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

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Abstract

In the current study, we used fiber photometry and optogenetics to make temporally precise observations and manipulations of locus coeruleus (LC) activity during learning in a trace fear conditioning task. Although the LC is typically thought to modulate neural activity via the release of norepinephrine (NE), several recent studies have suggested that it may also release dopamine (DA) into the hippocampus and other cortical regions. In some cases, it appears that DA release from LC into the HPC may be more important for memory than norepinephrine. The effects of DA 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). Here, we extend this data by providing direct evidence that LC activity increases both DA and NE content in the hippocampus and by showing that DA release is important for trace fear memory formation, but the release of NE is not.

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 commonly used to assess hippocampus (HPC)-dependent memory. 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:

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 has been described previously (Wilmot et al., 2019). 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. 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 and image acquisition:

The basic immunohistochemistry procedures have been described previously (Wilmot et al., 2019). Briefly, mice were transcardially perfused with 4% PFA. Following 24-48 hours of post-fixation, 40um coronal sections were cut. 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 room temperature in primary antibody (1:5000, ABE457, Millipore). Next, slices were placed in secondary antibody 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). Images were acquired at 10-20X magnification using a fluorescence slide scanner (BX61VS, Olympus).

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 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.

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.,

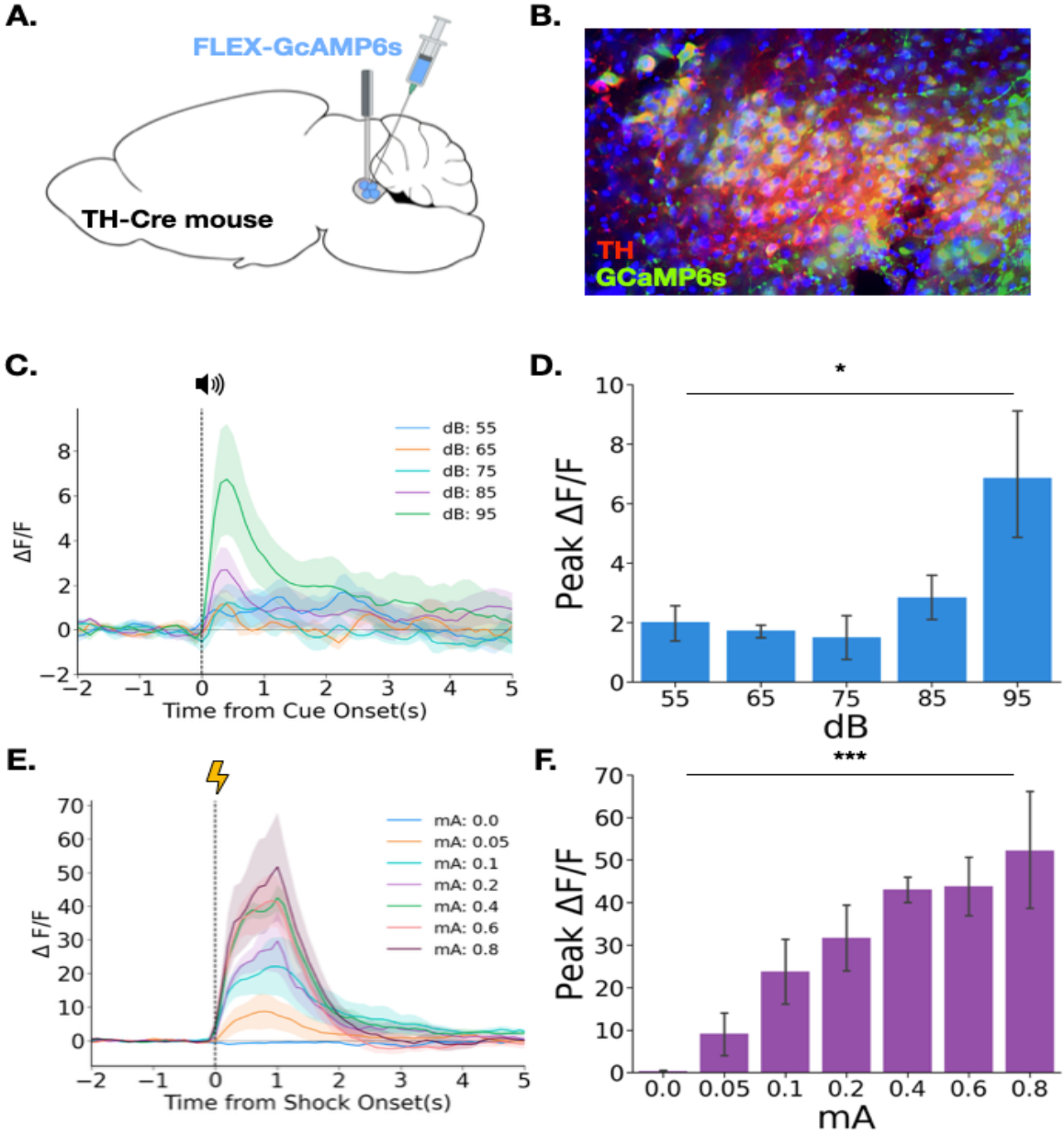

Figure 1. Locus coeruleus responses to neutral and aversive stimuli. **(A)** Schematic of virus infusion and fiber implant in LC. FLEX-GCaMP6s was infused into the LC of TH-Cre mice and an optical fiber was implanted just above the injection site. **(B)** GCaMP6s expression in LC. Green = GCaMP**(C)** 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.

