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The Hippocampus Contributes to Retroactive Stimulus Associations

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Publication Date

2022

Peer reviewed|Thesis/dissertation

The Hippocampus Contributes to Retroactive Stimulus Associations

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Psychology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

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Committee in Charge

2022

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Abstract

Trace fear conditioning (TFC) is a variant of Pavlovian conditioning in which the CS and US are separated by a temporal gap (aka trace interval). The hippocampus is commonly assumed to facilitate this type of learning by maintaining a memory of the CS until the US occurs. Prior work from our lab and others has demonstrated that optogenetic inhibition of CA1 during the tone and trace interval impairs the acquisition of TFC. However, there is currently little to no evidence that individual hippocampal neurons reliably maintain a memory of the CS during the trace interval. Here, we used fiber photometry to record bulk calcium activity in CA1 as mice underwent TFC. Similar to previous work, we found that the footshock US produced a large and prolonged increase in CA1 activity. To determine if this activity was important for learning, we optogenetically silenced CA1 after footshock and found that trace fear memory was significantly impaired. In contrast, silencing CA1 for an equivalent period during the intertrial interval had no effect, indicating that immediate, but not delayed post-shock activity is essential for memory formation. However, this was only true for new learning, as post-shock silencing on the second day of training did not disrupt a previously formed trace fear memory. Similar patterns of activity in CA1 have been observed in spatial studies when a reward US is encountered on a maze. In that case, reward induces replay during sharp wave-ripples that travel backwards in time to reactivate the path leading to food. We hypothesize that something similar may happen during TFC: the aversive US activates CA1 and causes it to replay the sequence of events that lead to footshock. This allows the animal to associate the aversive outcome with predictive stimuli that occurred tens of seconds earlier. Implications for models of trace conditioning and hippocampal function are discussed.

Acknowledgements

I am deeply indebted to my graduate advisor, Brian Wiltgen, for his direction and advice throughout my graduate career. Thank you for hiring me as your lab manager and providing me with the exceptional experience that allowed me to flourish in graduate school. I have learned so much more than I could've imagined during my time here. Thank you for encouraging my research interests and giving me the opportunity to establish new techniques in the lab. Thank you also for believing in me and providing me with the opportunity to grow and mature as neuroscientist.

Moreover, I would like to give my heartfelt thanks to my dissertation committee members: Charan Ranganath and Randall O'Reilly. Our many invaluable discussions during seminars and journal clubs helped me to have a more complete understanding of memory research at multiple levels. I would also like to thank the additional faculty members whose advice and encouragement helped me throughout my PhD – Andrew Fox, Lin Tian, Erie Boorman, Andy Yonelinas, Gene Gurkoff, and Brian Trainor.

I'm extremely grateful to all the incredible members of the Wiltgen Lab that I have had the pleasure of working with over the years: Jake Wilmot, Yusuke Ota, Ana Crestani, Jamie Krueger, Jalina Graham, and Anahita Hamidi. To Jake and Yusuke, thank you for the countless conversations we've had. Whether commiserating over failed experiments, discussing papers and research ideas, or just venting about the common stressors of graduate school, I am truly indebted to you guys. It's hard to imagine finishing this degree without your motivation and support.

I am also thankful for the remarkable lab alumni whom I've had the joy of overlapping with during my time here. Jamie, Jalina, and Anahita – thank you all for teaching me how to do everything in the lab, and for being so gracious with your time as

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I developed my independence in the lab. Jamie, thank you for being a source of joy and humor in the lab. I always enjoyed our talks about the levity of "science twitter". Working with you made the day-to-day working in the lab a fun experience. Jalina, not only did I value our scientific conversations, but thank you also for making the lab a more fun and quirkier place – may your love of lab tape never die.

Thanks also to the former Wiltgen Lab managers I had the pleasure of training and getting to know: Amanda Aguilo, Maribel Anguiano, and Marrisa Lafreniere. Thank you for doing a phenomenal job running the lab and assisting with experiments. The lab wouldn't have been operational without you.

To my closest friends and fellow Psych PhD grads – Jake, Jordan, and Mackenzie – you guys were instrumental in my success. I am deeply thankful and fortunate that I got to experience this journey with you guys. As Psychology PhD students at the Center for Neuroscience together we were uniquely positioned to understand and relate to each other in ways nobody else could. You have all become my closest friends and I will cherish our relationships for the rest of my life. It has truly been an honor to study and grow alongside you guys. I am so excited to see all the fantastic things we will go on to achieve in the future.

I would also like to thank my family for their unwavering love and support. I want to thank my mom and grandmother for always believing in me and encouraging me to pursue my aspirations. To my dad, thank you for teaching me about the importance of education, and instilling in me a love of learning about the world. To my in-laws, John & Ann, thank you also for being supportive and for welcoming me into your family – I could

not have asked for better in-laws. I am truly lucky to have had all your love and support during graduate school.

Lastly, to the love of my life, Megan: words cannot express how grateful I am to have you by my side. There is no way I could have achieved this without your unwavering love and support. Thank you for sparking my love for travel; we have been fortunate to visit so many incredible places over the past six years. Thank you for being my rock – no matter how difficult my day was, I could smile and take comfort in knowing that I was coming home to you. I love you, Meg.

Part 1:

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

Preface
Part 1 of this dissertation is published:

Wilmot J*, **Puhger K*,** Wiltgen B (2019). **[*co-first author]** Acute disruption of the dorsal hippocampus impairs the encoding and retrieval of trace fear memories. *Frontiers in Behavioral Neuroscience* https://doi.org/10.3389/fnbeh.2019.00116 PMCID: 31191269

      This chapter is the result of collaboration with Jacob Wilmot. We contributed

equally to all aspects of the publication: experimental design, execution of behavioral

experiments, statistical analysis of behavioral data, immunostaining, and quantification

of c-fos, and manuscript writing. Authorship order was randomly determined by coin flip.

The manuscript has been slightly modified for readability.

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).

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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.

Materials and Methods

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).

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.

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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 x 10^{13}, Penn Vector Core) and AAV9-CaMKIIa-eGFP (250nl/hemisphere, titer:3.49 x 10^{13}, Penn Vector Core). For inhibition experiments, the constructs were AAV5-CaMKIIa-ArchT-GFP (350nl/hemisphere, titer:5.2 x 10^{12}, UNC Vector Core) and AAV5-CaMKIIa-GFP (350nl/hemisphere, titer:5.3 x 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).

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

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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.

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 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 s. Mice were removed from the chamber 120 s 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.

Experiment-Specific Methods

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

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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.

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.

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 s. 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.

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 s. 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.

<u>Immunohistochemistry</u>

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).

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.

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.

