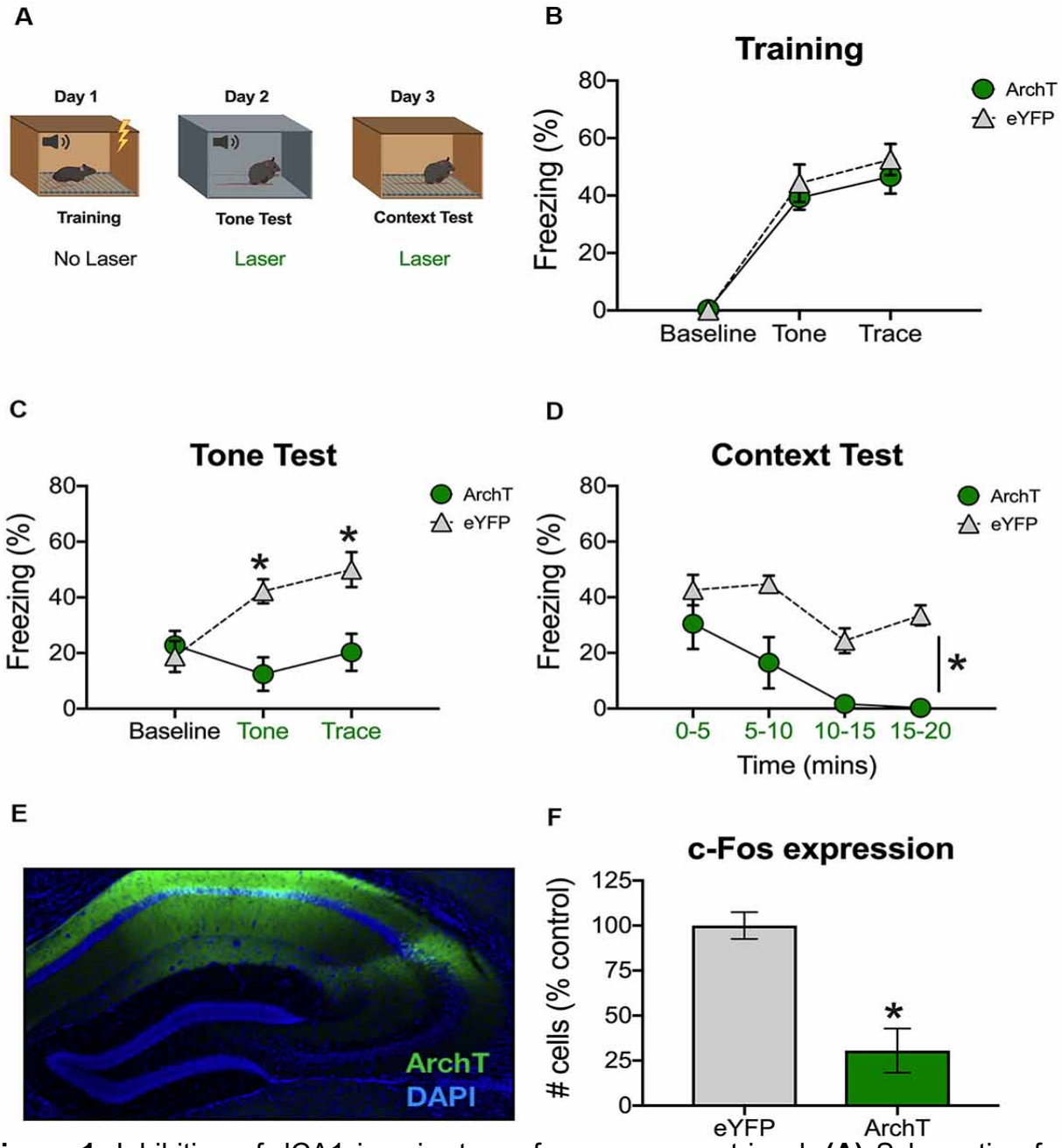

Figure 1. Inhibition of dCA1 impairs trace fear memory retrieval. **(A)** Schematic of behavioral paradigm. On day 1, animals underwent trace fear conditioning without laser stimulation. The next day, mice underwent a tone memory test in a novel context with green light delivered to dorsal CA1 during each trial. Twenty-four hours later, mice were placed back in the conditioning environment for a context memory test. Green light was delivered to dCA1 during the entire context test. **(B)** Freezing during the training phase of trace fear conditioning (Mean±SEM) **(C)** Freezing during the tone test (Mean±SEM). **(D)** Freezing during the context test (Mean±SEM). **(E)** Example of virus expression. Green = ArchT; Blue = DAPI. **(F)** c-Fos expression in eGFP and ArchT mice after the context test. Green x-axis labels denote periods during which the laser was delivered. In all panels, green represents the ArchT group and gray represents the control group. *p < 0.05 relative to control.

3.2. Stimulation of dCA1 impairs trace fear memory retrieval

To examine the effects of dCA1 stimulation on retrieval, we expressed the excitatory opsin ChR2 in pyramidal neurons under control of the αCaMKII promoter. Animals were trained and tested using the same procedure described in the previous experiment (Figure 2A). During training, freezing increased during the tone and trace intervals relative to the baseline period and no differences were observed between ChR2 and eGFP groups (Main effect of stimulus period $F(2, 14) = 59.71$, $p < .05$; No effect of group, $F(1, 7) = 0.82$, $p > .05$, No stimulus period x group interaction $F(2, 14) = 0.63$, $p > .05$) (Figure 2B).

Animals received a tone test the next day, during which blue light (20Hz) was delivered to dCA1 during the tone and trace intervals (Figure 2A). There were no group differences at baseline, but ChR2 stimulation significantly reduced freezing during the subsequent tone and trace intervals (Group x stimulus period interaction $F(2, 14) = 43.7$, $p < .05$; Bonferroni post-hoc tests, BL ($p > .05$), tone and trace ($p < .05$) (Figure 2C). Twenty-four hours later, the mice were put back in the original training environment to assess context fear. This test began with a 3-minute laser off period (BL) followed by 3-minutes of blue light stimulation and 3-minutes of no stimulation. It ended with a second 3-minute period of blue light stimulation. During BL, the groups froze at similar levels indicating that both had acquired context fear memories. However, when dCA1 was stimulated, freezing was significantly reduced in ChR2 mice relative to eGFP controls. Freezing remained low in this group after the laser turned off and did not recover for the remainder of the test session (Group x stimulus period interaction $F(3,$

21) = 12.34, $p < .05$; Bonferroni post-hoc tests, BL ($p > .05$) all subsequent laser on and laser off periods ($p < .05$) (Figure 2D).

To examine virus expression and determine the effects of dCA1 stimulation on neural activity, mice were perfused 90 minutes after the context test. As expected, we observed robust expression of ChR2 (Figure 2E) and stimulation produced a large increase in the number c-Fos positive dCA1 neurons relative to eGFP controls ($t (7) = 18.78$, $p < .05$) (Figure 2F). These data demonstrate that stimulation of dCA1 neurons impairs the retrieval of both trace and context fear memories.

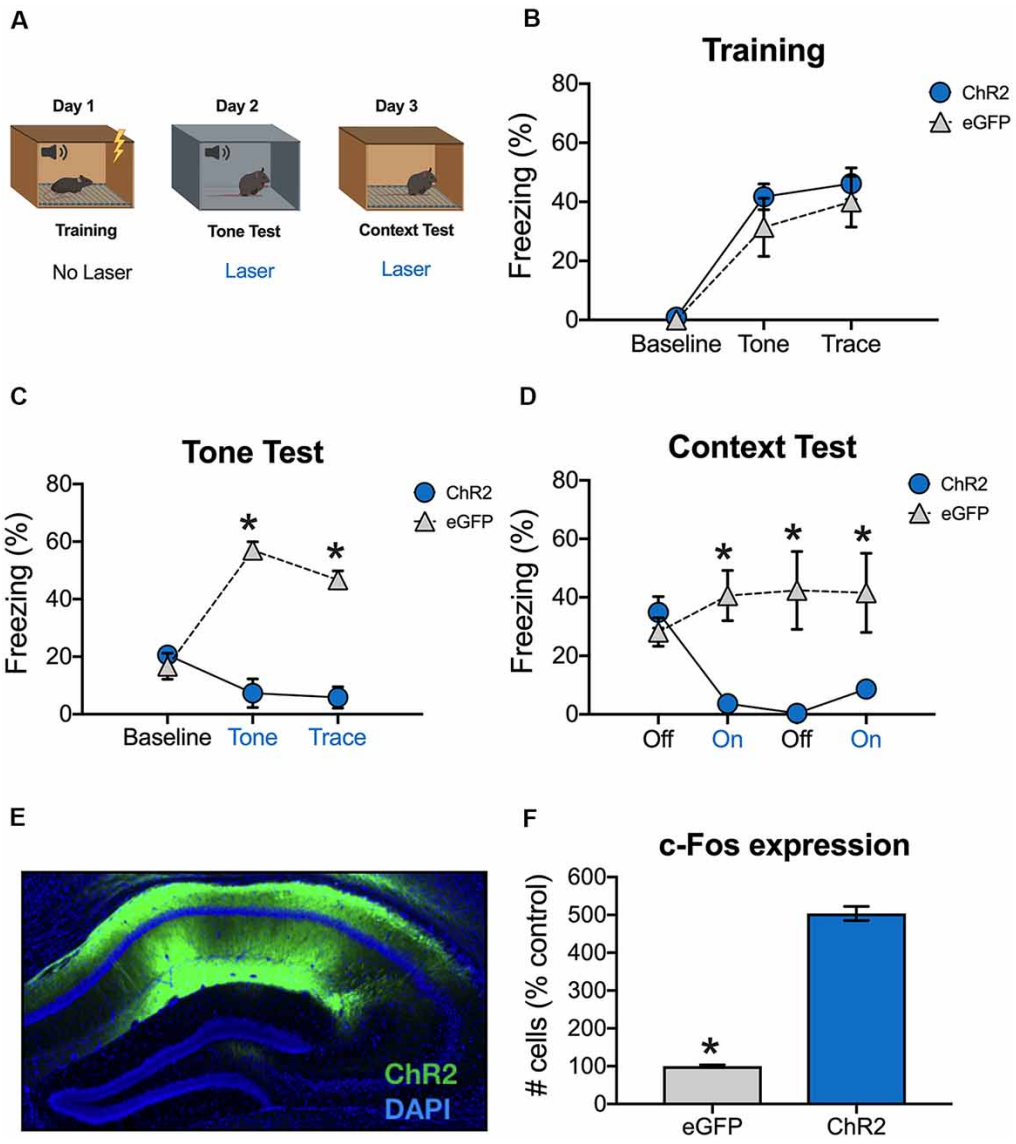

Figure 2. Stimulation of dCA1 impairs trace fear memory retrieval. **(A)** Schematic of behavioral paradigm. On day 1, animals underwent trace fear conditioning without laser stimulation. The next day, mice underwent a tone memory test in a novel context with blue light delivered (20Hz) to dorsal CA1 during each trial. Twenty-four hours later, mice were placed back in the conditioning environment for a context memory test. The laser was not turned on for the first 3 minutes of the context test. Then, blue light was delivered to dCA1 for the next 3 minutes, followed by another 3 minute laser off period, and a last 3-minute laser on epoch. **(B)** Freezing during the training phase of trace fear conditioning (Mean±SEM) **(C)** Freezing during the tone test (Mean±SEM). **(D)** Freezing during the context test (Mean±SEM). **(E)** Example of virus expression. Green = ChR2; Blue = DAPI. **(F)** c-Fos expression in eGFP and ChR2 mice after the context test. Blue x-axis labels denote periods during which the laser was delivered. In all panels, blue represents the ChR2 group and gray represents the control group. *p < 0.05 relative to control.

3.3. Stimulation of dCA1 impairs the acquisition of trace fear conditioning

We next determined the effects of stimulation on encoding by delivering blue light to dCA1 during each training trial (tone-trace interval-shock) (Figure 3A). There were no group differences during the baseline period, but ChR2 stimulation significantly reduced freezing during the tone and trace intervals (Group x stimulus period interaction $F(2, 20) = 18.2$, $p < .05$; Bonferroni post-hoc tests, BL ($p > .05$), tone and trace ($p < .05$) (Figure 3B). The same effects were observed the next day when mice received a tone test in the absence of blue light stimulation (Group x stimulus period interaction $F(2, 20) = 8.09$, $p < .05$; Bonferroni post-hoc tests, BL ($p > .05$), tone and trace ($p < .05$) (Figure 3C). Twenty-four hours after the tone test, context memory was assessed by returning the mice to the training context. Blue light was not delivered during this session. Similar to the tone test data, context fear was significantly reduced in ChR2 mice relative to eGFP controls (Main effect of group $F(1, 10) = 14.52$, $p < .05$; Main effect of time $F(3, 30) = 1.07$, $p < .05$; No group x time interaction $F(3, 30) = 0.96$, $p > .05$) (Figure 3D). Together, these data demonstrate that both trace and context fear memories are disrupted when dCA1 is stimulated during encoding.