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 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

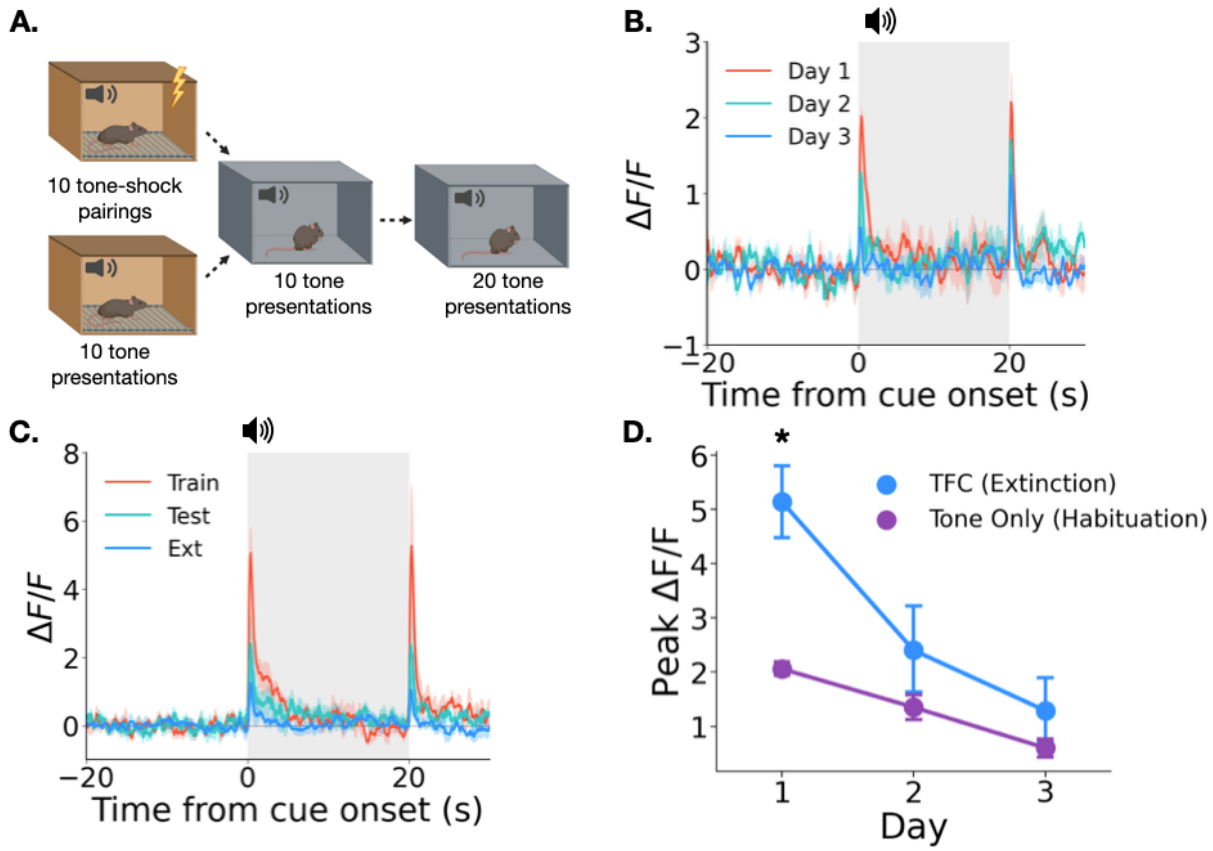

Figure 2. Locus coeruleus responses are modified by learning. **(A)** Schematic of behavioral conditions. On Day 1, mice received either 10 tone presentations or 10 tone-shock pairings (trace fear conditioning) in Context A. On Day 2, all mice received 10 tone presentations in Context B. On Day 3, all 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.

(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).

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

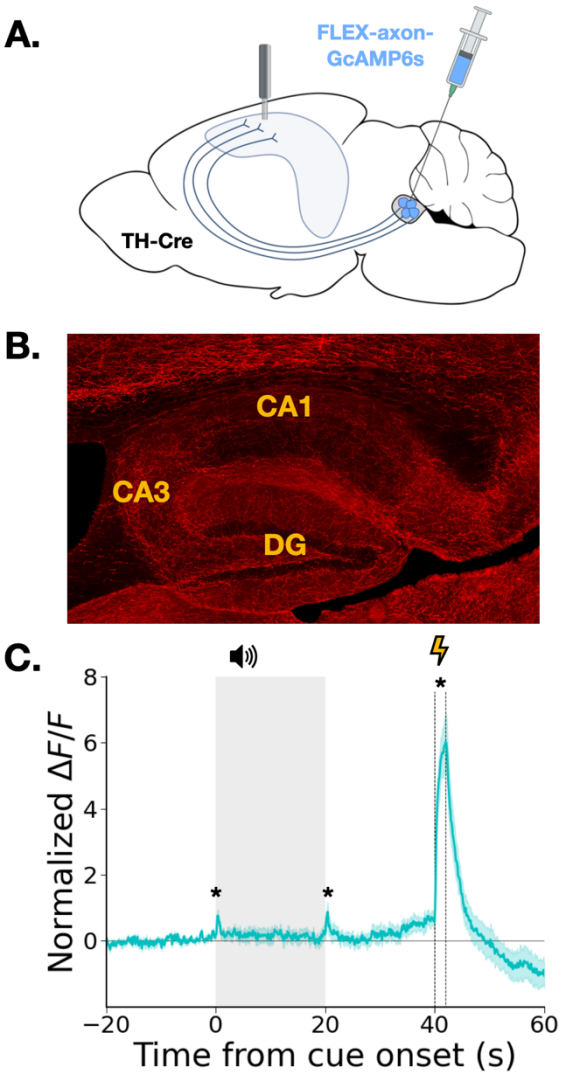

Figure 3. LC projections to dHPC respond to trace conditioning. **(A)** Schematic of surgical procedures. **(B)** TH staining in dorsal hippocampus is densest in dentate gyrus (DG), CA3, and stratum lacunosum moleculare of CA1. **(C)** Fiber photometry traces of LC-HPC projections during trace fear conditioning.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

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.