Results

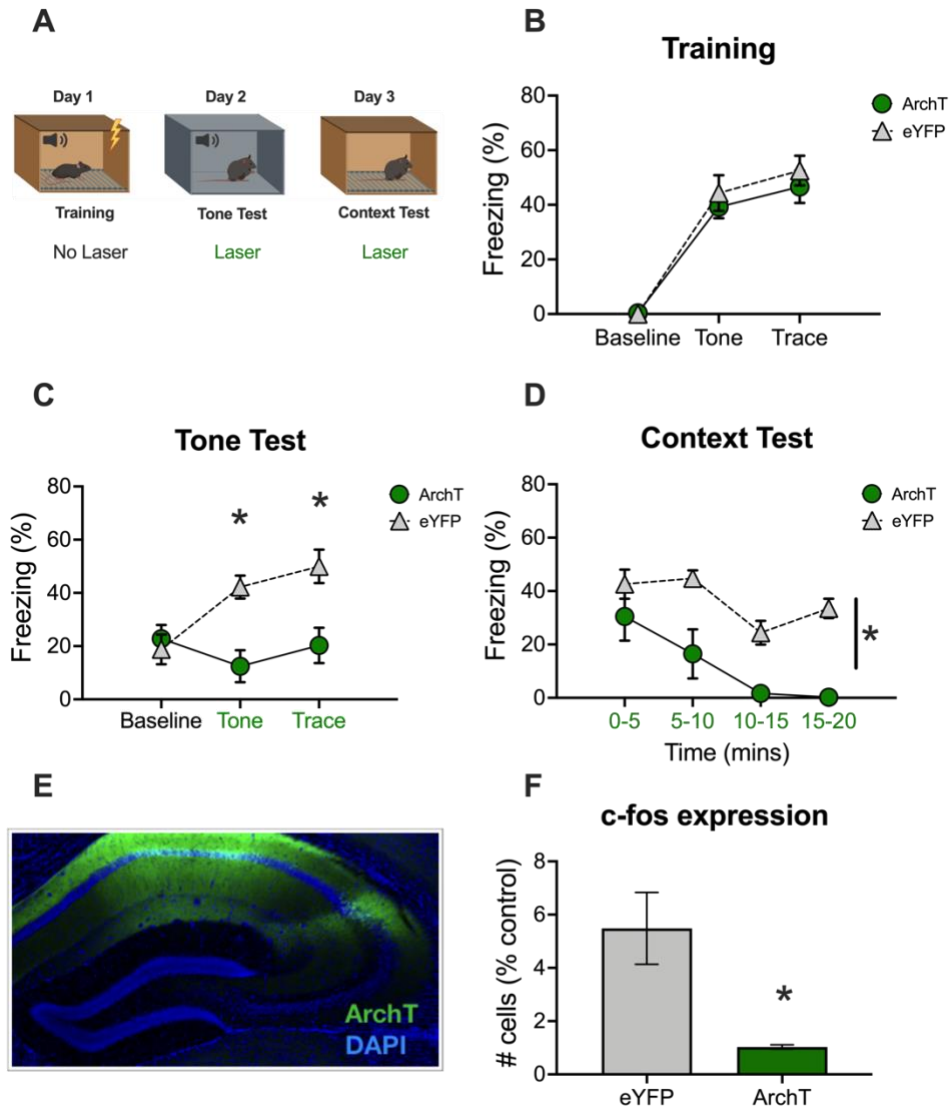### *Inhibition of dCA1 impairs trace fear memory retrieval*

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

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(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 dHPC 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
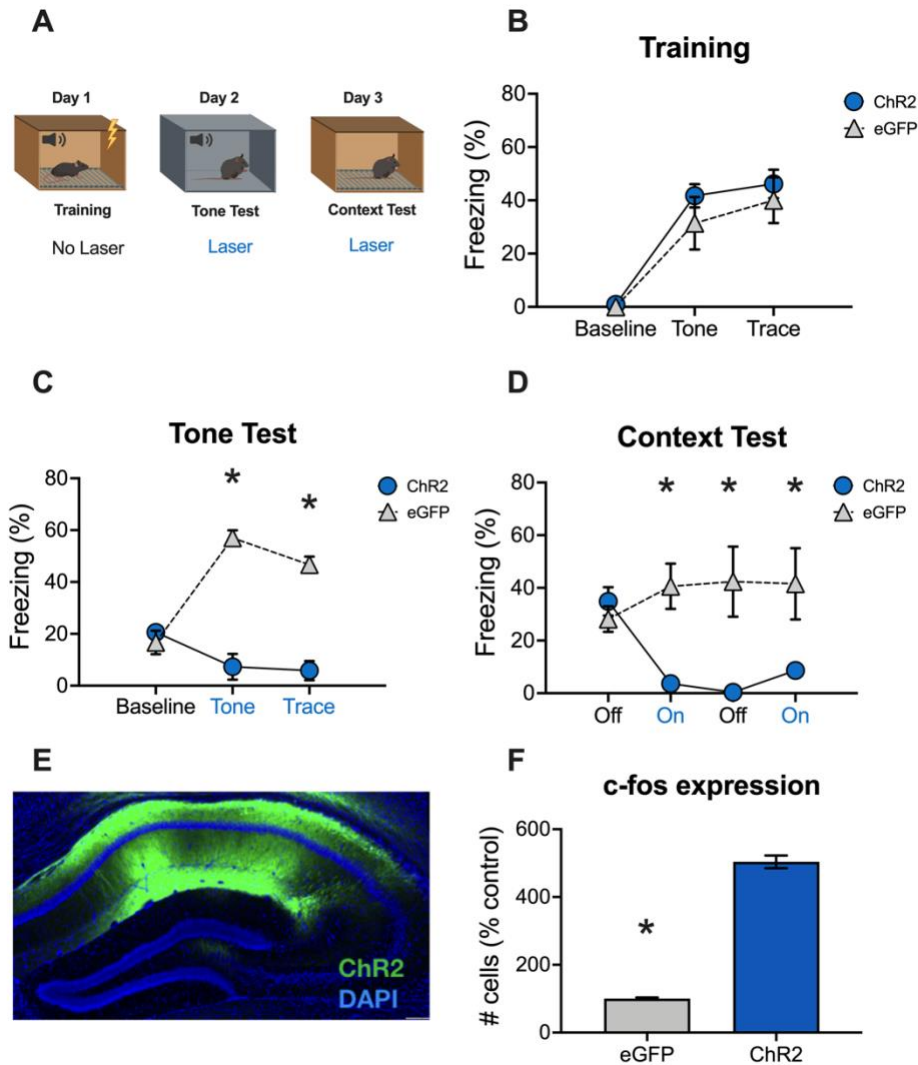
Stimulation of dCA1 impairs trace fear memory retrieval

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 dHPC 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.

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.
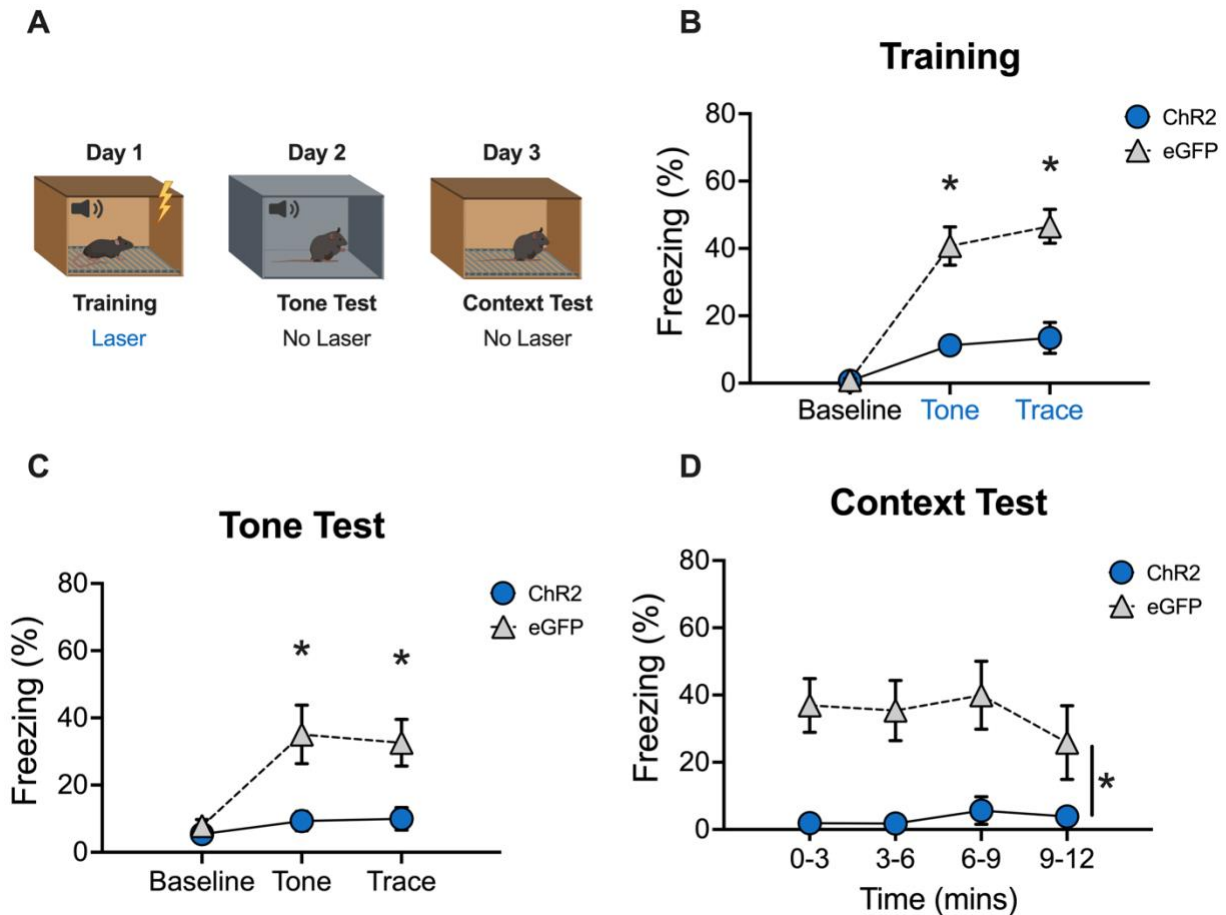
Figure 3. Stimulation of dHPC during trace fear encoding impairs memory acquisition.