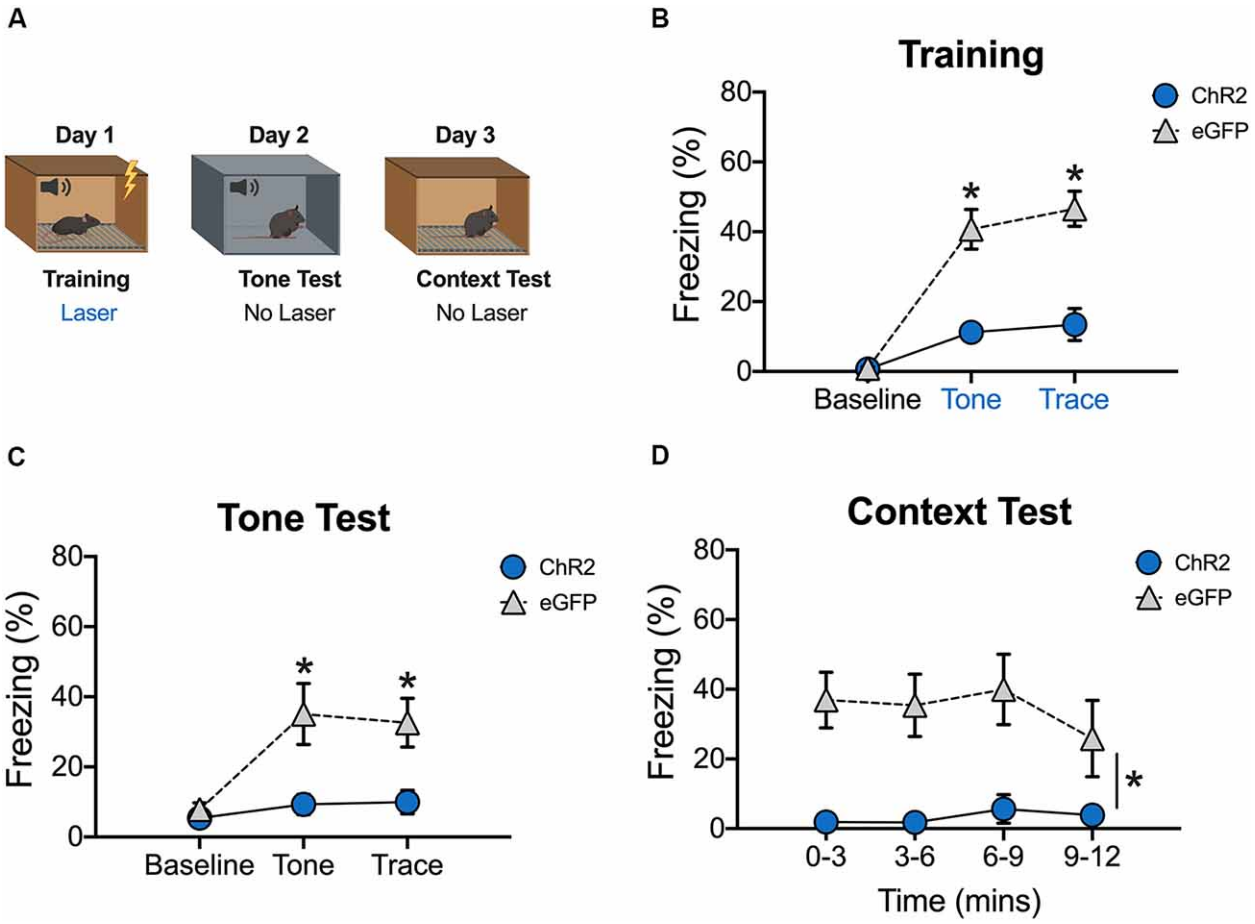

Figure 3. Stimulation of dCA1 during trace fear encoding impairs memory acquisition. **(A)** Schematic of behavioral paradigm. On day 1, animals underwent trace fear conditioning with blue light (20Hz) delivered to dCA1 during each training trial. The next day, mice underwent a tone memory test in a novel context with no laser stimulation. Twenty-four hours later, mice were placed back in the conditioning environment for a context memory test without light delivery. **(B)** Freezing during the training phase of trace fear conditioning (Mean±SEM) **(C)** Freezing during the tone test (Mean±SEM). **(D)** Freezing during the context test (Mean±SEM). Blue x-axis labels denote periods during which the laser was delivered. In all behavioral panels, blue represents the ChR2 group and gray represents the control group. *p < 0.05 relative to control.

3.4. Inhibition of dCA1 impairs the acquisition of trace fear conditioning

In our last experiment, we examined the effects of inhibition on trace fear encoding by stimulating ArchT during training (Figure 4A). As in the previous experiment, light was delivered to dCA1 during each conditioning trial (tone-trace interval-shock). Surprisingly, there were no differences between the ArchT and eGFP

groups during the baseline period or during the tone and trace intervals (No effect of group $F(1, 10) = 2.77$, $p > .05$; Main effect of stimulus period $F(2, 20) = 60.7$, $p < .05$; No Group x stimulus period interaction $F(2, 20) = 2.07$, $p > .05$) (Figure 4B). However, when memory was tested the next day (in the absence of light stimulation) ArchT animals froze significantly less than eGFP controls during all stimulus periods (Main effect of group $F(1, 10) = 29.74$, $p < .05$; Main effect of stimulus period $F(2, 20) = 41.33$, $p < .05$; No Group x stimulus period interaction $F(2, 20) = 0.29$, $p > .05$) (Figure 4C). Twenty-four hours after the tone test, context memory was assessed by returning the mice to the training environment. Green light was not delivered during this session. The ArchT and eGFP groups froze at similar levels during this test indicating that dCA1 inhibition did not affect the formation of a context fear memory (No effect of group $F(1, 10) = 0.53$, $p > .05$; No effect of time $F(3, 30) = 2.41$, $p > .05$; No group x time interaction $F(3, 30) = 0.74$, $p > .05$) (Figure 4D). These data are consistent with a recent report and suggest that reduced activity in dCA1 disrupts the acquisition of trace but not context fear memories (Sellami et al., 2017).

3.5. Altering dCA1 activity does not increase exploration or reduce the response to shock.

It is possible that our manipulations impaired trace fear conditioning because they induced hyperactivity or disrupted the animals' ability to process shock. This is unlikely given that optogenetic inhibition of dCA1 does not impair delay fear conditioning or increase activity in the open field (Goshen et al., 2011). In addition, optogenetic activation of dCA1 increases the ability of aged mice to acquire trace fear conditioning (Sellami et al., 2017).

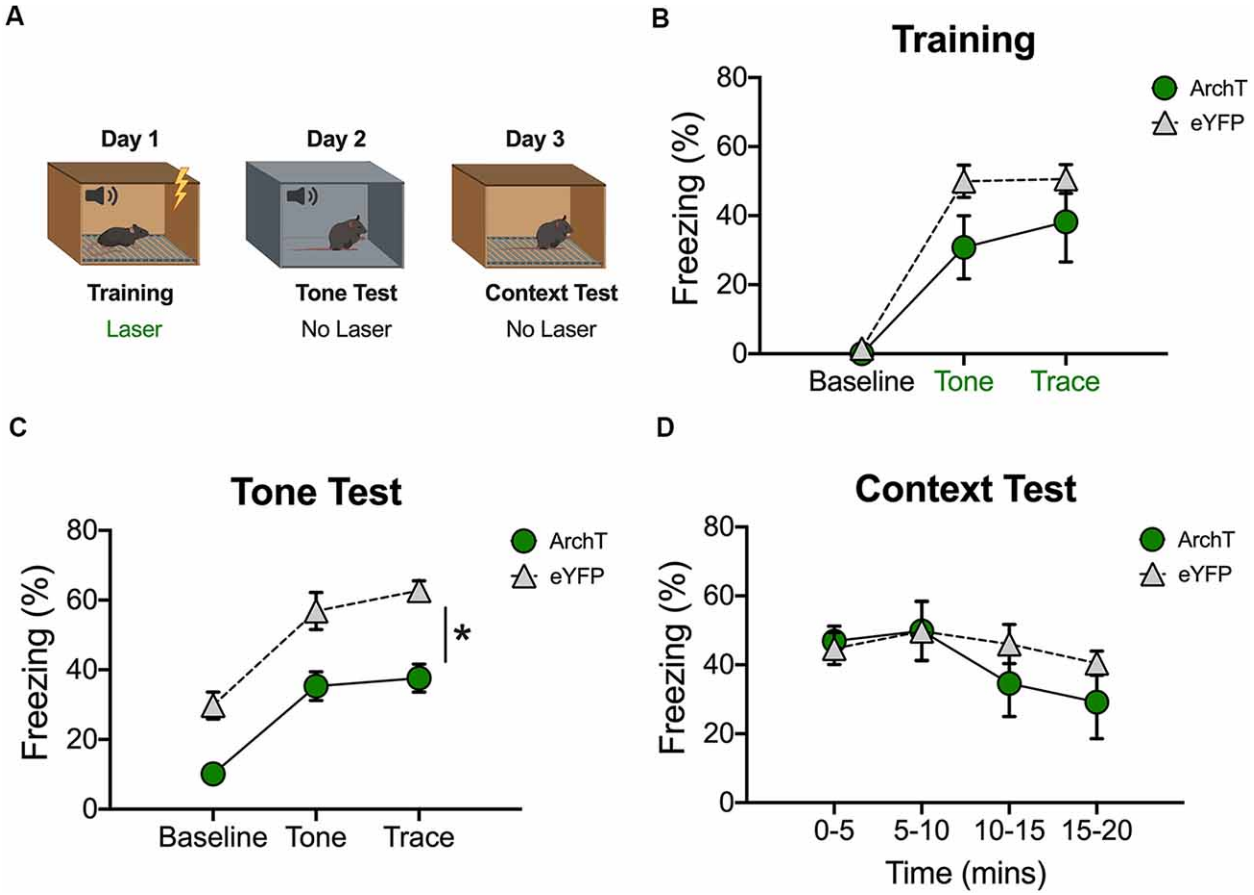

Figure 4. Inhibition of dCA1 during trace fear encoding impairs memory acquisition. **(A)** Schematic of behavioral paradigm. On day 1, animals underwent trace fear conditioning with green light delivered to dCA1 during each training trial. The next day, mice underwent a tone memory test in a novel context with no laser stimulation. Twenty-four hours later, mice were placed back in the conditioning environment for a context memory test without light delivery. **(B)** Freezing during the training phase of trace fear conditioning (Mean±SEM). **(C)** Freezing during the tone test (Mean±SEM). **(D)** Freezing during the context test (Mean±SEM). In all behavioral panels, green represents the ArchT group and gray represents the control group. Green x-axis labels denote periods during which the laser was delivered. *p < 0.05 relative to control.