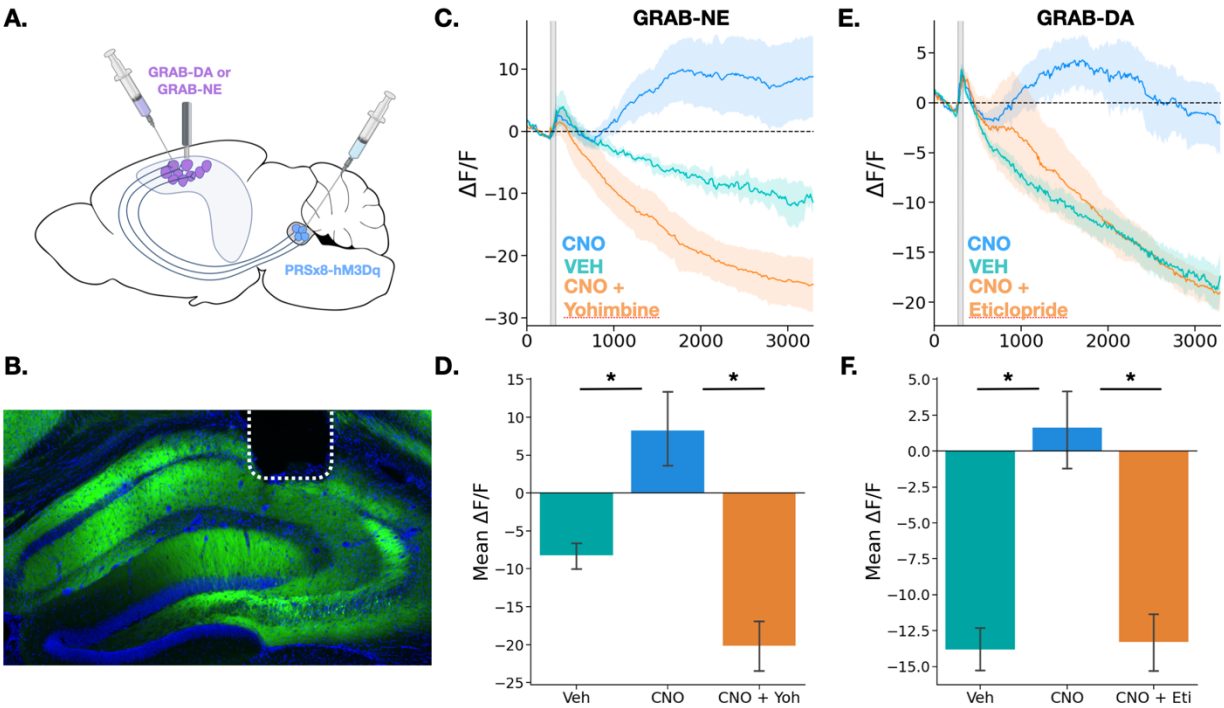

Figure 4. The locus coeruleus releases dopamine and norepinephrine into dorsal hippocampus. **(A)** Schematic of surgical procedures. AAV encoding GRAB-DA or GRAB-NE was infused into the dorsal hippocampus and an optical fiber was implanted in dorsal CA1. PRSx8-hM3D1 was infused into the locus coeruleus. **(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:

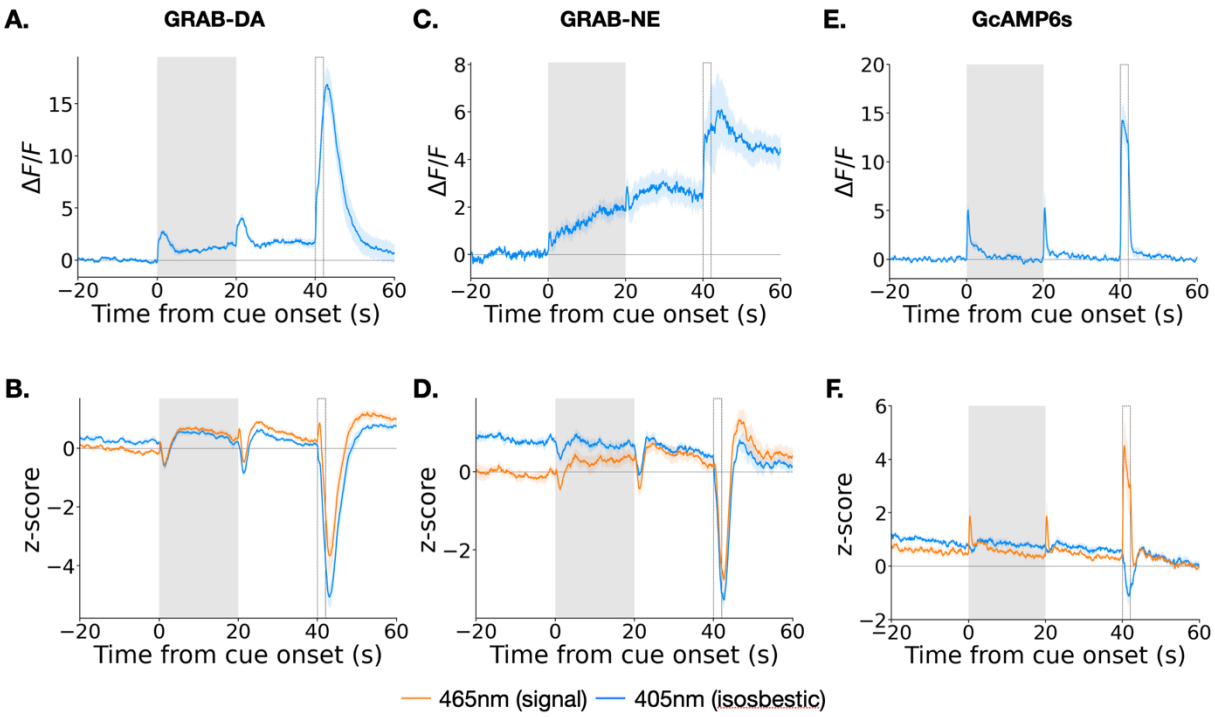

Figure 5. Measuring dopamine and norepinephrine release during trace fear conditioning. **(A)** dFF calculated using 405nm and 465nm signals as described in methods for GRAB-DA recorded during trace conditioning. **(B)** Z-scored raw fluorescence values of 405nm isosbestic and 465nm signal channels for 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,