(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).
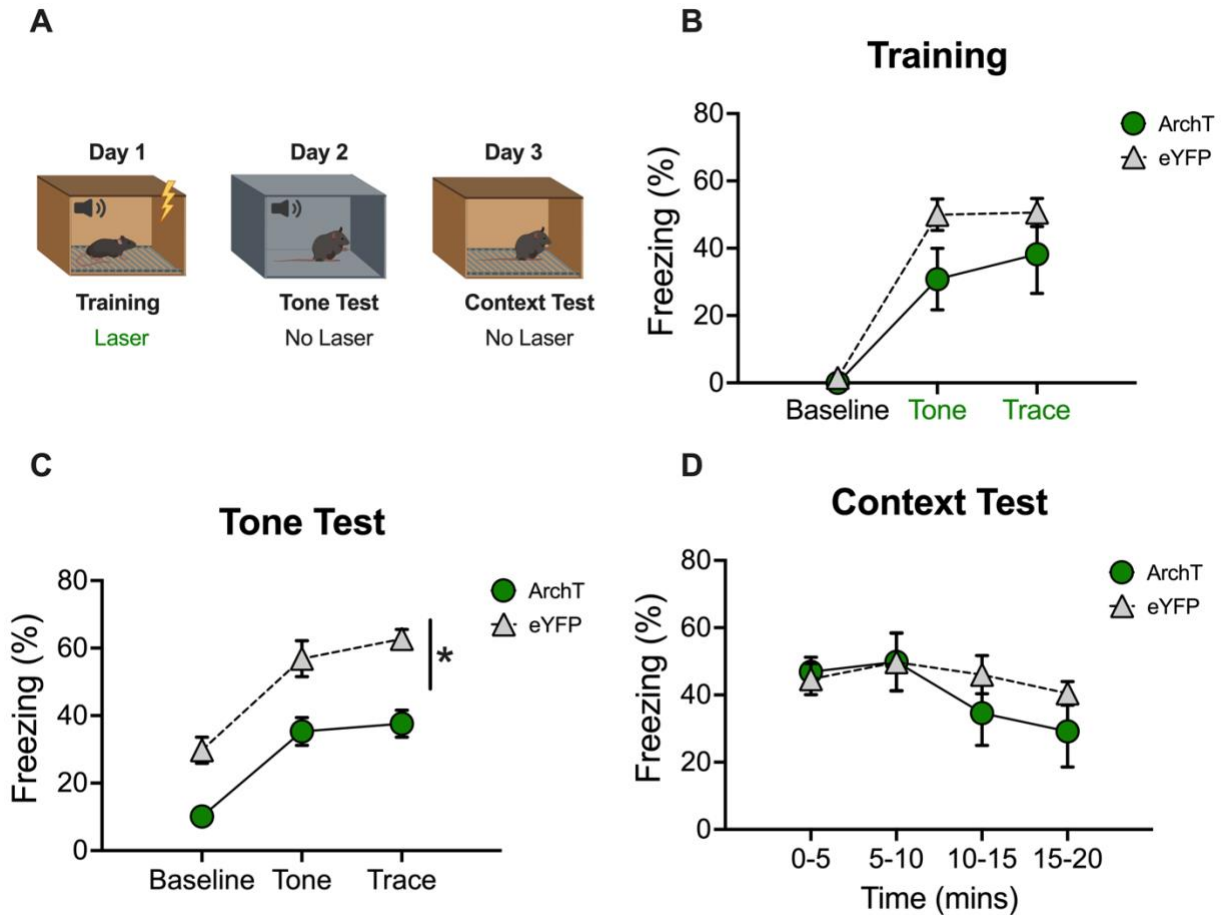
(C) Freezing during the tone test (Mean±SEM).

(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.

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).

Figure 4. Inhibition of dHPC 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.

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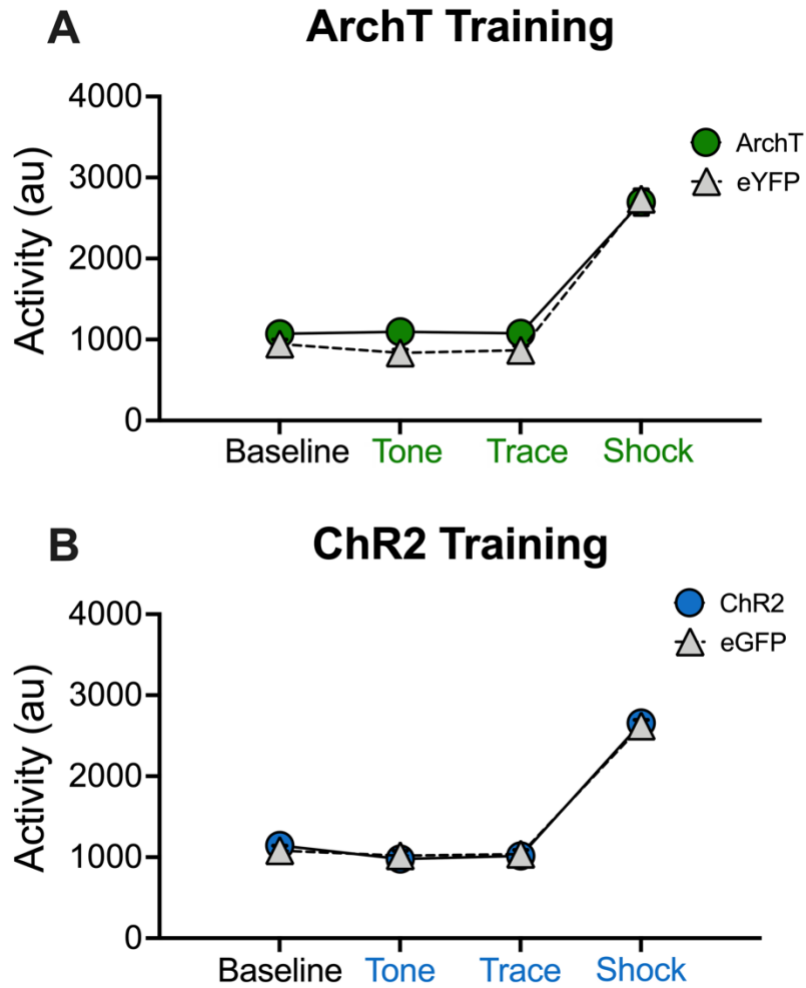
Figure 5. Stimulation and inhibition of dCA1 do not alter locomotor activity or shock responsivity. **(A)** Average motion (arbitrary units) during the last 20s of baseline and the first tone, trace, and shock periods in Experiment 3 (inhibition during trace fear encoding) (Mean±SEM). **(B)** Average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

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 unknown.