Nonetheless, we addressed this issue by determining if laser stimulation altered exploration or shock reactivity during the first conditioning trial (Figure 5). Only the first trial was analyzed because mice were exploring naturally and had not yet started freezing. In addition, endogenous opiates are released during fear conditioning and have been shown to reduce shock sensitivity (Fanselow & Baackes, 1982; Fanselow &

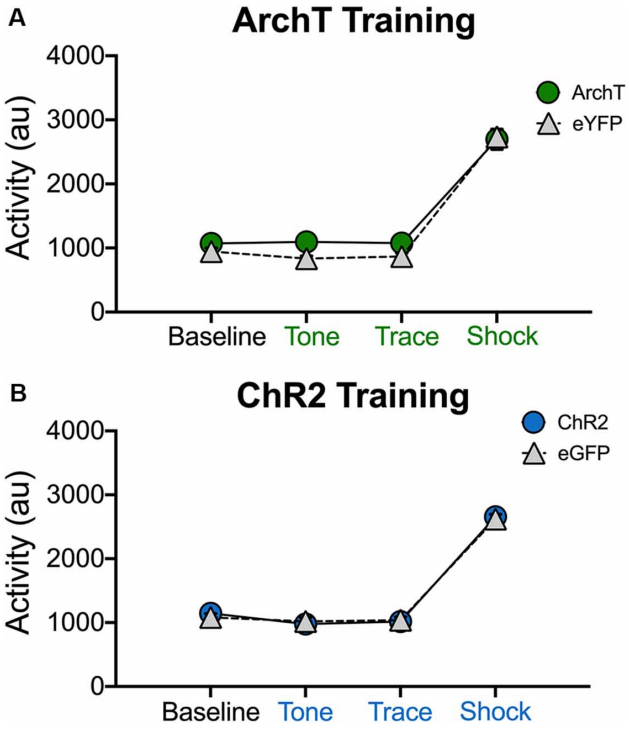

Figure 5. Stimulation and inhibition of dCA1 do not alter locomotor activity or shock responsivity. **(A)** Average motion (arbitrary units) during the last 20s of baseline and the first tone, trace, and shock periods in Experiment 3 (inhibition during trace fear encoding) (Mean±SEM). **(B)** Average motion during the last 20s of baseline and the first tone, trace, and shock periods in Experiment 4 (stimulation during trace fear encoding) (Mean±SEM).

Bolles, 1979). We quantified activity levels immediately before laser stimulation (BL) and then compared these to subsequent periods when the laser was on (tone, trace interval and shock). Analysis of our ArchT data revealed that activity levels were not altered when dCA1 was inhibited during the tone, trace interval or shock periods (No effect of group $F(1, 10) = 2.67$, $p > .05$; Main effect of stimulus period $F(3, 30) = 278.3$ $p < .05$; No group x stimulus period interaction $F(3, 30) = 1.59$, $p > .05$) (Figure 5A). Differences were also not observed when dCA1 was activated during these same periods via ChR2 stimulation (No effect of group $F(1, 10) = 0.03$, $p > .05$; Main effect of stimulus period $F(3, 30) = 330.2$, $p < .05$; No group x stimulus period interaction $F(3, 30) = 0.31$, $p > .05$) (Figure 5B). These results are consistent with previous reports and indicate that stimulation or inhibition of dCA1 does not impair trace fear conditioning by inducing hyperactivity or preventing the animals from processing shock.

5. Discussion

In this set of experiments, we compared the effects of optogenetic inhibition and stimulation of the dorsal hippocampus on the encoding and retrieval of trace fear memories. Our results demonstrate that intact dCA1 activity is required for the retrieval of both tone and context fear. This is true regardless of whether activity is decreased or increased. Although some previous work suggests that trace fear memories can be retrieved without the dorsal hippocampus (Cox et al., 2013; Czerniawski et al., 2009; Yoon & Otto, 2007), our results agree with previous studies that found lesions and pharmacological inactivation of this region impair trace fear expression (Chowdhury et al., 2005; Quinn et al., 2005; Raybuck & Lattal, 2011).

When dCA1 was inhibited during encoding, we found that tone fear memory was impaired, but memory for the training context remained intact. This is consistent with the fact that manipulations of the dorsal hippocampus during context fear learning often do not prevent memory formation (Frankland et al., 1998; Maren et al., 1997; Wiltgen et al., 2006). This finding is thought to reflect the ability of other brain areas (e.g. ventral hippocampus, prefrontal cortex) to compensate for the lack of dorsal hippocampus contributions to learning (Rudy et al., 2004; Wiltgen & Fanselow, 2003; Zelikowsky et al., 2013). In contrast, inactivation of the dorsal hippocampus after learning typically leads to robust retrograde amnesia for context fear (Anagnostaras et al., 1999; Kim & Fanselow, 1992; Maren et al., 1997; Matus-Amat et al., 2004), as seen in our retrieval experiments. Together, these data suggest that dCA1 is required for memory expression if this region is intact during learning (Moser & Moser, 1998; Rudy et al., 2004; Wiltgen & Fanselow, 2003).

Unlike inhibition, activation of dCA1 during training produced deficits in both tone and context fear memory. This more complete memory impairment suggests that the abnormal activity patterns induced by ChR2 stimulation disrupted encoding in brain regions that can normally compensate for the loss of the dorsal hippocampus. Consistent with this idea, stimulation of dCA1 has been shown to produce widespread increases in brain activity (Lebhardt et al., 2016; Takata et al., 2015). In contrast to our results, some studies have found that increases in CA1 activity during encoding enhance trace fear memory acquisition (Kitamura et al., 2014; Sellami et al., 2017). For example, Sellami et al. showed that direct stimulation of CA1 pyramidal cells during the trace interval attenuates trace fear conditioning deficits in aged mice (2017). However, this discrepancy may be explained by differences in age between studies. Young mice show learning-related increases in CA1 intrinsic excitability following trace fear conditioning that are reduced with aging (Oh et al., 2010). It is possible that CA1 stimulation during the trace interval rescues this physiological impairment in old mice, ameliorating their trace fear conditioning deficits, but adds noise to the already-excitable hippocampus in young animals. The effect of this noise on learning could be amplified by the higher stimulation frequency that was used in the current study (20Hz vs 5Hz).

The current results support the idea that dorsal CA1 is critically involved in forming and retrieving trace fear memories. Nonetheless, despite the extensive literature on this topic, the specific contribution of CA1 to these processes remains known. To better understand its role, future investigations will need to examine its unique physiological properties in more detail as well as characterize the type of

information it receives from brain areas like the prefrontal cortex, entorhinal cortex and the nucleus reunions.

Part 2

Phasic locus coeruleus activity facilitates hippocampus-dependent trace fear memory formation

1. Introduction

The locus coeruleus (LC) supports an array of cognitive processes by modulating long lasting brain-wide arousal states and responding to salient events in the environment. LC neurons accomplish this via two distinct modes of activity: tonic and phasic. Changes in the frequency of tonic activity are associated with corresponding changes in psychological state. Low tonic activity (~0-2Hz) is associated with drowsiness or sleep and increasing levels of tonic activity (~3-10Hz) are associated with increased arousal, progressing from exploration and task engagement to agitation and anxiety states (Aston-Jones & Cohen, 2005). Bouts of phasic activity (~10-20Hz) are most often observed during intermediate levels of tonic activity in response to salient environmental events (Aston-Jones et al., 1999). When locked to task-relevant events, these phasic responses are associated with improved cognitive performance across a variety of different tasks (Aston-Jones & Cohen, 2005 for review).

A large body of research supports the idea that the LC is critically involved in memory formation. Monoamine depletion in the LC, noradrenergic and dopaminergic antagonism in multiple brain regions, and direct inhibition of the locus coeruleus all impair memory across a variety of tasks (Giustino & Maren, 2018; J. E. Lisman & Grace, 2005; Selden et al., 1990; Uematsu et al., 2017; Wagatsuma et al., 2018). Conversely, LC stimulation as well as dopamine and norepinephrine agonism can enhance memory (Bach et al., 1999; Kempadoo et al., 2016; Packard & White, 1989, 1991; Sara & Devauges, 1988). However, studies examining the effect of LC on hippocampus-dependent memory have generally not distinguished between tonic and phasic LC activity. In some cases, this distinction is prevented by the use of temporally

imprecise manipulations like lesions and drug infusions. In others, it is difficult to determine the importance of event-locked phasic firing because of the use of spatial memory tasks that are known to depend on the hippocampus (HPC). Because space is a temporally diffuse stimulus, it is difficult to know the exact moments during spatial learning when LC phasic responses might be important. This does not rule out the possibility that phasic LC activity is important for spatial learning, but the lack of experimental control over the animals' sampling of the relevant stimuli makes this possibility opaque to examination.

Here, we used fiber photometry and optogenetics to make temporally precise observations and manipulations of LC activity during learning in a trace fear conditioning task. Trace conditioning is a form of Pavlovian conditioning in which the conditional stimulus is separated in time from the unconditional stimulus by a stimulus free period known as the trace interval. Although the reason for hippocampal involvement in trace conditioning is not known, we and others have demonstrated that intact hippocampal activity is required for trace fear encoding and retrieval (Raybuck & Lattal, 2014; Wilmot et al., 2019). Because trace fear conditioning involves learning about discrete, well-controlled stimuli, we were able to characterize the temporal dynamics of LC activity and LC-HPC communication during learning and examine the contribution of phasic LC activity during specific learning events to long-term memory formation.

Although the LC is typically thought to modulate neural activity via the release of norepinephrine, several recent studies have suggested that it may also release dopamine into the hippocampus and other cortical regions (Devoto & Flore, 2006; Smith & Greene, 2012). In some cases, it appears that dopamine release from LC into the

HPC may be more important for memory than norepinephrine (Kempadoo et al., 2016; Takeuchi et al., 2016; Wagatsuma et al., 2018). The effects of dopamine release on memory formation have primarily been studied using reward and spatial learning tasks. However, many studies have demonstrated that dopamine is released in several brain regions (eg. nucleus accumbens, prefrontal cortex, amygdala) in response to aversive stimulation (Badrinarayan et al., 2012; Bassareo et al., 2002; de Jong et al., 2019; Inoue et al., 1994; Stelly et al., 2019). Here, we extend this data by providing direct evidence that LC activity increases both dopamine and norepinephrine content in the hippocampus and by showing that dopamine release is important for trace fear memory formation, but the release of norepinephrine is not.

2. Materials and Methods

2.1. Subjects:

All subjects in this study were 2-4 month old male and female F1 hybrids generated by breeding TH-Cre mice maintained on a C57BL/6J background (Jackson Labs, Cat #008601) with 129S6 mice (Taconic). Mice were maintained on a 12h light/12h dark cycle with *ad libitum* access to food and water. All experiments were performed during the light portion (7 a.m-7 p.m.) of the light/dark cycle. Mice were group housed throughout the experiments. All experiments were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC).

2.2. Surgery:

Stereotaxic surgery was performed 2-3 weeks before behavioral experiments began for LC cell body experiments and 10-14 weeks before behavioral experiments for LC projection experiments. Mice were anesthetized with isoflurane (5% induction, 2% maintenance) and placed into a stereotaxic frame (Kopf Instruments). An incision was made in the scalp and the skull was adjusted to place bregma and lambda in the same horizontal plane. Small craniotomies were made above the desired injection site in each hemisphere. AAV was delivered at a rate of 2nl/s to the locus coeruleus (AP – 5.5mm and ML ± 0.9mm from bregma; DV -3.6mm from dura) or dorsal CA1 (AP -2.0mm and ML ± 1.5mm; DV – 1.25mm from dura) through a glass pipette using a microsyringe pump (UMP3, World Precision Instruments).

For optogenetic stimulation experiments, the AAV used was AAV9-EF1A-DIO-hChR2(E123T/T159C)-eYFP (300nl/hemisphere, titer:8.96 x 10^{13}, Addgene). For DREADDs stimulation epxperiments, the AAV used was AAV9-PRSx8-hM3Dq-HA (300nl, titer: 2.24x10^{14}, gift from Gary Aston-Jones). For photometry experiments, the constructs were AAV5-FLEX-hSyn-GCaMP6s (300nl, gift from Lin Tian), AAV9-hSyn-FLEX-axonGCaMP6s (300nl, Addgene), AAV9-hSyn-GRAB-NE1h (250nl, Addgene), or AAV9-hSyn-GRAB-DA2h (250nl, Addgene).

After AAV infusions, an optical fiber (200um diameter for optogenetics, Thorlabs; 400um diameter for photometry, Doric) was implanted above dorsal CA1 (dCA1) (AP -2.0mm and ML ± 1.5mm from bregma; DV -1.0mm from dura for optogenetics, DV – 1.25mm from dura for photometry) or locus coeruleus (AP -5.5mm and ML -0.9mm from bregma; DV -3.5mm). The fiber implants were secured to the skull using dental adhesive (C&B Metabond, Parkell) and dental acrylic (Bosworth Company).

2.3. Apparatus:

The behavioral apparatus was the same as that described in Part 1 of this dissertation.