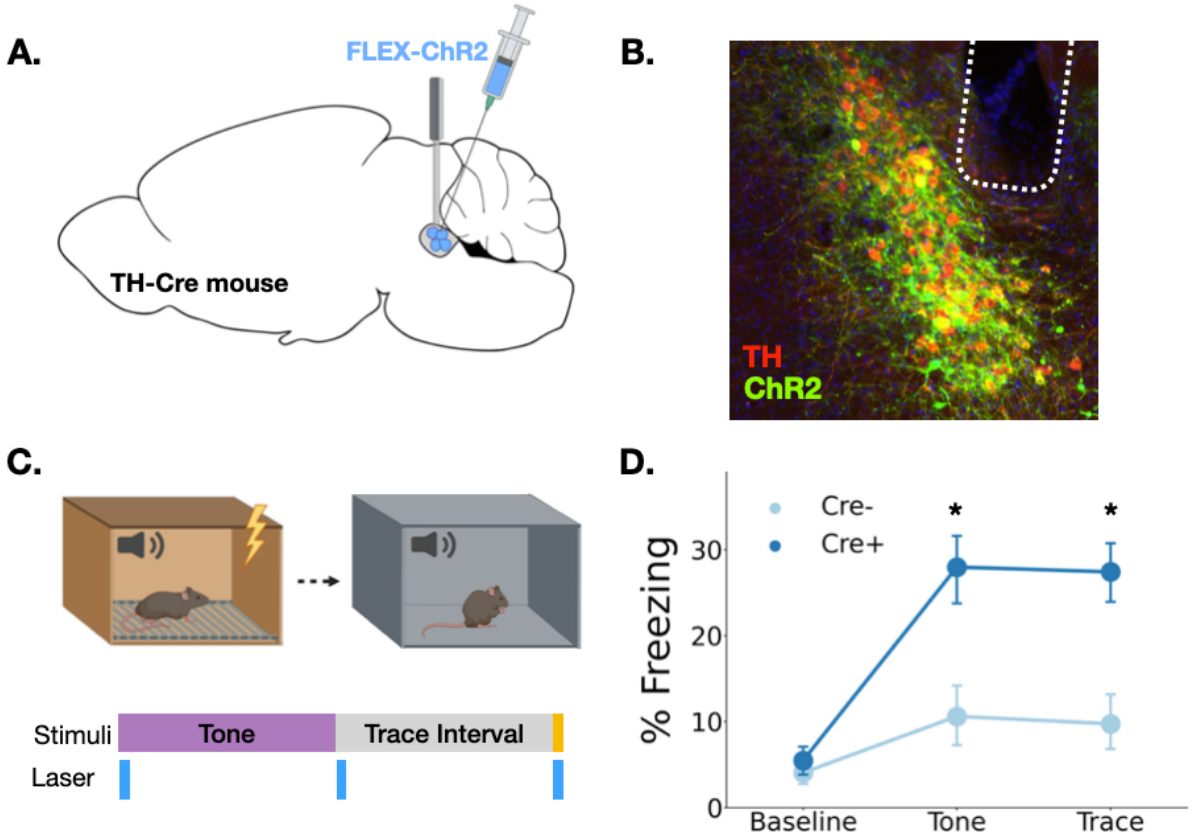

Figure 6. Phasic activation of the locus coeruleus enhances long-term memory formation. **(A)** Schematic of surgical procedures. Cre-dependent ChR2 was infused into the LC of TH-Cre mice and optical fibers were implanted above the infusion site. **(B)** Expression of ChR2 in LC. Green = ChR2-eYFP, Red = 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 $F(6, 42) = 0.70$, $p>.05$).