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The hippocampus contributes to retroactive stimulus association in trace fear conditioning

Introduction

A fundamental goal of memory research is to discern the mechanisms by which the brain stores and retrieves information. The amygdala is believed to process the emotional valence of memory (Cahill et al., 1995; Bechara et al., 1995; McGaugh, 2004; Kensinger & Corkin, 2004) while the hippocampus is thought to encode episodic memory by integrating sequences of events that occur within a particular spatial and temporal context (Wallenstein et al., 1998; Eichenbaum, 2017; Yonelinas et al., 2019). Consistent with this idea, animal studies have shown that the hippocampus represents spatiotemporal information (Allen et al., 2016; Brun et al., 2002; Eichenbaum, 2014), and is important for spatial and temporal learning (Anagnostaras et al., 1999; Steele & Morris, 1999; Bangasser et al., 2006; Dupret et al., 2010; Jacobs et al., 2013; Kitamura et al., 2014; Sellami et al., 2017). In contrast, the hippocampus is not required for learning simple cue relationships such as associating a conditional stimulus (CS) with an unconditional stimulus (US), which is mediated by other neural circuits (e.g., the amygdala) (J. J. Kim & Fanselow, 1992).

One example of this is Pavlovian delay conditioning: Animals with dorsal hippocampal damage can learn to associate an auditory stimulus that co-terminates with a footshock but those with amygdala damage cannot (J. J. Kim & Fanselow, 1992; Kochli et al., 2015). However, if the US occurs after a temporal gap (trace conditioning) the hippocampus is required to associate them (Chowdhury et al., 2005; Raybuck & Lattal, 2014). In trace conditioning, it is often assumed that the HPC is needed to bridge the temporal gap and maintain a memory of the CS until the US is presented. However, neurophysiological recordings in the hippocampus have not observed persistent activity

after the tone CS similar to what is observed in the prefrontal cortex during working memory tasks (Fuster, 1973; Jung et al., 1998; McEchron & Disterhoft, 1999).

Alternatively, the hippocampus might not persistently represent the CS throughout the trace interval but instead provide a sequential temporal code that permits the association between the CS and US (Kitamura et al., 2015; Sellami et al., 2017). This idea is supported by the observations that neural ensembles in CA1 fire sequentially during temporal delays in both spatial and non-spatial tasks to support memory (Pastalkova et al., 2008; MacDonald et al., 2011, 2013; Robinson et al., 2017; but see Sabariego et al., 2019). Computational models of trace eyeblink conditioning suggest that activity in the hippocampus generates a temporal code that spans the trace interval to associate the CS and US (Rodriguez & Levy, 2001; Yamazaki & Tanaka, 2005; Kryukov, 2012). While there is some evidence for this idea in trace *eyeblink* conditioning – where the trace interval is relatively short (300-700 ms) (McEchron & Disterhoft, 1997; Modi et al., 2014) – imaging and recording studies of the hippocampus during trace *fear* conditioning (TFC), where the interval is 10-30 s, do not find any evidence for a temporal code that bridges the CS and US across the trace interval (Gilmartin & McEchron, 2005; Ahmed et al., 2020). However, these studies also report a large increase in US-evoked CA1 activity which is consistent with model predictions that US-related firing important for learning (Rodriguez & Levy, 2001). This raises the possibility that hippocampal activity after the footshock might also contribute to TFC learning.

Initial theories of classical conditioning argue that CS-US associations are formed in large part due to their temporal proximity (Pavlov, 1927). However, this view was

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challenged by results from behavioral experiments which led to the idea that the US drives learning to the extent that the US is surprising or unexpected. According to this view learning is driven by prediction errors about the US (Rescorla & Wagner, 1972). For example, the phenomenon of blocking demonstrates that if a US is fully predicted by a CS (e.g., a light), then additional training with a compound stimulus (e.g., tone + light) will not support learning the new CS-US association (Kamin, 1969; Mackintosh & Turner, 1971). According to these later theories, the US initiates post-trial retroactive processing of recent stimuli to support learning. Consistent with this idea, when animals experience a surprising post-trial event after the US (e.g., presentation of a non-reinforced CS+) they do not learn the CS-US association because the surprising post-trial event induces competing retroactive processing that interferes with CS-US learning (Wagner et al., 1973).

In the current study, we use fiber photometry and optogenetics to elucidate a novel role of dorsal CA1 in retroactive processing during TFC to facilitate memory formation. First, we demonstrate that the footshock US induces a large increase in CA1 population activity. Next, using optogenetic inhibition of CA1, we show that this US-induced activity is necessary for TFC learning.

Materials and Methods

Subjects

      Subjects in this study were 8–16-week-old male and female mice (C57BL/6J, Jackson Labs; B6129F1, 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 of the light/dark cycle (0700-1900). Mice were group housed throughout the duration of the experiment. All experiments were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC).

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 - 2.0 mm and ML ± 1.5 mm from bregma; DV -1.25 mm from dura) through a glass pipette using a microsyringe pump (UMP3, World Precision Instruments). For the optogenetic inhibition experiments, the constructs were AAV5-CaMKIIa-eArchT3.0-EYFP (250 nl/hemisphere, titer: 4 x 10^{12}, diluted 1:10, UNC Vector Core) and AAV5-CaMKIIa-GFP (250 nl/hemisphere, titer: 5.3 x 10^{12}, diluted 1:10, UNC Vector Core). After AAV infusions, an optical fiber (optogenetics: 200 μm diameter, RWD Life Science, fiber photometry: 400 μm diameter, Thorlabs) was implanted above dorsal CA1 (AP -2.0 mm and ML ± 1.5 mm from bregma; DV -1.0 mm from dura). The fiber implants were secured to the skull using dental adhesive (C&B Metabond, Parkell) and dental acrylic (Bosworth Company).

Optogenetic inhibition and fiber photometry recordings took place ~2-3 weeks after surgery.

<u>*Behavioral apparatus*</u>

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 consists 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 70% 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.

<u>*Trace fear conditioning*</u>

All behavioral experiments took place during the light phase of the light-dark cycle. Prior to the start of each experiment, mice were habituated to handling and tethering to the optical fiber patch cable for 5 mins/day for 5 days. Next, mice underwent trace fear conditioning (TFC) in context A. For optogenetic inhibition experiments, mice were allowed to explore the conditioning chamber during training for 240 s before receiving three conditioning trials. Each trial consisted of a 20-second pure tone (85 dB, 3 kHz), a 20 s stimulus-free trace interval, and a 2 s footshock (0.4 mA) followed by an

intertrial interval (ITI) of 240 s. The following day, mice were placed in a novel context

(context B) for a tone memory test consisting of a 240 s baseline period followed by six

20 s CS presentations separated by a 260 s ITI. Twenty-four hours later mice were

returned to the training context A for 600 s to test their context memory. For fiber

photometry experiments, mice were allowed to explore the conditioning chamber during

training for 120 s before receiving ten conditioning trials. Each trial consisted of a 20-

second pure tone (85 dB, 3 kHz), a 20-second stimulus-free trace interval, and a 2-

second footshock (0.3 mA) followed by an intertrial interval (ITI) of 120 s. Freezing

behavior was measured using VideoFreeze software (Med Associates) and processed

using custom python scripts.

Optogenetic inhibition

For optogenetic inhibition experiments green light (561 nm, ~10 mW) was

delivered continuously for 40 s during each training trial. No light was delivered during

the tone or context memory tests. For both the post-shock silencing experiments light

was delivered immediately after termination of the footshock. For the ITI silencing

experiment light was delivered 140 s after termination of the footshock.

Fiber photometry

Fiber photometry enables the measurement of bulk fluorescence signal from a

genetically defined population of cells in freely-moving, behaving mice. To characterize

bulk CA1 pyramidal cell bulk calcium activity, we expressed GCaMP6f under the

CaMKII promoter and a 400 μm 0.37 NA low autofluorescence optical fiber was

implanted above the injection site. The fiber photometry system (Doric) consisted of an

FPGA based data acquisition system (Fiber Photometry Console, Doric) and a

programmable 2-channel LED Driver (Doric) to control two connectorized light-emitting

diodes (LED): a 465 nm LED (to measure calcium-dependent changes in GCaMP

fluorescence) and a 405 nm LED (an isosbestic control channel that measures calcium-

independent changes in fluorescence). LED power was set to ~40 µW, and the LEDs

were modulated sinusoidally (465 nm at 209 Hz, 405 nm at 311 Hz) to allow for lock-in

demodulation of the source signals. Light was passed through a sequence of dichroic

filters (Fluorescent Mini Cube, Doric) and transmitted into the brain via the implanted

optical fiber. Bulk GCaMP fluorescence from pyramidal cells beneath the optical fiber

was collected and passed through a GFP emission filter (500-540 nm) and collected on

a femtowatt photoreceiver (Newport 2151). Doric Neuroscience Studio software was

used to modulate the LEDs and sample signals from the photoreceiver at 12 kHz, apply

a 12 Hz low-pass filter, and decimate the signal to 120 Hz before writing the data to the

hard drive. The start and end of every behavioral session were timestamped with TTL

pulses from the VideoFreeze software and were recorded by photometry acquisition

system to sync the photometry and behavioral data.