2.4. Behavioral Procedures:

2.4.1. Trace Fear Conditioning

All behavioral testing occurred during the light portion of the light/dark cycle. Mice were habituated to handling and optical fiber connection for 3-5 minutes/day for 5 days before the beginning of behavior. Next, the mice underwent trace fear conditioning in context A. During training, mice were allowed to explore the conditioning chamber for 3 minutes before conditioning trials began. For optogenetics and pharmacology experiments, the animals then receive 3 trace conditioning trials. For photometry experiments, animals received 10 conditioning trials. Each trial consisted of a 20 second pure tone (85dB, 3000Hz) and a 2 second shock (0.3mA for optogenetics and pharmacology, 0.2mA for photometry) separated by a 20 second stimulus-free trace interval. The intertrial interval (ITI) was 180 seconds. Mice were removed from the chamber 180 seconds after the last trial. Twenty-four hours later, the mice were placed in context B for a tone test consisting of a 3 minute baseline period followed by 6 20-second tone presentations separated by a 180 second ITI for optogenetics and pharmacology experiments and 10 20-second tone presentations separated by a 140 second ITI for photometry experiments. In photometry experiments, mice underwent an extinction test the next day in which they received 20 more tone presentations

separated by 90 second ITI in context B. Freezing behavior was used to index fear and measured automatically using VideoFreeze software (Med Associates). The next day, mice were placed back in the original conditioning chamber (context A) for a 10 minute context test (data not shown).

2.4.2 DREADDS stimulation behavior

For DREADDS experiments, mice were placed in the same apparatus used for trace fear conditioning experiments. Baseline fluorescence was acquired for 10 minutes, after which mice were briefly removed from the chamber to receive an injection of either CNO or vehicle. After injection, mice were immediately placed back inside the chamber and remained there for 50 more minutes. This procedure was repeated the next day with animals that received CNO on the first day receiving saline on the second and vice versa.

2.4.3. Shock Response Curve

For the LC shock response curve experiment, mice were placed in the conditioning chamber and allowed to explore freely for 180 seconds. Then 1 second shocks were presented in three blocks of seven trials. Each block consisted of seven trials of different shock intensities (0mA, 0.05ma, 0.1mA, 0.2mA, 0.4mA, 0.6mA, and 0.8mA) in pseudorandom order separated by a 30s ITI. Trial blocks were separated by 180s and mice were removed from the chamber 180 seconds after the last trial.

2.4.4 *Tone Response Curve*

Mice were placed in the conditioning chamber and allowed to explore freely for 180 seconds. Then 25 twenty second tones of varying intensity (55dB, 65dB, 75dB,

85dB, 95dB) were presented in pseudorandom order with a 60 second ITI between tones. Mice were removed from the chamber 60 seconds after the last tone.

2.5 Optogenetics:

Blue light (465nm, 10mW measured at fiber tip) was delivered (20Hz, 5ms pulse width) to LC or dorsal CA1 in 2 second epochs during the training session. Light onset was simultaneous with onset of the tone, termination of the tone, and onset of the shock. No light was delivered during the tone or context tests.

2.6 Fiber Photometry:

The photometry system (Doric) consists of a fluorescence mini-cube that transmits light from a 465nm LED sinusoidally modulated at ~209 Hz that passed through a 465-nm or 560-nm bandpass filter, and a 405nm LED modulated at ~308 Hz that passed through a 405-nm bandpass filter. LED power was set at ~80 µW. The fluorescence from neurons below the fiber tip was transmitted via this same cable back to the mini-cube, where it was passed through a emission filter, amplified, and focused onto a femtowatt photoreceiver (Newport). The signals generated by the two LEDs were demodulated and decimated to 120 Hz for recording to disk.

All preprocessing and analysis was conducted using custom Python scripts. For preprocessing of GCaMP data, a least-squares linear fit was used to predict 465-nm signal from the 405-nm isosbestic signal. To calculate a dF/F, the predicted values were then subtracted from the true 465nm signal and this value was divided by the predicted value. In short, the dF/F was calculated as below:

$$\frac{\Delta F}{F} = \frac{465 - 465_{\,predicted}}{465_{\,predicted}} \times 100$$

For trial analyses in fear conditioning experiments, the dF/F was normalized to the baseline of the trial (the 20 seconds preceding delivery of the tone for fear conditioning, 2 seconds preceding the tone for tone and shock response experiments). Data was analyzed as the peak dF/F during the first 2 seconds of each stimulus period analyzed.

For GRAB-NE and GRAB-DA experiments, dF/F calculation did not use the isosbestic channel due to differential bleaching between the isosbestic and signal channels over the long time scales used in those experiments. Instead, dF/F was calculated via baseline normalization (i.e. subtracting the mean 465nm signal during a 5 minute baseline period from the 465nm signal and dividing the resulting value by the standard deviation of the 465nm during the baseline period).

2.7 Drugs:

For DREADDs experiments, animals received 5mg/kg I.P. injections of clozapine-N-oxide (CNO, Tocris) dissolved in 2% DMSO in sterile 0.9% saline. Vehicle injections were 2% DMSO in sterile 0.9% saline. All other drugs were dissolved in 0.9% sterile saline. SCH23390 (Sigma) was administered at 0.1mg/kg I.P. 30 minutes before behavioral experiments. Propranolol was administered at 20mg/kg I.P. 30 minutes before the behavioral session. Combined injections of propranolol and prazosin were administered at either 0.5mg/kg prazosin and 5mg/kg propranolol or 1mg/kg prazosin and 10mg/kg propranolol 30 minutes prior to the behavioral session. Yohimbine hydrochloride (Sigma) was administered at 2mg/kg I.P. 10 minutes after the start of

photometry recordings. Eticlopride hydrochloride (Sigma) was administered at 2mg/kg I.P. 10 minutes after the start of photometry recordings.

2.8 Immunohistochemistry:

Basic immunohistochemistry procedures were the same as described in Part 1 of this dissertation. Primary antibodies used included anti-c-Fos rabbit primary antibody (1:5000, ABE457, Millipore), anti-c-Fos goat primary antibody (1:5000, SC52, Santa Cruz), and anti-TH rabbit primary antibody (1:5000, AB152, Sigma). Secondary antibodies included biotinylated donkey-anti-rabbit (1:500, Jackson ImmunoResearch), biotinylated donkey-anti-goat (1:500, Jackson ImmunoResearch), and donkey-anti-rabbit-Alexa555 (1:500, Fisher). Detection was performed with Streptavidin-Cy3 (1:500, Jackson ImmunoResearch) or Streptavidin-Cy5 (1:500, Jackson ImmunoResearch).

2.9. Image Acquisition:

Images were acquired at 10-20X magnification using a fluorescence slide scanner (BX61VS, Olympus) as described in Part 1 of this dissertation.

2.10 Statistical Analyses:

For analysis of behavioral data from training and tone test sessions, freezing scores in each phase type (baseline, tone, trace) were averaged for each animal. All data were analyzed using repeated-measures ANOVA or t-tests as appropriate. ANOVA was followed by Bonferroni-corrected *post hoc* comparisons when necessary. A threshold of $p < .05$ was used to determine statistical significance. All data are shown as

mean ± SEM. Data were analyzed with GraphPad Prism (v8) or custom Python scripts and figures were generated using custom Python scripts and BioRender.

3. Results

3.1 The locus coeruleus responds to neutral and aversive environmental stimuli

Before examining the activity of the locus coeruleus during trace fear conditioning, we sought to characterize its responses to the neutral and aversive stimuli used in the task in isolation. We began by observing phasic LC responses to neutral auditory stimuli. Because previous research indicates the LC may be particularly involved in responses to salient environmental stimuli (Aston-Jones & Bloom, 1981; Aston-Jones & Cohen, 2005; Vazey et al., 2018), we examined the responses of the locus coeruleus to auditory stimuli of varying intensities (55dB-95dB).

To observe LC responses to environmental stimuli, we expressed the genetically encoded calcium indicator GCaMP6s specifically in the locus coeruleus of TH-Cre transgenic mice and implanted an optical fiber above the injection site to allow fiber photometric recordings of bulk LC activity (Figure 1A and Figure 1B). After recovery from surgery, GCaMP fluorescence in LC was measured as the mice were exposed to five interleaved presentations of a 3000 Hz pure tone at each dB level (25 total presentations). Small phasic responses were seen at each dB level, but responses increased with tone intensity, with responses to the 95dB tone most pronounced ($F(4,12) = 3.36$, $p < .05$) (Figure 1C and 1D), confirming that the magnitude of the LC response to an auditory stimulus is modulated by its intensity.

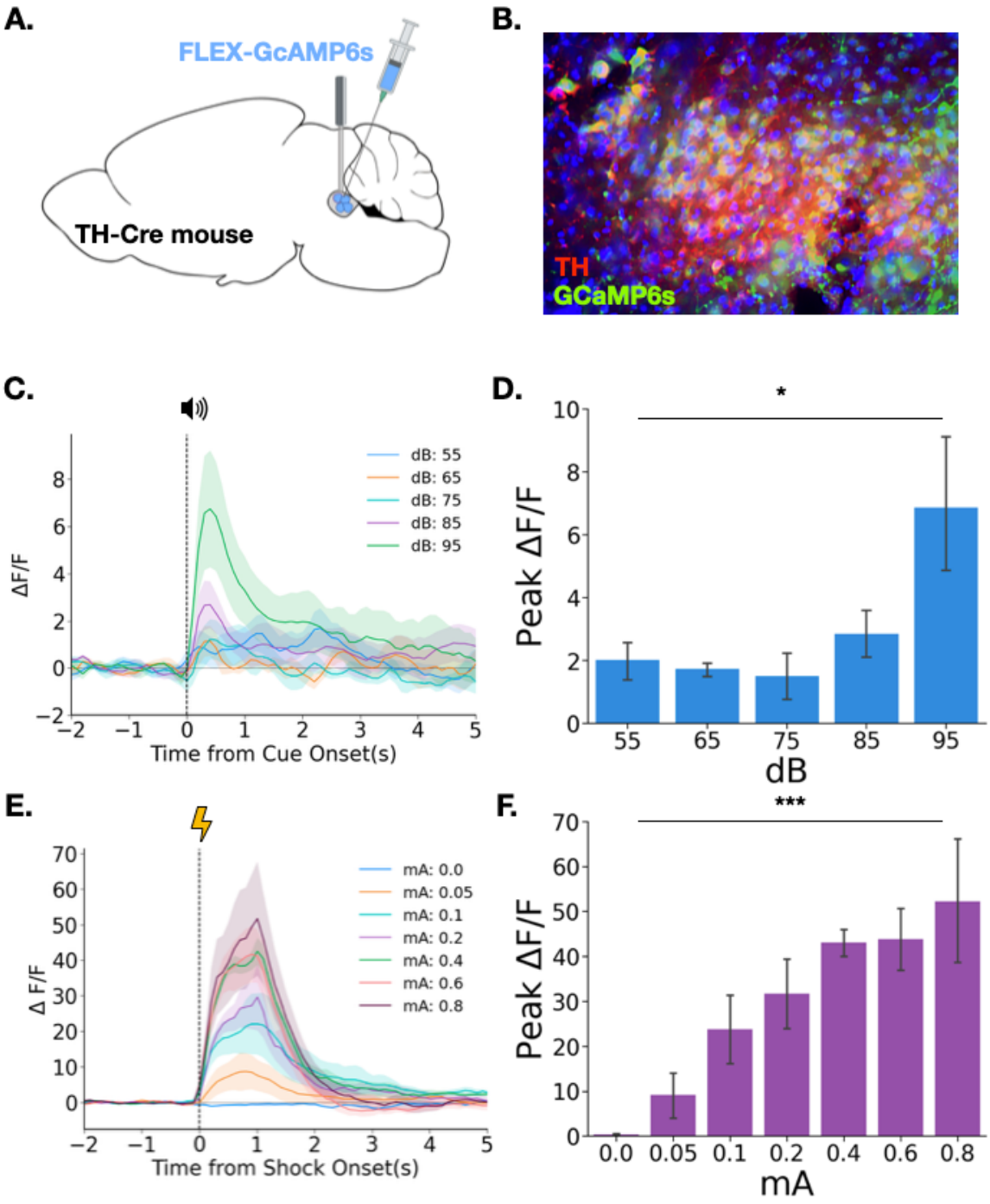

Figure 1. Locus coeruleus responses to neutral and aversive stimuli. **(A)** Schematic of virus infusion and fiber implant in LC. FLEX-GCaMP6s was infused into the LC of TH-Cre mice and an optical fiber was implanted just above the injection site. **(B)** GCaMP6s expression in LC. Green = GCaMP**(C)** Fiber photometry traces of LC responses to tone onset at varying dB levels. Dashed line indicates tone onset. **(D)** Peak dFF during tone onset at each dB level. Mean +/- SEM. **(E)** Fiber photometry traces of LC responses to shock onset at varying mA levels. Dashed line indicates shock onset. **(F)** Peak dFF during shock onset at each shock intensity. Mean +/- SEM. *p < 0.05.