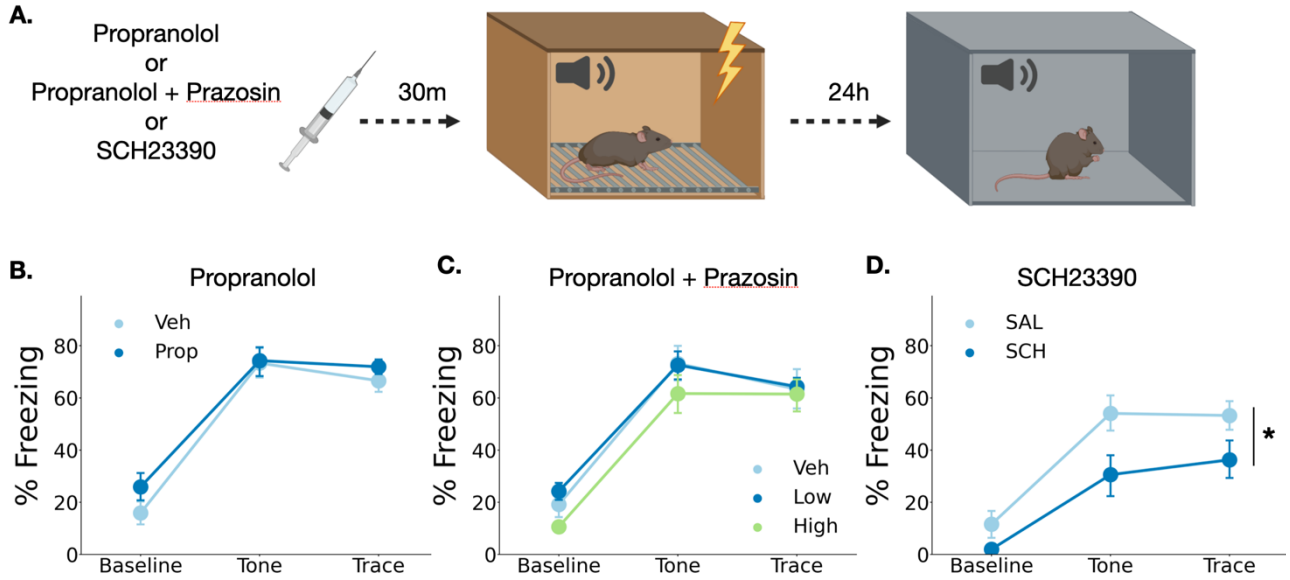

Figure 7. Dopamine, not norepinephrine, is required for trace fear memory formation. **(A)** Schematic of behavioral experiments. Mice were injected with either propranolol (20mg/kg) or SCH23390 (0.1mg/kg) thirty minutes before undergoing trace fear conditioning. The next day, mice were tested for their 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.

References

Abbott, S. B. G., Stornetta, R. L., Fortuna, M. G., Depuy, S. D., West, G. H., Harris, T. E., & Guyenet, P. G. (2009). Photostimulation of Retrotrapezoid Nucleus Phox2b-Expressing Neurons In Vivo Produces Long-Lasting Activation of Breathing in Rats. *Journal of Neuroscience*, *29*(18), 5806–5819. https://doi.org/10.1523/JNEUROSCI.1106-09.2009

Alexander, G. M., Rogan, S. C., Abbas, A. I., Armbruster, B. N., Pei, Y., Allen, J. A., Nonneman, R. J., Hartmann, J., Moy, S. S., Nicolelis, M. A., McNamara, J. O., & Roth, B. L. (2009). Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. *Neuron*, *63*(1), 27–39. https://doi.org/10.1016/j.neuron.2009.06.014

Aston-Jones, G., & Bloom, F. E. (1981). Nonrepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. *Journal of Neuroscience*, *1*(8), 887–900. https://doi.org/10.1523/JNEUROSCI.01-08-00887.1981

Aston-Jones, G., & Cohen, J. D. (2005). AN INTEGRATIVE THEORY OF LOCUS COERULEUS-NOREPINEPHRINE FUNCTION: Adaptive Gain and Optimal Performance. *Annual Review of Neuroscience*, *28*(1), 403–450. https://doi.org/10.1146/annurev.neuro.28.061604.135709

Aston-Jones, G., Rajkowski, J., & Cohen, J. (1999). Role of locus coeruleus in attention and behavioral flexibility. *Biological Psychiatry*, *46*(9), 1309–1320. https://doi.org/10.1016/S0006-3223(99)00140-7

Aston-Jones, G., Rajkowski, J., Kubiak, P., & Alexinsky, T. (1994). Locus coeruleus neurons in monkey are selectively activated by attended cues in a vigilance task. *Journal of Neuroscience*, *14*(7), 4467–4480. https://doi.org/10.1523/JNEUROSCI.14-07-04467.1994

Bach, M. E., Barad, M., Son, H., Zhuo, M., Lu, Y.-F., Shih, R., Mansuy, I., Hawkins, R. D., & Kandel, E. R. (1999). Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the cAMP signaling pathway. *Proceedings of the National Academy of Sciences*, *96*(9), 5280–5285. https://doi.org/10.1073/pnas.96.9.5280

Badrinarayan, A., Wescott, S. A., Weele, C. M. V., Saunders, B. T., Couturier, B. E., Maren, S., & Aragona, B. J. (2012). Aversive Stimuli Differentially Modulate Real-Time Dopamine Transmission Dynamics within the Nucleus Accumbens Core and Shell. *Journal of Neuroscience*, *32*(45), 15779–15790. https://doi.org/10.1523/JNEUROSCI.3557-12.2012

Bassareo, V., De Luca, M. A., & Di Chiara, G. (2002). Differential Expression of Motivational Stimulus Properties by Dopamine in Nucleus Accumbens Shell versus Core and Prefrontal Cortex. *The Journal of Neuroscience*, *22*(11), 4709–4719. https://doi.org/10.1523/JNEUROSCI.22-11-04709.2002

Bissière, S., Humeau, Y., & Lüthi, A. (2003). Dopamine gates LTP induction in lateral amygdala by suppressing feedforward inhibition. *Nature Neuroscience*, *6*(6), 587–592. https://doi.org/10.1038/nn1058

Bliss, T. V., Goddard, G. V., & Riives, M. (1983). Reduction of long-term potentiation in the dentate gyrus of the rat following selective depletion of monoamines. *The*

Journal of Physiology, *334*(1), 475–491. https://doi.org/10.1113/jphysiol.1983.sp014507

Bouret, S., & Sara, S. J. (2004). Reward expectation, orientation of attention and locus coeruleus-medial frontal cortex interplay during learning. *European Journal of Neuroscience*, *20*(3), 791–802. https://doi.org/10.1111/j.1460-9568.2004.03526.x

Broussard, G. J., Liang, Y., Fridman, M., Unger, E. K., Meng, G., Xiao, X., Ji, N., Petreanu, L., & Tian, L. (2018). In vivo measurement of afferent activity with axon-specific calcium imaging. *Nature Neuroscience*, *21*(9), 1272–1280. https://doi.org/10.1038/s41593-018-0211-4

Chen, F.-J., & Sara, S. J. (2007). Locus coeruleus activation by foot shock or electrical stimulation inhibits amygdala neurons. *Neuroscience*, *144*(2), 472–481. https://doi.org/10.1016/j.neuroscience.2006.09.037