Fiber photometry analysis

Fiber photometry data were analyzed using a custom python analysis pipeline.

The fluorescence signals from 405-nm excitation and 465-nm excitation were

downsampled to 10 Hz before calculating $\Delta F/F$. Briefly, a linear regression model was

fit to the 405 nm signal to predict the 465 nm signal. The predicted 465 nm signal was

then used to normalize the actual 465 nm signal:

$$\Delta\text{F/F} = \frac{465\text{nm}_{\text{actual}} - 465\text{nm}_{\text{predicted}}}{465\text{nm}_{\text{predicted}}} \times 100$$

For analysis, individual TFC trials were extracted from the whole-session recording data, where each trial begins 20 s prior to CS onset and ends 100 s after the footshock. For each trial, $\Delta F/F$ values were z-scored using the 20 s baseline period prior to CS onset $((\Delta F/F - \mu_{baseline})/\sigma_{baseline})$.

Trial-averaged GCaMP responses were smoothed with loess regression for visualization purposes only; all statistical analyses were performed on the non-smoothed data. For statistical analysis, mean fluorescence values were calculated during the trace interval ("pre-shock", 20-40 s from CS onset) and after the footshock ("post-shock", 42-62 s from CS onset).

Statistical analysis

For analysis of the training and tone test behavioral data, freezing was measured during each trial epoch (session baseline, tone, trace, ITI) and averaged across trials for each animal. All behavioral data were analyzed using Two-Way Repeated Measures ANOVA followed by *post hoc* comparisons adjusted with the Bonferroni-Sidak method when appropriate. For the context test session, freezing was computed across the entire session and analyzed using Welch's unpaired t-test. For the fiber photometry a paired t-test was used to compare pre-shock and post-shock fluorescence within subjects. A threshold of $p < 0.05$ was used to determine statistical significance. All data are shown as mean ± SEM. All statistical analyses were performed in python, and all figures were generated in python and BioRender.

Histology

To verify viral expression and optical fiber location, mice were deeply anesthetized with isoflurane and transcardially perfused with cold phosphate buffered

saline (1X PBS) followed by 4% paraformaldehyde (PFA) in 1X PBS. Brains were

extracted and post-fixed with PFA overnight at room temperature. The following day 40

μm coronal sections were taken on a vibratome (Leica Biosystems) and stored in a

cryoprotectant solution. Finally, slices containing the dorsal hippocampus were washed

for 5 mins with 1X PBS three times before staining the slices for 10 minutes with DAPI

(1:1,000, Life Technologies) and mounted on slides with Vectashield (Vector Labs).

Images were acquired at 10x magnification on a fluorescence virtual slide microscope

system (Olympus).

Results

Footshock elicits a large increase in CA1 calcium activity

In order to examine neural activity in CA1 during trace fear conditioning we used fiber photometry to measure bulk calcium fluorescence, an indirect readout of population activity. Mice were injected with CaMII-GCaMP6f (n = 11) which expresses the calcium indicator GCaMP6f in CA1 pyramidal cells (Fig. 1A). Mice underwent a single session of TFC training consisting of 10 training trials. Consistent with previous studies we did not find any significant GCaMP response to the CS onset, CS offset, or during the trace interval (Fig. 1B) (Ahmed et al., 2020; Gilmartin & McEchron, 2005). However, we find a large, sustained increase in GCaMP fluorescence elicited by the US (Fig. 1B). The trial-averaged mean fluorescence was significantly greater during the 20 s after the footshock (post-shock) than during the 20 s prior to the shock (pre-shock) (Fig 1C; $t_{(10)}$ = -6.256, $p < 0.05$). These data demonstrate a large US-elicited increase in CA1 activity, raising the possibility that US-induced activity also contributes to TFC learning. Next, we will test this hypothesis by using optogenetic inhibition to selectively silence CA1 after the footshock during TFC. We predict that silencing CA1 immediately after the footshock, but not later during the ITI, will impair memory.
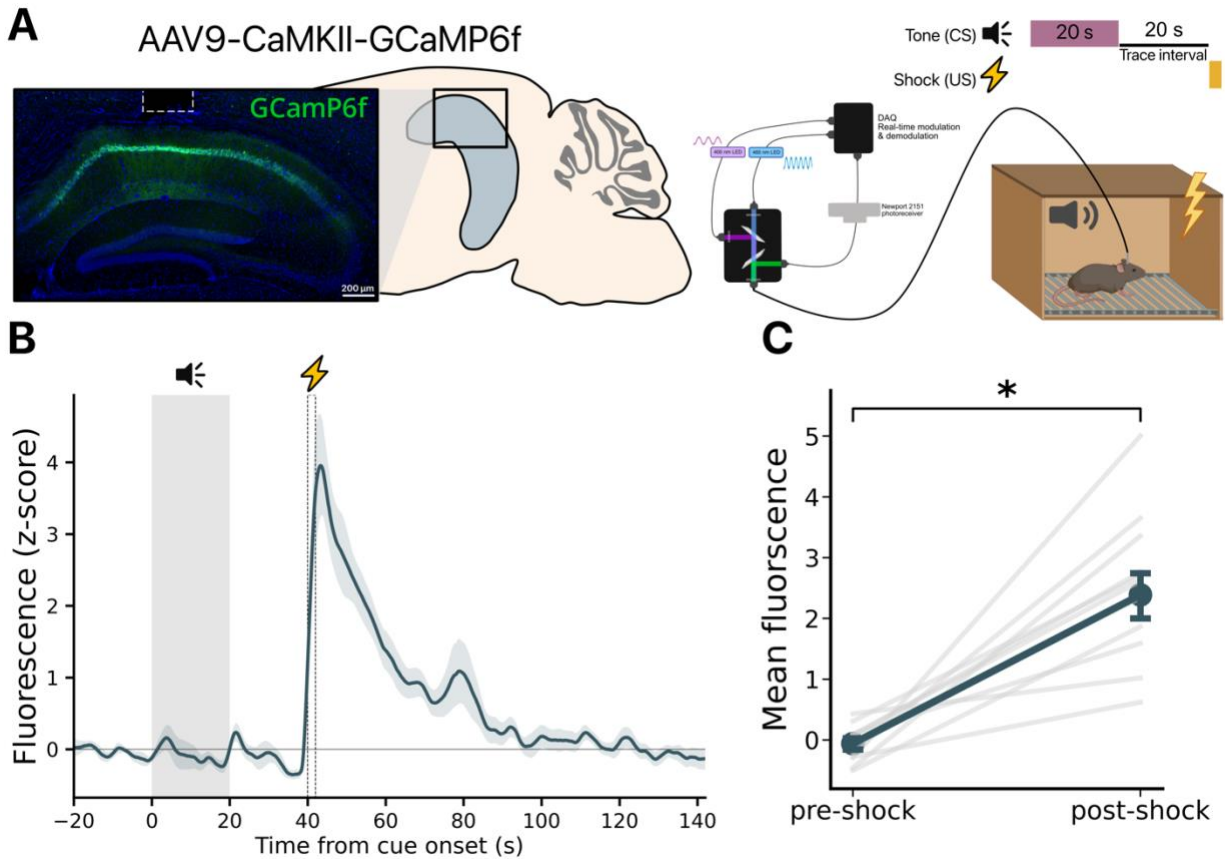
Figure 1. US-elicited increase in population-level GCaMP activity in CA1 during TFC.
(A) *Left*: Representative image of *post hoc* validation of GCaMP6s expression and optical fiber placement (white dotted lines) targeting CA1. *Right:* Schematic of the fiber photometry system used to measure bulk fluorescence during TFC training.
(B) Bulk calcium response during TFC training trials show a large increase in activity elicited by the footshock. Gray rectangle indicates when tone is presented. Dotted rectangle indicates footshock presentation.
(C) GCaMP fluorescence is significantly increased after the shock. Light gray lines represent each animal's mean fluorescence for the 20 seconds before the shock (pre-shock) and the 20 seconds after the shock (post-shock). Dark line represents the mean pre-shock and post-shock fluorescence averaged over all subjects.
All data are expressed as mean ± SEM. *$p < 0.05$.