The locus coeruleus is also known to respond robustly to aversive stimuli (Chen & Sara, 2007; Hirata & Aston-Jones, 1994; Rasmussen & Jacobs, 1986; Uematsu et al., 2017). To determine whether these LC responses are modulated by the intensity of the aversive stimulus, we recorded calcium activity in the LC while mice were exposed to foot shocks of varying intensity (0-0.8mA). Consistent with the idea that LC encodes information about the salience of environmental stimuli, we found that LC responses to foot shock were much larger than the previously observed responses to neutral auditory stimuli (Figure 1 C-F). Additionally, the size of the LC response to footshock increased with foot shock intensity ($F(6,18) = 6.46$, $p < .001$) (Figure 1E and 1F).

The results of these initial experiments demonstrate that the LC responds to both neutral and aversive stimuli and that the size of its responses is positively correlated with the salience and/or valence of the stimulus. Additionally, this data provides evidence that the LC exhibits phasic responses to the types of stimuli used in trace fear conditioning, even without an explicit requirement for learning.

3.2 Locus coeruleus responses change with learning

Having demonstrated that the locus coeruleus is responsive to salient environmental events, we next sought to determine whether its responses change across learning. Again, we began by using fiber photometry to monitor LC responses to a neutral auditory stimulus. If the LC responds to novelty or salience, we would expect the responses to diminish over repeated experiences with a stimulus as the animal habituates to its presence (Thompson & Spencer, 1966). To test this idea, we recorded activity in the LC over the course of 3 days as animals were repeatedly exposed to the

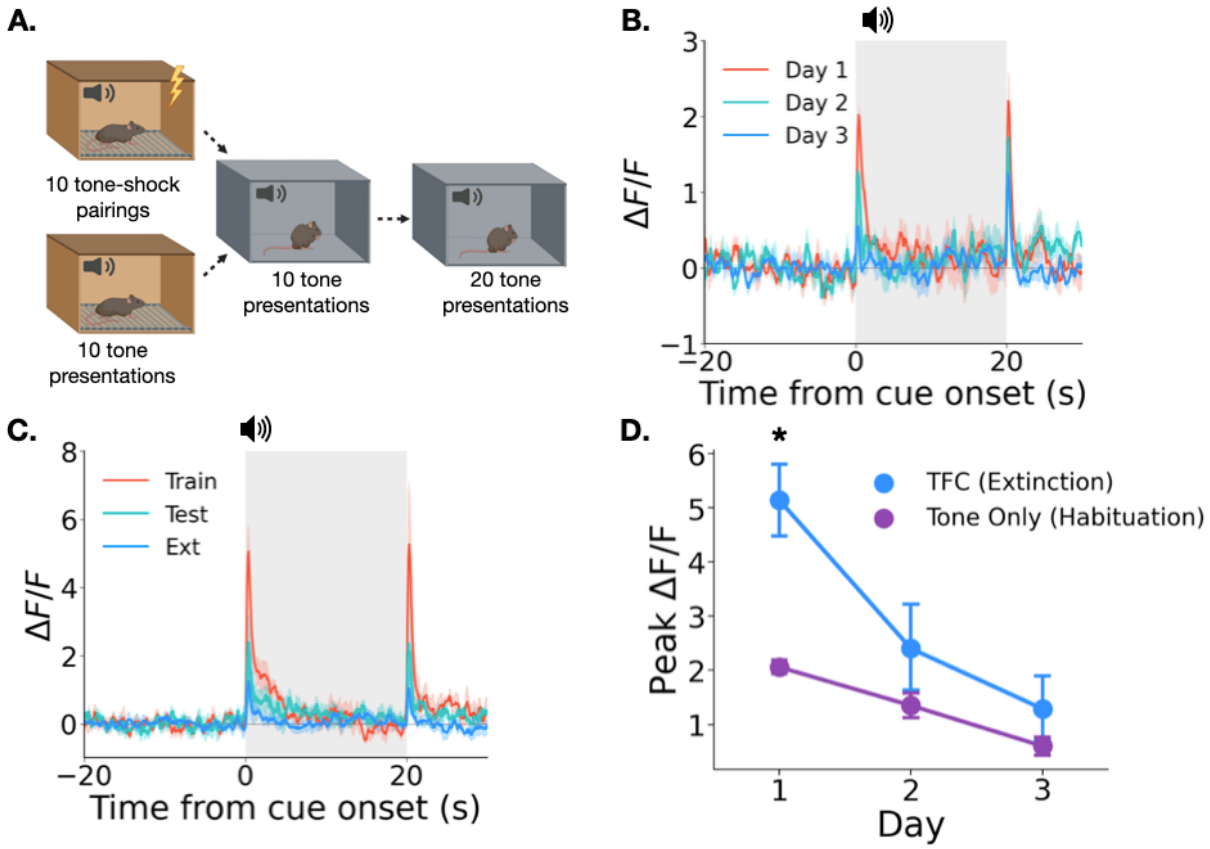

Figure 2. Locus coeruleus responses are modified by learning. **(A)** Schematic of behavioral conditions. On Day 1, mice received either 10 tone presentations or 10 tone-shock pairings (trace fear conditioning) in Context A. On Day 2, all mice received 10 tone presentations in Context B. On Day 3, all mice received 20 tone presentations in Context B. **(B)** Fiber photometry traces of LC responses to tone onset and termination in tone only animals across the 3 experimental days. **(C)** Fiber photometry traces of LC responses to tone onset and termination in trace fear conditioned animals across the 3 experimental days (Train = Day 1; Test = Day 2; Ext = Day 3). **(D)** Peak dFF during tone onset for trace fear conditioned and tone only mice across the 3 experimental days. Mean +/- SEM. *p < 0.05.

same tone (85dB, 3KHz) (Figure 2A). As expected, LC responses to the tone were largest on the first day and gradually reduced in magnitude over the next two days (Main effect of day $F(2,14) = 25.6$, $p<.05$)(Figure 2B and C). The LC also responded to the termination of each tone and this response habituated across repeated tone presentations (Main effect of day $F(2,14) = 13.5$, $p<.05$). This finding is consistent with prior work showing that animals process the termination of an auditory CS as a distinct,

salient event that itself can undergo conditioning(Sommer-Smith, 1967; Sommer-Smith et al., 1962). Therefore, across trials, the animals habituated to the entire pattern of events (tone one – 20 seconds – tone off), and LC responses to both the tone onset and termination were both reduced.

Next, we examined the effect of associative fear learning on LC responses in a second group of mice that underwent the same procedures, except that on the first day they received ten trace fear conditioning trials instead of unpaired tone presentations. Trace conditioning trials consisted of a 20-second tone followed by a 20-second stimulus free trace interval ending in a 2-second 0.2mA footshock. The LC responses in these animals followed a similar pattern to that observed in the tone only group, with activity on Day 1 being the largest and gradually decreasing across days as the mice extinguished. However, the magnitude of the LC responses was much larger in mice that underwent trace fear conditioning on Day 1(Day x Group interaction: $F(2,14) = 3.7$, $p<.05$; Day 1, TFC vs tone only: $t(7) = 4.21$, $p<.05$). These data suggest that LC responses can be modified by associative learning – as the neutral conditional stimulus becomes predictive of an aversive outcome, the LC response to the previously neutral stimulus is enhanced. Additionally, as this association is extinguished and the tone becomes less predictive of an aversive outcome, the LC response is reduced – there was no significant difference between conditioned and tone only mice on the second or third days of behavior when neither group was receiving footshocks (Day 2: $t(7) = 1.38$, $p>.05$; Day 3: $t(7) = 1.21$, $p>.05$). These results are consistent with previous recording studies that found individual LC neurons can acquire a learned response to the

conditional stimulus during training that disappears during extinction (Rasmussen & Jacobs, 1986; Sara & Segal, 1991).

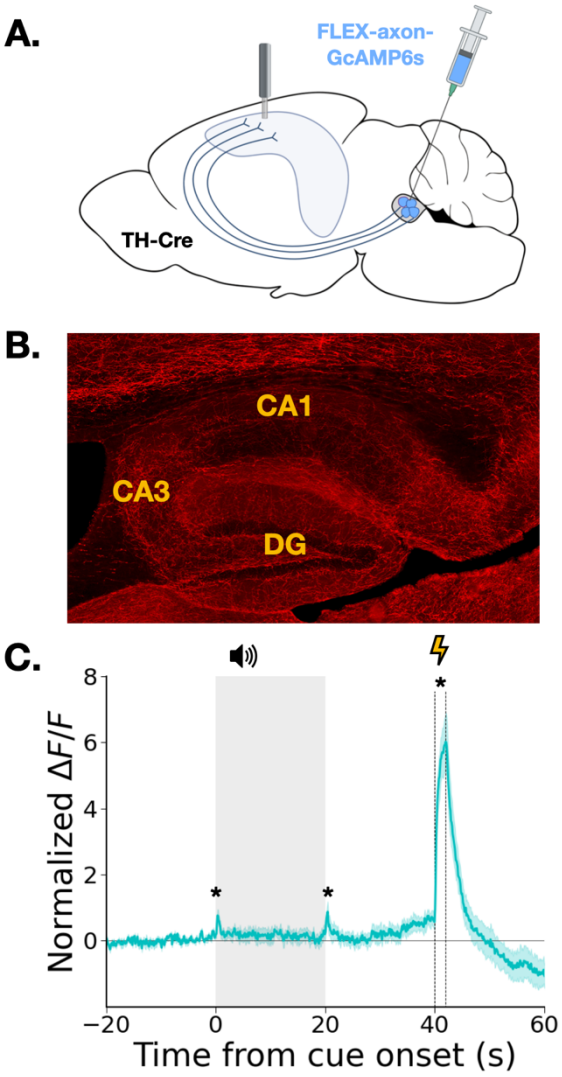

Figure 3. LC projections to dHPC respond to trace conditioning. **(A)** Schematic of surgical procedures. **(B)** TH staining in dorsal hippocampus is densest in dentate gyrus (DG), CA3, and stratum lacunosum moleculare of CA1. **(C)** Fiber photometry traces of LC-HPC projections during trace fear conditioning. *p < 0.05.

3.3 Locus coeruleus terminals in dorsal hippocampus exhibit phasic responses during trace fear conditioning

We next examined whether the specific projections from the LC to the dorsal hippocampus (dHPC) exhibit phasic responses during trace fear conditioning. To do this, we infused a cre-dependent AAV encoding axon-GCaMP6s into the LC of TH-Cre mice and implanted an optical fiber just above dorsal CA1 (Figure 3A). Axon-GCaMP6s is a calcium indicator modified to enrich the amount of GCaMP6s expressed specifically in axons, resulting in enhanced performance when imaging long-range projections, like those from LC to dHPC (Broussard et al., 2018). Although axon-GCaMP did allow us to record from LC-HPC projections *in vivo*, its fluorescence was too weak to obtain strong histology images *ex vivo*. However, it is known that LC projects throughout the hippocampus, particularly to dentate gyrus, CA3, and stratum lacunosum

moleculare layer of CA1 (SLM). We confirmed this pattern with immunohistochemistry by staining for tyrosine hydroxylase (Figure 3B). Although some of the stained fibers could also originate from the ventral tegmental area, previous work has demonstrated that the majority of TH+ fibers in dorsal hippocampus arise from LC projections and not from VTA (Kempadoo et al., 2016; Takeuchi et al., 2016; Wagatsuma et al., 2018).

When we recorded activity from LC-HPC projections during the same trace fear conditioning procedure used above, we found that these projections are active in response to the same learning-related events as the LC cell bodies: tone onset ($t(10) = 5.83$, $p<.05$), tone termination ($t(10) = 4.28$, $p<.05$), and shock ($t(10) = 8.34$, $p<.05$) (Figure 3C). These responses, especially to the tone, were weaker than the responses seen directly in LC, but this may be due to the overall weaker signal when recording from long range projections in comparison to cell bodies. These results confirm that the phasic responses observed in LC during trace conditioning are also present in the specific subset of LC axons that project directly to the dHPC.