Compton, D. M., Dietrich, K. L., Smith, J. S., & Davis, B. K. (1995). Spatial and non-spatial learning in the rat following lesions to the nucleus locus coeruleus. *Neuroreport: An International Journal for the Rapid Communication of Research in Neuroscience*, *7*(1), 177–182. https://doi.org/10.1097/00001756-199512000-00043

Dana, H., Sun, Y., Mohar, B., Hulse, B. K., Kerlin, A. M., Hasseman, J. P., Tsegaye, G., Tsang, A., Wong, A., Patel, R., Macklin, J. J., Chen, Y., Konnerth, A., Jayaraman, V., Looger, L. L., Schreiter, E. R., Svoboda, K., & Kim, D. S. (2019). High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. *Nature Methods*, *16*(7), 649–657. https://doi.org/10.1038/s41592-019-0435-6

de Jong, J. W., Afjei, S. A., Pollak Dorocic, I., Peck, J. R., Liu, C., Kim, C. K., Tian, L., Deisseroth, K., & Lammel, S. (2019). A Neural Circuit Mechanism for Encoding Aversive Stimuli in the Mesolimbic Dopamine System. *Neuron*, *101*(1), 133-151.e7. https://doi.org/10.1016/j.neuron.2018.11.005

Devoto, P., & Flore, G. (2006). On the Origin of Cortical Dopamine: Is it a Co-Transmitter in Noradrenergic Neurons? *Current Neuropharmacology*, *4*(2), 115–125. https://doi.org/10.2174/157015906776359559

Feng, J., Zhang, C., Lischinsky, J. E., Jing, M., Zhou, J., Wang, H., Zhang, Y., Dong, A., Wu, Z., Wu, H., Chen, W., Zhang, P., Zou, J., Hires, S. A., Zhu, J. J., Cui, G., Lin, D., Du, J., & Li, Y. (2019). A Genetically Encoded Fluorescent Sensor for Rapid and Specific In Vivo Detection of Norepinephrine. *Neuron*, *102*(4), 745-761.e8. https://doi.org/10.1016/j.neuron.2019.02.037

Foote, S. L., Aston-Jones, G., & Bloom, F. E. (1980). Impulse activity of locus coeruleus neurons in awake rats and monkeys is a function of sensory stimulation and arousal. *Proceedings of the National Academy of Sciences*, *77*(5), 3033–3037. https://doi.org/10.1073/pnas.77.5.3033

Frey, U., Schroeder, H., & Matthies, H. (1990). Dopaminergic antagonists prevent long-term maintenance of posttetanic LTP in the CA1 region of rat hippocampal slices. *Brain Research*, *522*(1), 69–75. https://doi.org/10.1016/0006-8993(90)91578-5

Giustino, T. F., & Maren, S. (2018). Noradrenergic Modulation of Fear Conditioning and Extinction. *Frontiers in Behavioral Neuroscience*, *12*. https://doi.org/10.3389/fnbeh.2018.00043

Grant, S. J., Aston-Jones, G., & Redmond, D. E. (1988). Responses of primate locus coeruleus neurons to simple and complex sensory stimuli. *Brain Research Bulletin*, *21*(3), 401–410. https://doi.org/10.1016/0361-9230(88)90152-9

Guiard, B. P., Mansari, M. E., & Blier, P. (2008). Cross-Talk between Dopaminergic and Noradrenergic Systems in the Rat Ventral Tegmental Area, Locus Ceruleus, and Dorsal Hippocampus. *Molecular Pharmacology*, *74*(5), 1463–1475. https://doi.org/10.1124/mol.108.048033

Hervé-Minvielle, A., & Sara, S. J. (1995). Rapid habituation of auditory responses of locus coeruleus cells in anaesthetized and awake rats. *Neuroreport*, *6*(10), 1363–1368. https://doi.org/10.1097/00001756-199507100-00001

Hirata, H., & Aston-Jones, G. (1994). A novel long-latency response of locus coeruleus neurons to noxious stimuli: Mediation by peripheral C-fibers. *Journal of Neurophysiology*, *71*(5), 1752–1761. https://doi.org/10.1152/jn.1994.71.5.1752

Hu, H., Real, E., Takamiya, K., Kang, M.-G., Ledoux, J., Huganir, R. L., & Malinow, R. (2007). Emotion Enhances Learning via Norepinephrine Regulation of AMPA-Receptor Trafficking. *Cell*, *131*(1), 160–173. https://doi.org/10.1016/j.cell.2007.09.017

Huang, Y.-Y., Martin, K. C., & Kandel, E. R. (2000). Both Protein Kinase A and Mitogen-Activated Protein Kinase Are Required in the Amygdala for the Macromolecular Synthesis-Dependent Late Phase of Long-Term Potentiation. *Journal of Neuroscience*, *20*(17), 6317–6325. https://doi.org/10.1523/JNEUROSCI.20-17-06317.2000

Hwang, D.-Y., Carlezon, W. A., Isacson, O., & Kim, K.-S. (2001). A High-Efficiency Synthetic Promoter That Drives Transgene Expression Selectively in Noradrenergic Neurons. *Human Gene Therapy*, *12*(14), 1731–1740. https://doi.org/10.1089/104303401750476230

Inoue, T., Tsuchiya, K., & Koyama, T. (1994). Regional changes in dopamine and serotonin activation with various intensity of physical and psychological stress in the rat brain. *Pharmacology Biochemistry and Behavior*, *49*(4), 911–920. https://doi.org/10.1016/0091-3057(94)90243-7

Jendryka, M., Palchaudhuri, M., Ursu, D., van der Veen, B., Liss, B., Kätzel, D., Nissen, W., & Pekcec, A. (2019). Pharmacokinetic and pharmacodynamic actions of clozapine-N-oxide, clozapine, and compound 21 in DREADD-based chemogenetics in mice. *Scientific Reports*, *9*(1), 4522. https://doi.org/10.1038/s41598-019-41088-2