CA1 inhibition after the footshock impairs TFC memory

After observing a large increase in US-elicited CA1 pyramidal cell activity, we next sought to determine whether CA1 activity during the post-shock period was necessary for TFC learning. To silence CA1 we infused AAV-CaMKII-eArchT3.0-eYFP (ArchT) into dorsal CA1 (n = 12 mice). Control mice (n = 12) received an infusion of AAV-CaMKII-eGFP (eGFP). During training, 561 nm light was delivered continuously for 40 s immediately after the footshock for all three CS-US pairings (Figure 2A). During training, there were no group differences in freezing during the baseline period prior to conditioning, but ArchT mice froze significantly less than eGFP mice during the trace interval and ITI (Figure 2B; Group x Phase interaction, $F_{(3, 66)}$ = 4.842, $p < 0.05$; *post hoc* Group comparisons: baseline and tone, $p > 0.05$; trace and ITI, $p < .05$). When tone memory was tested the next day in a novel context in the absence of laser stimulation, ArchT mice froze significantly less than eGFP controls (Figure 2C; Main effect of Group $F_{(1, 22)}$ = 11.32, $p < 0.05$). Twenty-four hours later we assessed context memory by returning the mice to the training context for 600 s. Surprisingly, contrary to previous reports (Sellami et al., 2017; Wilmot et al., 2019), ArchT mice froze significantly less than eGFP controls (Figure 2D; $t_{(22)}$ = -7.17, $p < 0.05$). These data indicate that CA1 activity immediately after the footshock supports tone memory and context memory in TFC.
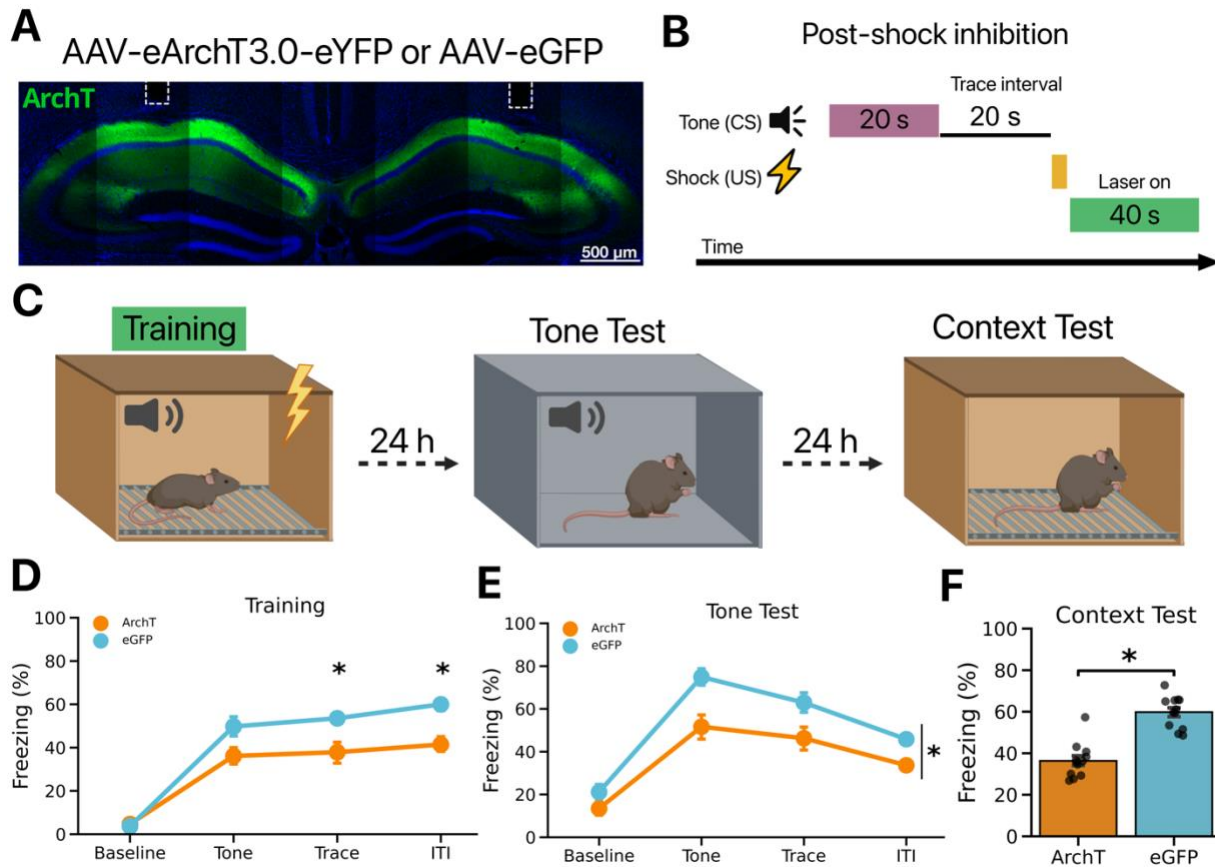
Figure 2. Effects of CA1 inhibition after the footshock on memory.
(A) Representative image of *post hoc* validation of AAV expression and optical fiber placement (white dotted lines) targeting CA1.
(B) Experimental design to silence CA1 after the footshock during learning.
(C) On the first day mice underwent trace fear conditioning while laser stimulation (561 nm) was delivered to CA1 continuously for 40 s immediately after the footshock on each training trial. The next day mice received a tone test in a novel context B. The following day contextual fear memory was tested in the original training context.
(D) During the training session, ArchT mice (n = 12) froze significantly less during the trace interval and ITI than the eGFP control group (n = 12). Data represent average freezing over 3 training trials.
**(E)** During the tone test, ArchT mice froze significantly less than eGFP controls. Data represent average freezing over 6 CS presentation trials.
(F) During the context test, ArchT mice froze significantly less than eGFP controls.
All data are expressed as mean ± SEM. *$p < 0.05$ relative to control.

Delayed CA1 inhibition during the ITI does not impair TFC memory

Prior work has demonstrated that CA1 activity during the trace interval is critical

for TFC memory (Kitamura et al., 2014; Sellami et al., 2017; Wilmot et al., 2019). Our

current results indicate that CA1 activity after the footshock is also necessary for TFC

memory formation (Figure 2B–C). In order to rule out any potential nonspecific effects of

CA1 inhibition during training, we repeated the previous optogenetic inhibition

experiment but delayed inhibition until later in the ITI. Mice received injections of ArchT

(n = 12) or eGFP (n = 12) into dorsal CA1. Three weeks later mice were trained as

described in the previous experiment, but laser stimulation was presented 140 s after

each footshock (Figure 3A). Delaying CA1 inhibition until later in the ITI did not affect

learning in either group (Figure 3B; Main effect of Phase $F_{(3, 66)}$ = 148.44, $p < 0.05$).