3.4 The locus coeruleus releases both dopamine and norepinephrine into the dorsal hippocampus

Our data suggest that LC projections to the hippocampus are activated by salient learning-related events. However, it remains unclear which neurotransmitter the LC releases into the dHPC during these responses. Canonically, the LC is known as the primary source of norepinephrine in the forebrain (Jones & Moore, 1977; Lindvall & Björklund, 1974; Pickel et al., 1974; Schwarz & Luo, 2015). However, recent evidence suggests that the LC can co-release norepinephrine and dopamine into the

hippocampus and other cortical areas (Devoto & Flore, 2006; Kempadoo et al., 2016; Smith & Greene, 2012; Takeuchi et al., 2016; Wagatsuma et al., 2018).

To test this possibility, we expressed the genetically encoded dopamine and norepinephrine sensors GRAB-DA and GRAB-NE, respectively, in the dorsal CA1 of separate groups of mice and implanted optical fibers above the injection site (Figure 4A). In the same animals, we expressed the excitatory DREADD hM3Dq in LC under the control of the PRSx8 promoter, which can be used to drive selective expression in LC neurons (Abbott et al., 2009; Hwang et al., 2001; Vazey & Aston-Jones, 2014). After the animals recovered from surgery, we recorded fluorescence as animals freely explored a conditioning chamber. After 10 minutes of baseline recording, we injected mice with either CNO, to activate LC neurons, or vehicle and continued to record fluorescence for 50 more minutes. Mice that received CNO the first day were treated with vehicle on the second day and vice versa.

Interestingly, the results for the dopamine and norepinephrine sensors were similar. First, in all groups of animals, there was a small increase in fluorescence immediately after the injection (Figure 4C and E), likely produced by a LC response to the highly salient I.P. injection process. However, after this increase subsided, fluorescence continued to decrease for the rest of the session in the vehicle groups for both sensors. This continued decrease is likely the result of a true reduction in the amount of dopamine and norepinephrine in the hippocampus (as the chamber becomes less novel and the animals explore less) in addition to any photobleaching that occurred over the 50 minute session. In contrast, CNO injections in mice expressing either sensor produced prolonged increases in fluorescence relative to the vehicle groups

beginning approximately 10 minutes after the injection (Figure 4C and E), indicating that stimulation of the LC increases both dopamine and norepinephrine concentrations in the dHPC (GRAB-DA, t(5) = 4.7, p<.05; GRAB-NE, t(4) = 3.4, p<.05). The time course of these increases is consistent with the latency of onset of hM3Dq effects on physiology and behavior in other studies (Alexander et al., 2009; Jendryka et al., 2019). These data confirm that the LC can co-release both dopamine and norepinephrine into the dHPC.

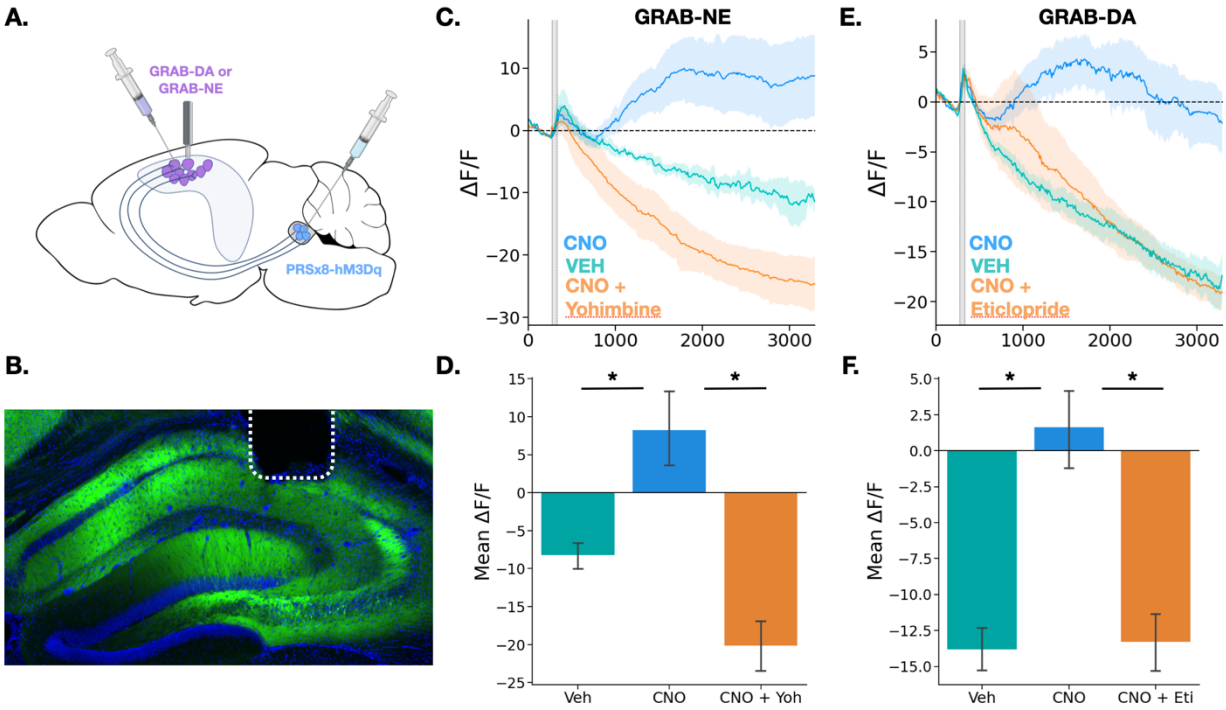

Figure 4. The locus coeruleus releases dopamine and norepinephrine into dorsal hippocampus. **(A)** Schematic of surgical procedures. AAV encoding GRAB-DA or GRAB-NE was infused into the dorsal hippocampus and an optical fiber was implanted in dorsal CA1. PRSx8-hM3D1 was infused into the locus coeruleus. **(B)** Example expression of GRAB-NE in dHPC. GRAB-NE = Green, DAPI = Blue. GRAB-DA expression was similar. **(C)** Fiber photometry traces of GRAB-NE recorded in dorsal CA1 with injections of CNO, vehicle (VEH), or CNO and yohimbine. Grey bar indicates approximate time of injection. **(D)** Mean GRAB-NE dFF for the final 40 minutes of the recording session with each injection type. **(E)** Fiber photometry traces of GRAB-DA recorded in dorsal CA1 with injections of CNO, vehicle, or CNO and eticlopride. Gray bar indicates approximate time of injection. **(F)** Mean GRAB-DA dFF for the final 40 minutes of the recording session with each injection type. Mean +/- SEM. *p < 0.05.

To further confirm these results and rule out nonspecific effects of CNO administration on sensor fluorescence, we next injected the mice with a combination of CNO and antagonists for the receptors that serve as the backbone of the sensors. Mice expressing GRAB-DA were injected with CNO and eticlopride hydrochloride, a selective dopamine D_2 receptor antagonist, which binds to the GRAB-DA sensor and prevents it from responding to the presence of dopamine (Sun et al., 2020). Mice expressing GRAB-NE were injected with CNO and yohimbine, a selective α-2 adrenergic receptor antagonist, which prevents GRAB-NE from responding to norepinephrine (Feng et al., 2019). In both cases, co-injection of CNO and a receptor antagonist prevented the increases in fluorescence observed with injections of CNO alone (GRAB-NE CNO vs + Yoh $t(4) = 7.2$, $p<.05$; GRAB-DA CNO vs CNO+Eti $t(5) = 3.5$, $p<.05$), providing further evidence that LC stimulation drives the release of both dopamine and norepinephrine into the dorsal hippocampus.

Having determined that the LC can release both dopamine and norepinephrine into the hippocampus, we next sought to determine which of these neurotransmitters is released during trace fear conditioning. We expressed GRAB-DA or GRAB-NE in dorsal CA1 and implanted optical fibers above the injection site. After the mice recovered from surgery, we made fiber photometry recordings of sensor fluorescence while mice were trained in trace fear conditioning. Traditional analysis of the data using the 405nm isosbestic control as the baseline, neurotransmitter-insensitive signal to calculate the dFF seemed to reveal that both dopamine and norepinephrine are released during trace fear conditioning (Figure 5A, C). In both cases, dFF increased at the same times that we observed phasic responses in LC cell bodies and LC terminals in the hippocampus:

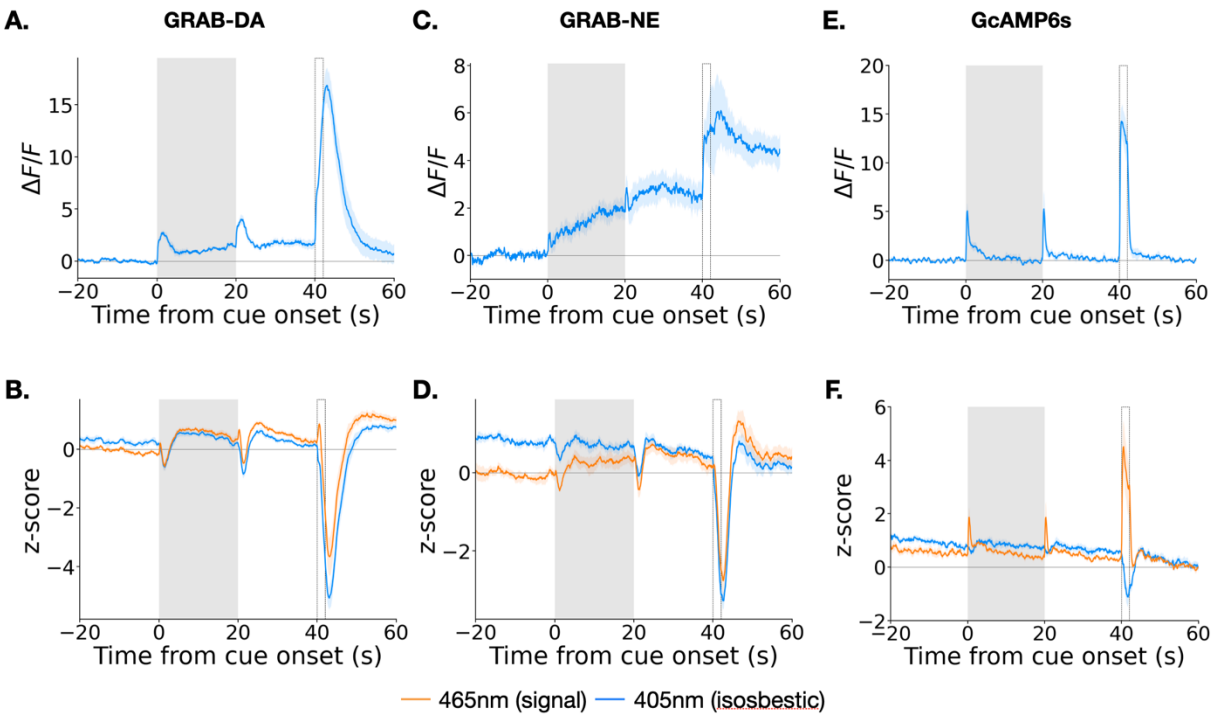

Figure 5. Measuring dopamine and norepinephrine release during trace fear conditioning. **(A)** dFF calculated using 405nm and 465nm signals as described in methods for GRAB-DA recorded during trace conditioning. **(B)** Z-scored raw fluorescence values of 405nm isosbestic and 465nm signal channels for GRAB-DA recorded during trace fear conditioning. **(C)** Same as (A), but for GRAB-NE. **(D)** Same as (B), but for GRAB-NE. **(E)** Same as (A) but for GCaMP6s. **(F)** Same as (B), but for GCaMP6s. Note the substantial increases in fluorescence in the 465nm channel that do not occur in the 405nm channel in this experiment. Grey bars indicate tone. Dashed box indicates shock. Mean +/- SEM. *p < 0.05.

tone onset, tone termination, and shock. In the GRAB-NE animals, especially, there appeared to be a step-wise increase in dFF at each phasic response as the dFF does not return to baseline between phasic responses.