Jones, B. E., & Moore, R. Y. (1977). Ascending projections of the locus coeruleus in the rat. II. Autoradiographic study. *Brain Research*, *127*(1), 23–53. https://doi.org/10.1016/0006-8993(77)90378-X

Kempadoo, K. A., Mosharov, E. V., Choi, S. J., Sulzer, D., & Kandel, E. R. (2016). Dopamine release from the locus coeruleus to the dorsal hippocampus promotes spatial learning and memory. *Proceedings of the National Academy of Sciences*, *113*(51), 14835–14840. https://doi.org/10.1073/pnas.1616515114

Labouesse, M. A., Cola, R. B., & Patriarchi, T. (2020). GPCR-Based Dopamine Sensors—A Detailed Guide to Inform Sensor Choice for In Vivo Imaging. *International Journal of Molecular Sciences*, *21*(21), 8048. https://doi.org/10.3390/ijms21218048

LaLumiere, R. T., Buen, T.-V., & McGaugh, J. L. (2003). Post-Training Intra-Basolateral Amygdala Infusions of Norepinephrine Enhance Consolidation of Memory for Contextual Fear Conditioning. *The Journal of Neuroscience*, *23*(17), 6754–6758. https://doi.org/10.1523/JNEUROSCI.23-17-06754.2003

Lemon, N., Aydin-Abidin, S., Funke, K., & Manahan-Vaughan, D. (2009). Locus coeruleus activation facilitates memory encoding and induces hippocampal LTD that depends on beta-adrenergic receptor activation. *Cerebral Cortex (New York, N.Y.: 1991)*, *19*(12), 2827–2837. https://doi.org/10.1093/cercor/bhp065

Likhtik, E., & Johansen, J. P. (2019). Neuromodulation in circuits of aversive emotional learning. *Nature Neuroscience*, *22*(10), 1586–1597. https://doi.org/10.1038/s41593-019-0503-3

Lindvall, O., & Björklund, A. (1974). The organization of the ascending catecholamine neuron systems in the rat brain as revealed by the glyoxylic acid fluorescence method. *Acta Physiologica Scandinavica. Supplementum*, *412*, 1–48.

Lisman, J. E., & Grace, A. A. (2005). The Hippocampal-VTA Loop: Controlling the Entry of Information into Long-Term Memory. *Neuron*, *46*(5), 703–713. https://doi.org/10.1016/j.neuron.2005.05.002

Lisman, J., Grace, A. A., & Duzel, E. (2011). A neoHebbian framework for episodic memory; role of dopamine-dependent late LTP. *Trends in Neurosciences*, *34*(10), 536–547. https://doi.org/10.1016/j.tins.2011.07.006

McCall, J. G., Al-Hasani, R., Siuda, E. R., Hong, D. Y., Norris, A. J., Ford, C. P., & Bruchas, M. R. (2015). CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. *Neuron*, *87*(3), 605–620. https://doi.org/10.1016/j.neuron.2015.07.002

McGaugh, J. L. (2004). The Amygdala Modulates the Consolidation of Memories of Emotionally Arousing Experiences. *Annual Review of Neuroscience*, *27*(1), 1–28. https://doi.org/10.1146/annurev.neuro.27.070203.144157

Murchison, C. F., Zhang, X.-Y., Zhang, W.-P., Ouyang, M., Lee, A., & Thomas, S. A. (2004). A Distinct Role for Norepinephrine in Memory Retrieval. *Cell*, *117*(1), 131–143. https://doi.org/10.1016/S0092-8674(04)00259-4

Novitskaya, Y., Sara, S. J., Logothetis, N. K., & Eschenko, O. (2016). Ripple-triggered stimulation of the locus coeruleus during post-learning sleep disrupts ripple/spindle coupling and impairs memory consolidation. *Learning & Memory*, *23*(5), 238–248. https://doi.org/10.1101/lm.040923.115

Packard, M. G., & White, N. M. (1989). Memory facilitation produced by dopamine agonists: Role of receptor subtype and mnemonic requirements. *Pharmacology Biochemistry and Behavior*, *33*(3), 511–518. https://doi.org/10.1016/0091-3057(89)90378-X

Packard, M. G., & White, N. M. (1991). Dissociation of hippocampus and caudate nucleus memory systems by posttraining intracerebral injection of dopamine agonists. *Behavioral Neuroscience*, *105*(2), 295–306. https://doi.org/10.1037//0735-7044.105.2.295

Pickel, V. M., Segal, M., & Bloom, F. E. (1974). A radioautographic study of the efferent pathways of the nucleus locus coeruleus. *Journal of Comparative Neurology*, *155*(1), 15–41. https://doi.org/10.1002/cne.901550103

Rasmussen, K., & Jacobs, B. L. (1986). Single unit activity of locus coeruleus neurons in the freely moving cat: II. Conditioning and pharmacologic studies. *Brain Research*, *371*(2), 335–344. https://doi.org/10.1016/0006-8993(86)90371-9

Raybuck, J. D., & Lattal, K. M. (2014). Bridging the interval: Theory and Neurobiology of Trace Conditioning. *Behavioural Processes*, *0*, 103–111. https://doi.org/10.1016/j.beproc.2013.08.016

Sara, S. J., & Devauges, V. (1988). Priming stimulation of locus coeruleus facilitates memory retrieval in the rat. *Brain Research*, *438*(1–2), 299–303. https://doi.org/10.1016/0006-8993(88)91351-0

Sara, S. J., & Segal, M. (1991). Plasticity of sensory responses of locus coeruleus neurons in the behaving rat: Implications for cognition. In C. D. Barnes & O. Pompeiano (Eds.), *Progress in Brain Research* (Vol. 88, pp. 571–585). Elsevier. https://doi.org/10.1016/S0079-6123(08)63835-2