Unlike the results from the immediate ITI inhibition, there were no differences in freezing

between ArchT and eGFP mice during training (Figure 3B; No Main effect Group $F_{(1, 22)}$

= 0.446, $p > 0.05$). Tone memory was tested the following day in a novel context both

groups of mice displayed similar levels of freezing to the tone (Figure 3C; Main effect of

Phase $F_{(3, 66)}$ = 125.41, $p < 0.05$, No main effect of Group $F_{(1, 22)}$ = 0.022, $p > 0.05$). This

is consistent with previous findings that CA1 disruption during the ITI does not affect

tone memory in TFC (Kitamura et al., 2014; Sellami et al., 2017). Similarly, context

memory was also unaffected when CA1 inhibition after the footshock was delayed

(Figure 3D; $t_{(22)}$ = -0.694, $p > 0.05$). These results provide evidence that CA1 is

selectively required immediately after the footshock but not later in the ITI.
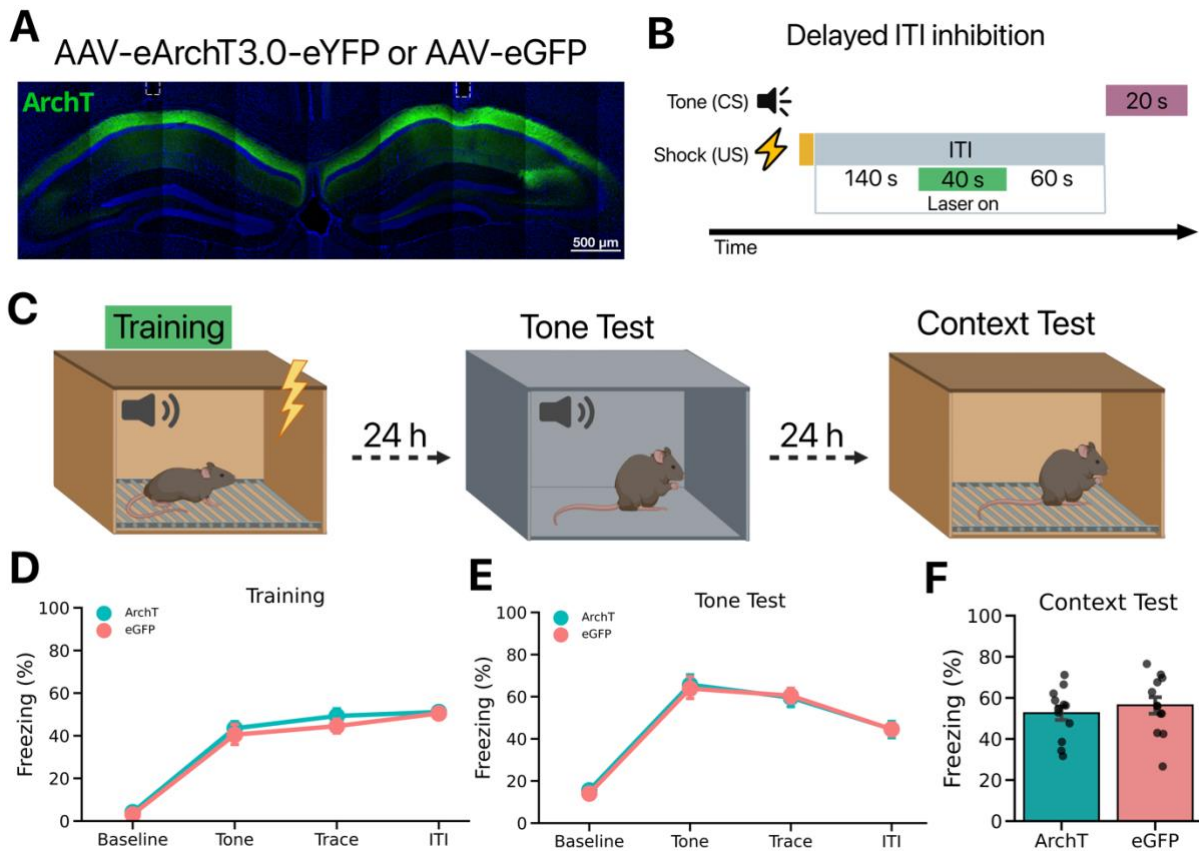
Figure 3. Effects of delayed ITI inhibition of CA1 on TFC memory.

(A) Representative image of *post hoc* validation of AAV expression and optical fiber placement (white dotted lines) targeting CA1.

(B) Experimental design to silence CA1 during the ITI. On the first day mice underwent TFC while laser stimulation (561 nm) was delivered to CA1 continuously for 40 s starting 140 s after the footshock on each training trial.

(C) On the first day mice underwent trace fear conditioning while laser stimulation (561 nm) was delivered to CA1 continuously for 40 s after a 140 s delay following termination of the footshock. The next day mice received a tone test in a novel context B. The following day contextual fear memory was tested in the original training context.

(D) ArchT mice and eGFP performed similarly during training.

(E) ArchT mice and eGFP did not differ in their freezing to the tone CS during the tone test.

(F) During the context test, both groups showed similar freezing responses to the training context.

All data are expressed as mean ± SEM. *$p < 0.05$ relative to control.

CA1 inhibition late in learning does not impair TFC memory

Our results thus far demonstrate that CA1 contributes to TFC learning by retroactively associating the US and CS. Next, we asked whether CA1 activity after the footshock was involved in the maintenance of previously consolidated memories. To test this idea, we injected mice with ArchT (n = 12) or eGFP (n = 12) as described in the previous experiments. On the first day, mice were given 3 TFC trials in the without laser stimulation. No group differences were observed during this session (Figure 4B; main effect of Phase $F_{(3, 66)}$ = 136.19, $p < 0.05$; no main effect of Group $F_{(1, 22)}$ = 0.147, $p > 0.05$). On training day 2 mice were given another 3 TFC trials, and laser stimulation was delivered 40 s immediately after the footshock. Contrary to post-shock CA1 inactivation during initial learning, silencing CA1 on the second day of training did not impair learning (Figure 4C; no main effect of Phase $F_{(3, 66)}$ = 0.690, $p > 0.05$, no main effect of Group $F_{(1, 22)}$ = 0.690, $p > 0.05$). During the tone test both groups of mice froze similarly in response to the tone (Fig. 4D; main effect of Phase $F_{(3, 66)}$ = 186.31 $p < 0.05$; no main effect of group $F_{(1, 22)}$ = 1.88, $p > 0.05$). Contextual fear memory was also similar between groups (Fig. 4E, $t_{(22)}$ = 0.944, $p > 0.05$). These results indicate that silencing CA1 immediately after the footshock do not impair previously a formed TFC memory. This is consistent with the view that CA1 activity after the footshock is required to initially learn the CS-US relationship but is not required when animals have already learned the CS-US association.
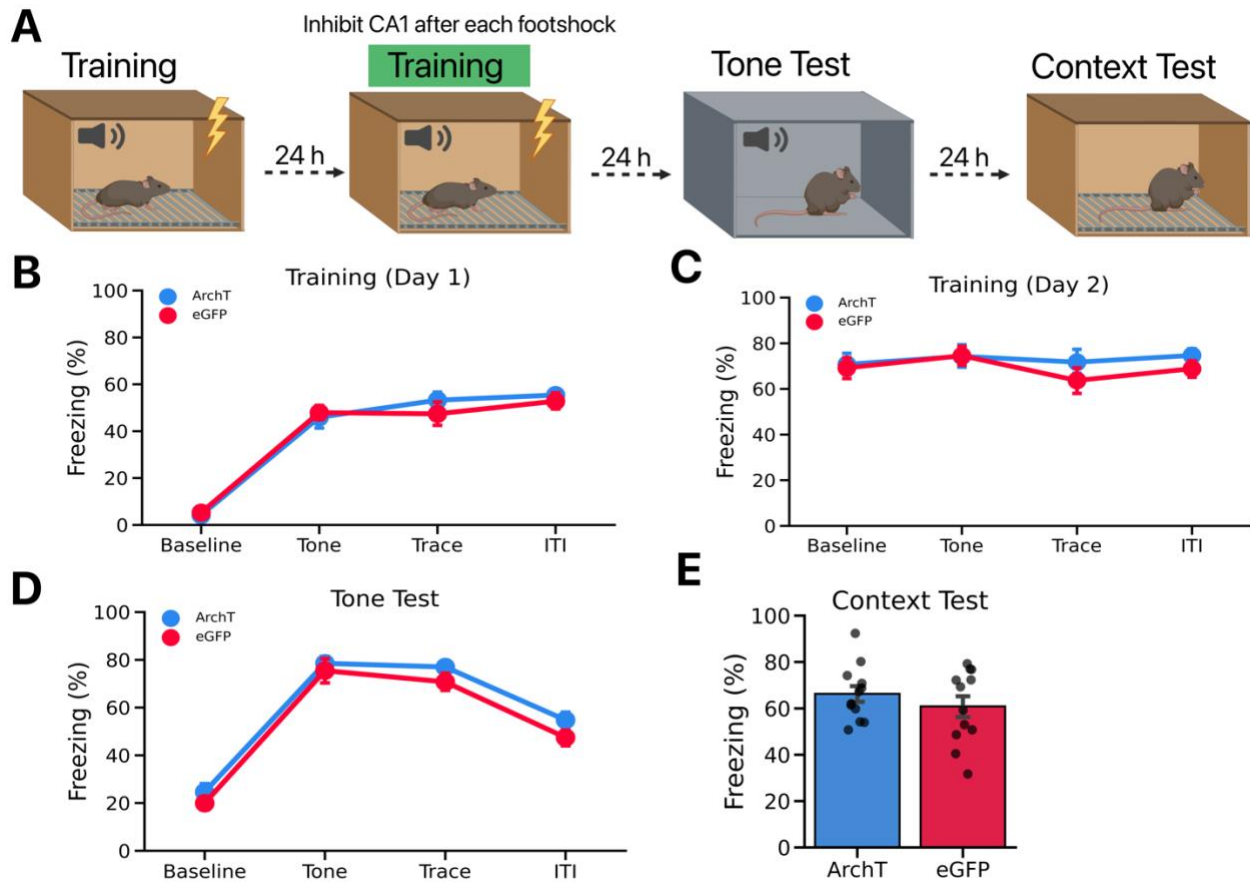
Figure 4. Effects of delayed ITI CA1 inhibition on TFC memory.
(A) Experimental design to silence CA1 after the footshock on the second training day. Both groups received TFC training on the first day without any laser stimulation. On the second training day, mice underwent trace fear conditioning while laser stimulation (561 nm) was delivered to CA1 continuously for 40 s immediately after the footshock on each training trial. The next day mice received a tone test in a novel context B. The following day contextual fear memory was tested in the original training context.
(B) ArchT mice and eGFP performed similarly during the first day of training.
(C) ArchT mice and eGFP performed similarly during the second training day.
**(D)** During the tone test, ArchT mice and eGFP did not differ in their freezing to the tone.
(E) During the context test, both groups showed similar freezing responses to the training context.
All data are expressed as mean ± SEM. *$p < 0.05$ relative to control.