Unfortunately, careful inspection of the data revealed data quality issues that preclude us from drawing strong conclusions about the dynamics of dopamine and norepinephrine release in the hippocampus during trace fear conditioning. When examining the z-scored raw data traces for the 405nm and 465nm signals, we discovered that the increased dFF observed during the tone and shock were mainly driven by artifacts present in both the 405nm and 465nm signal (Figure 5B, D). These

figures clearly show that the fluorescence in both channels drops at the onset of each salient event. During preprocessing and dFF calculation, these artifacts are inverted due to imperfect fitting of the 465nm to the 405nm channel. These same artifacts are not present in the 465nm signal for the GCaMP6s recordings – indeed, large transient increases in fluorescence are observed at each of the relevant time points. Similar, but smaller transient decreases are observed in the 405nm signal for GCaMP6s, but these likely represent true Ca2+ fluctuations, as 405nm is ~10-15nm shorter than the isosbestic wavelength for GCaMP which can result in inverted calcium responses in this channel (Dana et al., 2019). These issues have been noted elsewhere (Siciliano & Tye, 2019), but are not commonly discussed and dFF data is often presented without accounting for the source of fluctuations in the dFF, which could be changes in the 465nm signal, changes in the 405nm signal, or both. These same issues are not a concern in the DREADDs experiment because we were not examining time locked responses to specific events that may generate motion artifacts in the data. Additionally, to prevent any artifacts from differential bleaching in the isosbestic and signal channels across the long recording session, we computed dFF using a baseline subtraction method instead of the typical isosbestic channel subtraction method. These differences prevent the artifacts seen during trace fear conditioning from influencing our DREADDs photometry data.

3.5 Phasic locus coeruleus activation enhances trace fear conditioning

Our fiber photometry experiments provide strong correlational evidence that the phasic LC responses are involved in trace fear learning via the release of catecholamines, but whether this response enhances learning, as is often proposed,

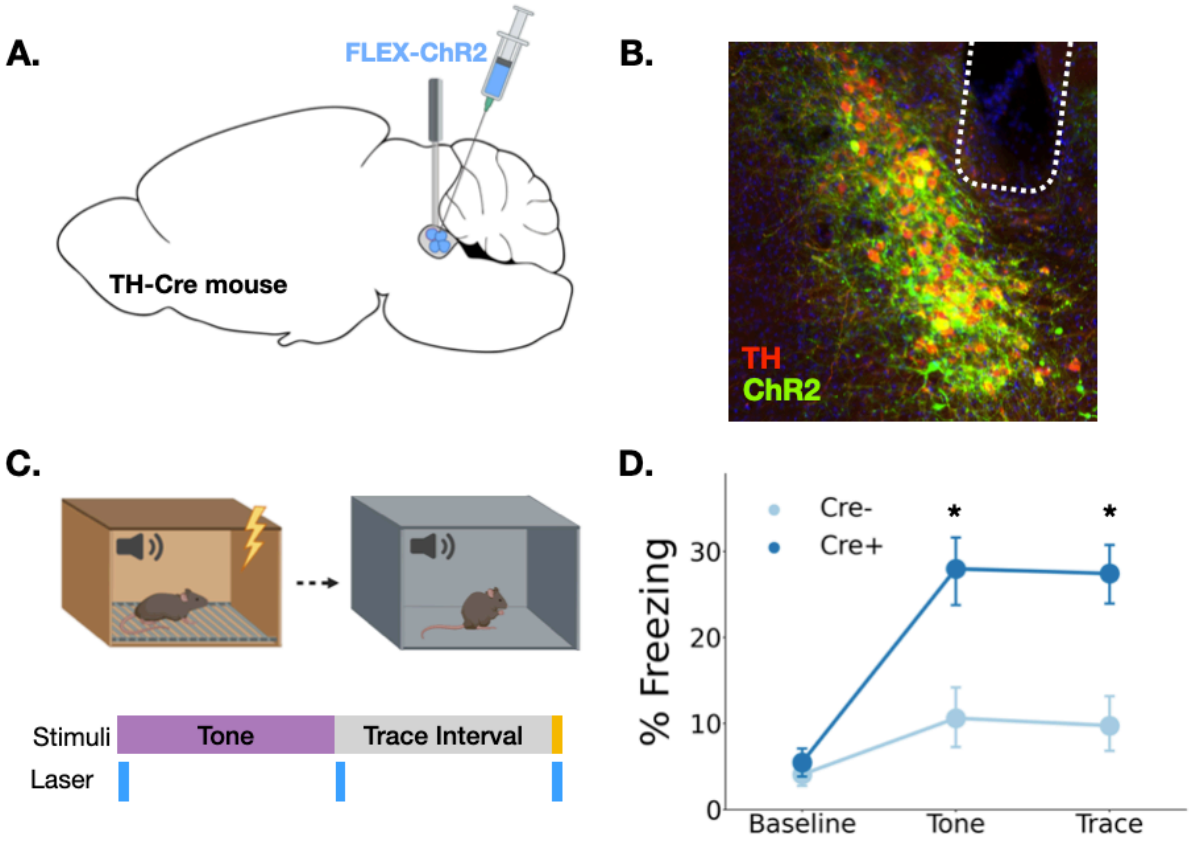

Figure 6. Phasic activation of the locus coeruleus enhances long-term memory formation. **(A)** Schematic of surgical procedures. Cre-dependent ChR2 was infused into the LC of TH-Cre mice and optical fibers were implanted above the infusion site. **(B)** Expression of ChR2 in LC. Green = ChR2-eYFP, Red = tyrosine hydroxylase stain. **(C)** Schematic of behavioral procedures. Animals were trained in trace fear conditioning in Context A on Day 1 and tested in Context B on Day 2. During training, 20Hz blue light was delivered to the locuse coeruleus for two seconds at the beginning and end of the tone as well as during the shock. **(D)** Memory performance for the two groups during the tone test as indexed by freezing behavior. The groups did not differ at baseline, but the Cre+ mice expressing ChR2 froze significantly more during the tone and trace intervals. Mean +/- SEM. *p < 0.05.

remains unknown (Giustino & Maren, 2018; Likhtik & Johansen, 2019; McGaugh, 2004; Sears et al., 2013; Takeuchi et al., 2016). To determine whether phasic activation of LC enhances trace fear conditioning, we infused a cre-dependent version of the excitatory opsin ChR2 into the LC of TH-Cre mice or their wild-type littermates and optical fibers were implanted above the infusion site (Figure 6A and 6B). After recovery from surgery,

the mice underwent trace fear conditioning using a protocol with low tone (65dB) and shock intensity (0.2mA) to produce weak learning that may allow us to uncover an enhancement with LC stimulation. These values were chosen based on the reduced LC responses to stimuli of these intensities in our tone- and shock-response curve experiments. During the training session, 20Hz blue light was delivered to the locus coeruleus in three two-second periods beginning at each of the trial events at which consistent LC responses were observed in fiber photometry experiments: tone onset, tone termination, and shock onset (Figure 6C).

The next day, animals underwent a memory test in a novel context in which they were repeatedly exposed to the training tone. Freezing during the tone and the post-tone period was used as an index of fear memory retrieval. Phasic LC stimulation at the onset of all learning-related events significantly enhanced long-term memory formation (significant Group x Trial Phase interaction: $F(2,24) = 5.28$, $p<.05$). Cre-positive mice expressing ChR2 froze significantly more in the memory test during both the tones ($t(12) = 3.08$, $p <.05$) and the 20-second post-tone period ($t(12) = 3.52$, $p<.05$) corresponding to the trace-interval on the training day. These data indicate that increased phasic activation of the LC at specific learning-related time points during trace conditioning is sufficient to enhance long term memory formation.

3.6 Dopamine, not norepinephrine, is required for trace fear memory formation

Although these data indicate that phasic responses in the locus coeruleus can enhance trace fear memory formation, it remained unclear whether the release of dopamine or norepinephrine is responsible for this effect. To test which of these

neurotransmitter systems contributes to trace fear memory formation, we trained animals in trace fear conditioning after the administration of either norepinephrine or dopamine receptor antagonists and tested their memory the next day (Figure 7A).

To determine whether norepinephrine is critically involved in trace fear conditioning, we administered a high dose (20mg/kg) of the β-adrenergic receptor antagonist propranolol 30 minutes before training. During the memory test the next day, we observed no significant differences in freezing between the propranolol-treated mice and the saline-treated controls (Figure 7B), suggesting that norepinephrine acting through β-adrenergic receptors is not required for trace fear memory (drug main effect $F(1,14) = 0.85$, $p>.05$; drug x epoch interaction $F(2,42) = 0.53$, $p>.05$). This result is consistent with prior work showing that the activation of β-adrenergic receptors is important for the retrieval, but not the acquisition or consolidation of hippocampus-dependent context fear conditioning (Murchison et al., 2004).

To provide a more complete blockade of the effects of norepinephrine, we also tested whether simultaneous antagonism of both β- and α-adrenergic receptors affects trace fear conditioning. We administered combined injections of propranolol and the α-adrenergic receptor antagonist prazosin at two different doses (low = 0.5mg/kg prazosin + 5mg/kg propranolol; high = 1mg/kg prazosin + 10mg/kg propranolol) 30 minutes before training. We found no significant effect of the drug treatments on memory retrieval the next day (Drug main effect $F(2,14) = 2.1$, $p>.05$; Drug x epoch interaction $F(6, 42) = 0.70$, $p>.05$).

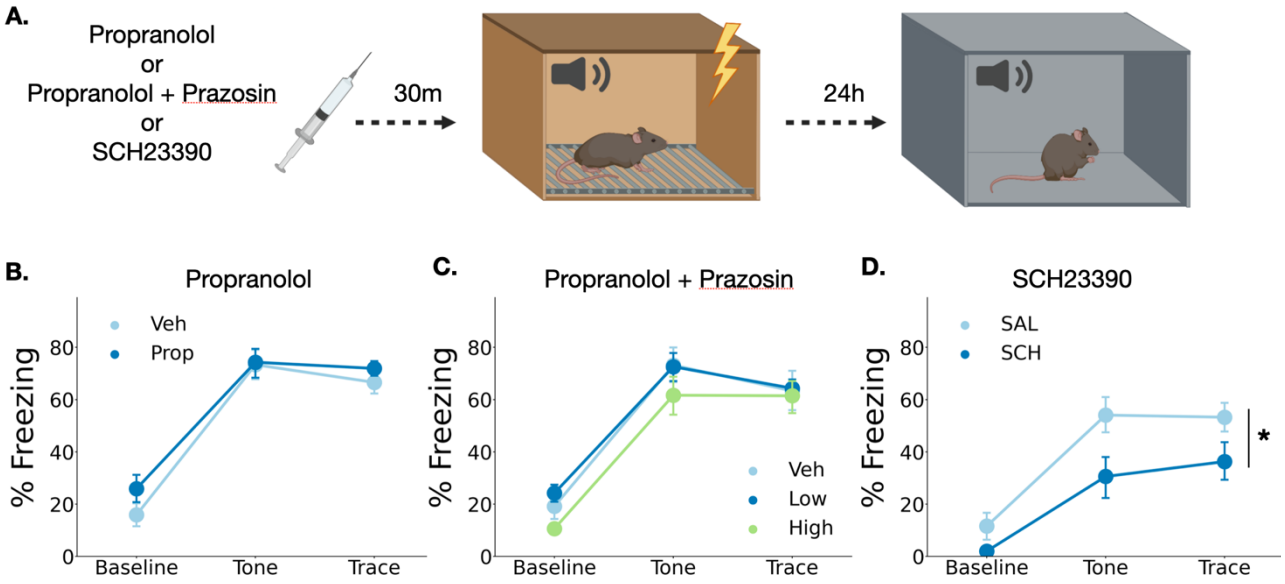

Figure 7. Dopamine, not norepinephrine, is required for trace fear memory formation. **(A)** Schematic of behavioral experiments. Mice were injected with either propranolol (20mg/kg) or SCH23390 (0.1mg/kg) thirty minutes before undergoing trace fear conditioning. The next day, mice were tested for their memory of the tone-shock association in a novel context. **(B)** Freezing behavior during the tone test in animals that received propranolol injections (Prop) and vehicle controls (Veh). **(C)** Freezing behavior during the tone test in animals that received low or high doses of propranolol and prazosin and in vehicle controls. **(D)** Freezing behavior during the tone test in animals that received SCH23390 (SCH) injections and in saline controls (SAL). Data presented as Mean+/- SEM. *p<.05.

To test whether dopamine is required for trace fear memory formation, we administered the dopamine D1 receptor antagonist SCH23390 (0.1mg/kg) 30 minutes before training. During the memory test the next day, SCH-treated mice froze significantly less than the saline controls (Main effect of drug $F(1,14) = 6.14$, $p<.05$)(Figure 7C). Taken together, these data indicate that dopamine, but not norepinephrine, is critical for trace fear memory formation.