Schwarz, L. A., & Luo, L. (2015). Organization of the Locus Coeruleus-Norepinephrine System. *Current Biology*, *25*(21), R1051–R1056. https://doi.org/10.1016/j.cub.2015.09.039

Sears, R. M., Fink, A. E., Wigestrand, M. B., Farb, C. R., de Lecea, L., & LeDoux, J. E. (2013). Orexin/hypocretin system modulates amygdala-dependent threat learning through the locus coeruleus. *Proceedings of the National Academy of Sciences*, *110*(50), 20260–20265. https://doi.org/10.1073/pnas.1320325110

Selden, N. R., Robbins, T. W., & Everitt, B. J. (1990). Enhanced behavioral conditioning to context and impaired behavioral and neuroendocrine responses to conditioned stimuli following ceruleocortical noradrenergic lesions: Support for an attentional hypothesis of central noradrenergic function. *Journal of Neuroscience*, *10*(2), 531–539. https://doi.org/10.1523/JNEUROSCI.10-02-00531.1990

Siciliano, C. A., & Tye, K. M. (2019). Leveraging calcium imaging to illuminate circuit dysfunction in addiction. *Alcohol*, *74*, 47–63. https://doi.org/10.1016/j.alcohol.2018.05.013

Smith, C. C., & Greene, R. W. (2012). CNS Dopamine Transmission Mediated by Noradrenergic Innervation. *Journal of Neuroscience*, *32*(18), 6072–6080. https://doi.org/10.1523/JNEUROSCI.6486-11.2012

Sommer-Smith, J. A. (1967). Tone cessation as a conditioned stimulus II. Inhibition. *Electroencephalography and Clinical Neurophysiology*, *23*(5), 439–448. https://doi.org/10.1016/0013-4694(67)90186-1

Sommer-Smith, J. A., Galeano, C., Piñeyrúa, M., Roig, J. A., & Segundo, J. P. (1962). Tone cessation as conditioned signal. *Electroencephalography and Clinical Neurophysiology*, *14*(6), 869–877. https://doi.org/10.1016/0013-4694(62)90137-2

Stanton, P. K., & Sarvey, J. M. (1985). Depletion of norepinephrine, but not serotonin, reduces long-term potentiation in the dentate gyrus of rat hippocampal slices. *Journal of Neuroscience*, *5*(8), 2169–2176. https://doi.org/10.1523/JNEUROSCI.05-08-02169.1985

Stelly, C. E., Haug, G. C., Fonzi, K. M., Garcia, M. A., Tritley, S. C., Magnon, A. P., Ramos, M. A. P., & Wanat, M. J. (2019). Pattern of dopamine signaling during aversive events predicts active avoidance learning. *Proceedings of the National Academy of Sciences*, *116*(27), 13641–13650. https://doi.org/10.1073/pnas.1904249116

Sun, F., Zhou, J., Dai, B., Qian, T., Zeng, J., Li, X., Zhuo, Y., Zhang, Y., Wang, Y., Qian, C., Tan, K., Feng, J., Dong, H., Lin, D., Cui, G., & Li, Y. (2020). Next-generation GRAB sensors for monitoring dopaminergic activity in vivo. *Nature Methods*, *17*(11), 1156–1166. https://doi.org/10.1038/s41592-020-00981-9

Takeuchi, T., Duszkiewicz, A. J., Sonneborn, A., Spooner, P. A., Yamasaki, M., Watanabe, M., Smith, C. C., Fernández, G., Deisseroth, K., Greene, R. W., & Morris, R. G. M. (2016). Locus coeruleus and dopaminergic consolidation of everyday memory. *Nature*, *537*(7620), 357. https://doi.org/10.1038/nature19325

Thompson, R. F., & Spencer, W. A. (1966). Habituation: A model phenomenon for the study of neuronal substrates of behavior. *Psychological Review*, *73*(1), 16–43. https://doi.org/10.1037/h0022681

Tully, K., Li, Y., Tsvetkov, E., & Bolshakov, V. Y. (2007). Norepinephrine enables the induction of associative long-term potentiation at thalamo-amygdala synapses. *Proceedings of the National Academy of Sciences*, *104*(35), 14146–14150. https://doi.org/10.1073/pnas.0704621104

Uematsu, A., Tan, B. Z., Ycu, E. A., Cuevas, J. S., Koivumaa, J., Junyent, F., Kremer, E. J., Witten, I. B., Deisseroth, K., & Johansen, J. P. (2017). Modular organization of the brainstem noradrenaline system coordinates opposing learning states. *Nature Neuroscience*, *20*(11), 1602–1611. https://doi.org/10.1038/nn.4642

Vazey, E. M., & Aston-Jones, G. (2014). Designer receptor manipulations reveal a role of the locus coeruleus noradrenergic system in isoflurane general anesthesia. *Proceedings of the National Academy of Sciences*, *111*(10), 3859–3864. https://doi.org/10.1073/pnas.1310025111

Vazey, E. M., Moorman, D. E., & Aston-Jones, G. (2018). Phasic locus coeruleus activity regulates cortical encoding of salience information. *Proceedings of the National Academy of Sciences*, *115*(40), E9439–E9448. https://doi.org/10.1073/pnas.1803716115

Wagatsuma, A., Okuyama, T., Sun, C., Smith, L. M., Abe, K., & Tonegawa, S. (2018). Locus coeruleus input to hippocampal CA3 drives single-trial learning of a novel context. *Proceedings of the National Academy of Sciences*, *115*(2), E310–E316. https://doi.org/10.1073/pnas.1714082115

Wilmot, J. H., Puhger, K., & Wiltgen, B. J. (2019). Acute Disruption of the Dorsal Hippocampus Impairs the Encoding and Retrieval of Trace Fear Memories. *Frontiers in Behavioral Neuroscience*, *13*. https://doi.org/10.3389/fnbeh.2019.00116