Discussion

 The dorsal hippocampus has a long-established role in trace fear conditioning (Bangasser et al., 2006; McEchron et al., 1998; Raybuck & Lattal, 2014). However, the specific role of CA1 in TFC acquisition is not fully understood. Recent optogenetic studies have found that CA1 activity during the trace interval is necessary for learning (Kitamura et al., 2014; Sellami et al., 2017). In this study, we used fiber photometry and optogenetics to measure and manipulate activity in the dorsal hippocampus during trace fear conditioning. Our results expand our understanding of the role of CA1 in TFC by demonstrating that US-evoked activity in CA1 is also required for TFC acquisition.

 In the first experiment, we used fiber photometry to record bulk calcium fluorescence from CA1 during TFC. We found no change in bulk calcium fluorescence in response to the CS or during the trace interval. This lack of CS-evoked or trace interval population-level activity measured via fiber photometry is consistent with previous studies of hippocampal activity during TFC. Single-unit recording of CA1 during TFC found little change in firing in response to the CS or during the trace interval (Gilmartin & McEchron, 2005). A recent study utilizing head-fixed two-photon imaging of CA1 during TFC also found a negligible change in activity, both at the single-cell and population level, elicited by the CS or the trace interval (Ahmed et al., 2020). However, they found that the CS was reliably encoded by CA1 when activity rates were assessed on a longer timescale. In contrast, our fiber photometry recordings showed that footshock elicits a large increase in CA1 activity. These data are consistent with prior work showing that pyramidal cells in CA1 are excited by aversive unconditional stimuli (Ahmed et al., 2020; Gilmartin & McEchron, 2005).

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We next sought to determine whether the increased post-shock activity in CA1 was causally involved in the acquisition of trace fear conditioning. First, we found that optogenetic inhibition of CA1 during the period when activity is elevated by footshock led to a marked memory impairment for both the tone and training context. This is consistent with behavioral studies of eyeblink conditioning where surprising or unexpected post-trial events are thought interfere with learning by disrupting a post-trial "rehearsal" process. (Wagner et al., 1973). These data suggest CA1 plays a role in retroactive processing of the CS-US relationship.

Next, we found that delaying inactivation until 140 s after the footshock did not impair TFC memory. This is consistent with previous findings showing CA1 inactivation during the ITI does not affect trace fear acquisition (Kitamura et al., 2014; Sellami et al., 2017). We also found that this post shock activity is most important early in learning while US prediction errors are largest. When mice were given a training session 24 hours prior to a second training session with post-shock inactivation, there was no effect on memory. These data raise the interesting possibility that dorsal CA1 might play an active role in memory encoding early in learning during trace fear conditioning rather than merely associating sequences of stimuli that are temporally adjacent but discontiguous.

The discovery of place cells in the hippocampus led to the idea that the main function of the hippocampus was to generate a "cognitive map" of the environment the animal could use to guide subsequent behavior (O'Keefe & Dostrovsky, 1971). Decades of subsequent recording studies support the view that the hippocampus binds spatial and non-spatial information together to generate an internal model of the world (i.e. a

"cognitive map") wherein space is only one of several relevant dimensions (Eichenbaum et al., 1999; O'Keefe & Nadel, 1978; O'Reilly & Rudy, 2001; Schiller et al., 2015; Tolman, 1948; Yonelinas et al., 2019). Several studies have found that the hippocampus encodes non-spatial information such as temporal information (Allen et al., 2016; Eichenbaum, 2014; MacDonald et al., 2011; Pastalkova et al., 2008) odors, sound frequencies, and abstract variables such as evidence accumulation when relevant to an animal's behavior (Aronov et al., 2017; Nieh et al., 2021; Terada et al., 2017; Wood et al., 1999). These data are consistent with the idea that the hippocampus supports learning by actively selecting the most important internal and external stimuli to optimize long-term memory formation (Terada et al., 2022).

The hippocampus can also reactivate or replay behavioral sequences of activity during large bursts of population activity known as sharp wave-ripples (SWRs) (Buzsáki, 2015). Importantly, these events can be replayed in the forward and reverse order which could support prospective and retrospective temporal associations during behavior (Diba & Buzsáki, 2007; Foster & Wilson, 2006; Karlsson & Frank, 2009; Ólafsdóttir et al., 2017). Although hippocampal replay is often studied in the context of spatial behaviors, recent work has extended this to non-spatial tasks. For example, in a sensory preconditioning task it was found that neurons in CA1 representing reward outcome fired before neurons that represented the sensory cue during SWRs (Barron et al., 2020).

SWRs are thought to coordinate activity throughout the brain. We hypothesize that US-induced increases in CA1 activity are driven by SWRs, which facilitate communication between the hippocampus and other brain areas like the amygdala

(Girardeau et al., 2017). It is possible, therefore, that SWRs transmit CS information to the amygdala at the end of each trial, allowing it to become associated with the US. The precise timing of this signal may not be important, as amygdala activity remains elevated for several seconds after an aversive event occurs (Grewe et al., 2017; E. J. Kim et al., 2018; Pelletier et al., 2005; Rosen et al., 1998). Consequently, the convergence of SWRs with elevated amygdala activity could promote synaptic strengthening and allow memory representations in the HPC to drive defensive behaviors like freezing. Future work should focus on investigating single-unit activity in the hippocampus and amygdala during trace fear conditioning. These data will shed light on how interactions between these two regions during the post-shock period support retroactive learning of the CS-US relationship.

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