4. Discussion

The locus coeruleus and its interaction with the hippocampus are known to be critical for hippocampus-dependent memory formation (Compton et al., 1995;

Kempadoo et al., 2016; Lemon et al., 2009; Takeuchi et al., 2016; Wagatsuma et al., 2018). However, due to the predominant use of spatial memory tasks in which the learned information is experienced diffusely in time, the precise temporal dynamics of learning-related LC and LC-HPC activity remain poorly understood. In these experiments, we used trace fear conditioning to examine the contribution of precisely timed phasic LC and LC-HPC activity to long-term memory formation.

In the first experiment, we demonstrated that the LC exhibits phasic responses to both neutral and aversive stimuli. This data is consistent with previous reports (Aston-Jones & Bloom, 1981; Hirata & Aston-Jones, 1994; Rasmussen & Jacobs, 1986) and confirms that phasic responses in the LC can be detected using fiber photometry recordings of the genetically encoded calcium indicator GCaMP6s. Our data also demonstrate that the size of the LC phasic response is modulated by the intensity, or salience, of sensory stimuli. This relationship between salience and LC response magnitude was particularly pronounced for the aversive foot-shock. Across all intensities, LC responses were larger to the aversive stimuli than to neutral auditory stimuli, suggesting that the LC may encode some information about the emotional valence of stimuli in addition to the simple sensory salience – though to determine this conclusively would require equating the salience of stimuli across the somatosensory and auditory modalities while varying the emotional valence, which may not be possible, especially in a rodent model. These data are consistent with previous work suggesting a role for LC in signaling salience (Foote et al., 1980; Grant et al., 1988; Vazey et al., 2018) and extend them by providing a parametric characterization of LC responses to stimuli of varying intensities.

We also found that phasic LC responses change with learning. First, consistent with a role for LC in signaling salience or novelty, LC responses to a neutral auditory stimulus habituate across repeated exposure to a stimulus, as demonstrated previously (Hervé-Minvielle & Sara, 1995). Additionally, we demonstrated that the LC response to the same auditory stimulus is larger when the stimulus is associated with an aversive outcome. Previous work has shown that LC neurons can respond more to conditioned than non-conditioned stimuli (Aston-Jones et al., 1994; Bouret & Sara, 2004; Uematsu et al., 2017); however, to our knowledge, this is the first demonstration of increased LC responding to the CS in a trace conditioning task where the CS and US are separated by an extended interval. This may indicate some level of top-down influence over the LC by the hippocampus, as the hippocampus is required for learning in this task (Wilmot et al., 2019).

Our results also confirmed that the direct projections from the LC into the dHPC are phasically activated by all learning-relevant stimuli during trace conditioning. This data provides support for the idea that phasic LC input to the hippocampus is involved in memory formation. We did not observe obvious changes in tonic LC-HPC activity in our data, though these changes may be more subtle and occur over longer time scales that would be difficult to detect using fiber photometry. The presence of the same responses in the LC-HPC axon terminals that were seen in LC cell bodies fits with the canonical view of the LC as a largely homogenous structure whose neurons' axons are highly collateralized. However, it was important to test whether LC-HPC projections respond during trace conditioning directly, as more recent work suggests the LC may be composed of many distinct "modules" of neurons that project to separate regions and it

is not yet known whether these modules exhibit different response properties (Schwarz & Luo, 2015; Uematsu et al., 2017). Because we only recorded from LC projections to the HPC, our data could be consistent with either a homogenous or modular LC organization. Future work could distinguish between these possibilities by recording LC projections in an array of downstream targets.

Because a number of recent studies have indicated that the LC neurons co-release dopamine and norepinephrine (Devoto & Flore, 2006; Kempadoo et al., 2016; Smith & Greene, 2012; Takeuchi et al., 2016; Wagatsuma et al., 2018), we also sought to determine which of these neurotransmitters is released during the phasic LC-HPC responses observed during trace conditioning. Using DREADDs, we were able to show that direct LC stimulation drives increases in both dopamine and norepinephrine in the HPC. Due to the low number of VTA/SN fibers in dorsal HPC, is likely that the observed increase in dopamine during LC stimulation is due to direct release from the LC. However, it is also possible that HPC dopamine is increased indirectly via LC projections to dopaminergic midbrain regions (Guiard et al., 2008).

Unfortunately, were not able to confidently detect changes in norepinephrine or dopamine concentration at physiological levels during learning. This result is most likely due to the very low concentrations of dopamine and norepinephrine in the hippocampus relative to areas, like striatum, where fluorescent neurotransmitter sensors have been used successfully (Labouesse et al., 2020) and highlights a need for more sensitive sensors. Additionally, our results with the fluorescent neurotransmitter sensors GRAB-DA and GRAB-NE (as well as dLight and nLight, data not shown), underscore the need for careful inspection of fiber photometry data to determine the source of fluctuations in

the reported dFF. Currently, the standard preprocessing of photometry data involves some form of subtracting an isosbestic control signal from the “true” neurotransmitter-dependent signal. Although this method can be useful for removing non-neurotransmitter-dependent sources of variability in the photometry signal, it can also introduce artifacts into the data in cases where there is a poor fit between the control and signal channels. In some cases, these artifacts can appear very similar to real fluctuations in the dFF. Therefore, it is imperative that the raw photometry data is scrutinized before preprocessing and analysis to ensure accurate capture of neurotransmitter/calcium dynamics.

A wide body of evidence suggests that LC activation enhances memory acquisition (Kempadoo et al., 2016), consolidation (LaLumiere et al., 2003; Novitskaya et al., 2016; Takeuchi et al., 2016), and retrieval (Murchison et al., 2004; Sara & Devauges, 1988). Here, we extended this data by showing that precisely timed phasic activation of the locus coeruleus enhances memory formation. Specifically, our data indicate that phasic LC activation can enhance memory formation when the relevant stimuli are not salient enough to produce significant learning on their own.

There are multiple possibilities as to the mechanism underlying this enhancement. First, both dopamine (Frey et al., 1990; J. Lisman et al., 2011) and norepinephrine (Bliss et al., 1983; Hu et al., 2007; Stanton & Sarvey, 1985) enhance synaptic plasticity in the hippocampus and in other structures important for trace fear conditioning, including the amygdala (Bissière et al., 2003; Huang et al., 2000; Tully et al., 2007). Given our data showing that dopamine, and not norepinephrine, is required for trace fear memory formation, the most direct possibility is that the release of

dopamine from the LC enhances learning-related plasticity in regions supporting trace fear conditioning. Our finding that systemic β-adrenergic receptor antagonism has no effect on trace fear memory formation was surprising in light of previous work demonstrating memory impairments after infusions of adrenergic antagonists into the hippocampus or amygdala (Giustino & Maren, 2018 for review). However, our data is consistent with recent findings from several other groups (Kempadoo et al., 2016; Murchison et al., 2004; Takeuchi et al., 2016; Wagatsuma et al., 2018). In particular, the work of Thomas et al. used several different methods to demonstrate that β-adrenergic receptors in the dHPC are required for retrieval but not learning or memory consolidation (Murchison et al., 2004). Based on this previous work and our data showing learning-related activity in LC-HPC projections, we believe that LC release of dopamine into the hippocampus is at least partially responsible for our observed effects. However, future work manipulating this projection directly and/or using intra-hippocampal infusions of dopamine agonists and antagonists would be required to make this conclusion.

The LC may also assert its effect on memory formation indirectly via its influence over sensory processing, attention, or valence processing, all of which are affected by phasic LC activity (Aston-Jones & Cohen, 2005; Bouret & Sara, 2004; McCall et al., 2015; Vazey et al., 2018). Under this hypothesis, the LC could facilitate memory by enhancing the responses of neural populations involved in processing, attending, or assigning valence to the learning-relevant stimuli. By reducing the intensity threshold gating these neurons' responses, phasic LC activity could allow the relatively weak stimuli used in this experiment to capture the animals' attention and produce learning

when they otherwise would not. Distinguishing between the plasticity and cognitive modulation accounts of LC-driven memory enhancements would be difficult as many of the same cellular mechanisms are likely involved in both processes. Indeed, it is likely that LC enhancements of plasticity and cognitive function both contribute to its effects on memory.

Conclusions and Future Directions

In this study, we have shown that memory formation relies on precisely timed neural activity in both the hippocampus and the locus coeruleus. Because most studies of hippocampal function use spatial or contextual memory tasks in which the learned information is experienced diffusely in time, relatively little research has homed in on the processes governing memory formation at the key moments when the relevant learning is occurring. Here, we used trace fear conditioning, a hippocampus-dependent task that involves learning an association between temporally discrete stimuli, to make more precise observations and manipulations of neural activity at these moments of learning.

In Part 1 of this dissertation, we showed that intact hippocampal activity is required for both memory retrieval and encoding specifically during the presentation of the relevant stimuli (CS and US). Although not presented in this study, our lab has also collected data showing that disrupting hippocampal activity outside of the relevant time points, such as in the middle of the intertrial interval, has no effect on memory formation (Puhger et al., unpublished data). Our data also suggest that specific temporal patterns of hippocampal activity during the learning experience are required for memory formation and retrieval, as either decreasing or increasing hippocampal activity results in severe memory impairments. In fact, in our experiments, increasing hippocampal activity appeared to disrupt memory even more severely than inhibiting it. This finding contrasts with previous work suggesting that increasing activity in the hippocampus may enhance memory formation (Kitamura et al., 2014; Sellami et al., 2017), though significant differences in stimulation and behavioral protocols exist between these studies. Indeed, several other studies suggest that artificially or pathologically (as in the case of epilepsy) increased hippocampal activity impairs learning and memory (Krueger

et al., 2020; Palop et al., 2007; Viskontas et al., 2000; Zhao et al., 2014). Future studies could seek to resolve these discrepancies by examining the effect of stimulating different cell types and/or inputs to the dorsal hippocampus with a variety of stimulation protocols.

While we have shown that hippocampal activity specifically during the entirety of CS-trace interval-US sequence is important for memory formation, future studies could determine the time course of hippocampal involvement more precisely. These manipulations may provide some insight into the specific contribution of the hippocampus to learning in trace conditioning tasks. For example, it is possible that hippocampal activity is only required during one of these parts of the learning process. If the HPC is specifically required for maintaining a representation of the CS during the trace interval so that it can then be associated with the US, its activity may be required throughout the entire trial so that CS information can enter the hippocampus, be maintained throughout the trace interval, and become associated with the US. However, if the HPC is responsible for retrieving a memory of the CS at the time of the US, HPC activity may only be required during the stimulus presentations and not during the trace interval (though disrupting hippocampal activity immediately after CS presentation may impair the consolidation of the CS memory). These questions and others could be answered using the same techniques we used in Part 1 of this dissertation but with more temporally restricted periods of optogenetic inhibition.

In Part 2 of this dissertation, we showed that the locus coeruleus and its projections to the hippocampus are activated phasically at the beginning of each part of the trace conditioning sequence (CS, trace interval, and US) and that these phasic

responses can enhance memory formation, most likely via the release of dopamine. This data serves as a strong foundation upon which future studies should build in a number of directions. First, future studies could improve upon the circuit selectivity of these experiments. While we obtained precise, circuit-specific observational data showing phasic activation of LC-HPC terminals during trace fear memory formation, our manipulation experiments were limited to direct manipulations of the locus coeruleus and systemic injections of norepinephrine and dopamine antagonists. To confirm that the LC-HPC projections, specifically, facilitate memory formation, future experiments should manipulate LC axon terminals directly in the hippocampus. Alternatively, or additionally, to more conclusively demonstrate that LC dopamine release in the HPC is facilitates memory formation, it would be necessary to simultaneously manipulate LC terminals in the HPC while blocking dopamine receptors in the same location. This experiment would be technically challenging, but it is within the realm of possibility.

In addition to increasing the spatial selectivity of the manipulations, future work could also further delineate the contributions of the phasic LC-HPC responses observed at different moments during trace conditioning. In the current study, we asked the general question of whether any phasic LC activation can enhance memory and therefore stimulated LC responses during CS onset, CS termination, and US onset. However, it is possible that only one or a subset of these responses is responsible for the observed memory enhancements. This issue could be easily addressed by performing several versions of the same experiment with stimulation restricted to only one event in each. This type of work would allow some insight into the specific contribution of LC phasic activity to memory formation. For example, if only the US

response enhances memory, the LC may be involved in signaling the occurrence of emotional events that should be remembered. If CS responses also enhance memory, the LC may have a more general role in signaling the occurrence of salient environmental events which may warrant increased attention and plasticity.

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