The hippocampus contributes to retroactive stimulus associations in trace fear conditioning

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Abstract

Animals with damage to the dorsal hippocampus can acquire fear to an auditory stimulus (CS) that co-terminates with a footshock (US) but those with amygdala damage cannot. However, if the US occurs after a temporal gap (trace conditioning) a functioning hippocampus is required to associate them. It is often assumed this is the case because the dorsal hippocampus maintains a memory of the CS until the US is presented. However, neurophysiological recordings have not observed persistent activity after the tone CS in the hippocampus similar to that observed in the prefrontal cortex during working memory tasks. Therefore, in the current study, we tested a novel hypothesis about the contribution of the hippocampus to trace fear conditioning (TFC). We propose that CS and US representations may become linked in the hippocampus after a learning trial ends. To test the idea, we first used fiber photometry to examine GCaMP activity in dorsal CA1 during TFC. We found a large and sustained increase in activity after the footshock that lasted for approximately 40-sec. To determine if this increase was important for TFC, we inactivated dorsal CA1 during this period using the inhibitory opsin ArchT. We found that memory was impaired when dorsal CA1 was silenced for 40-sec immediately after the footshock had terminated. The same manipulation had no effect when it occurred during the intertrial interval. Finally, we found that post-shock activity in dorsal CA1 was required early, but not late in training. These data suggest that the dorsal hippocampus may link events that are separated in time by reactivating memories of them after they occur.

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

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.

Behavioral 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 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.

Trace fear conditioning

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 CS presentations separated by a 240 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 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:

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

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 ($(\Delta \mathrm{F}/\mathrm{F} - \mu_{\mathrm{baseline}})/\sigma_{\mathrm{baseline}}$).

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.

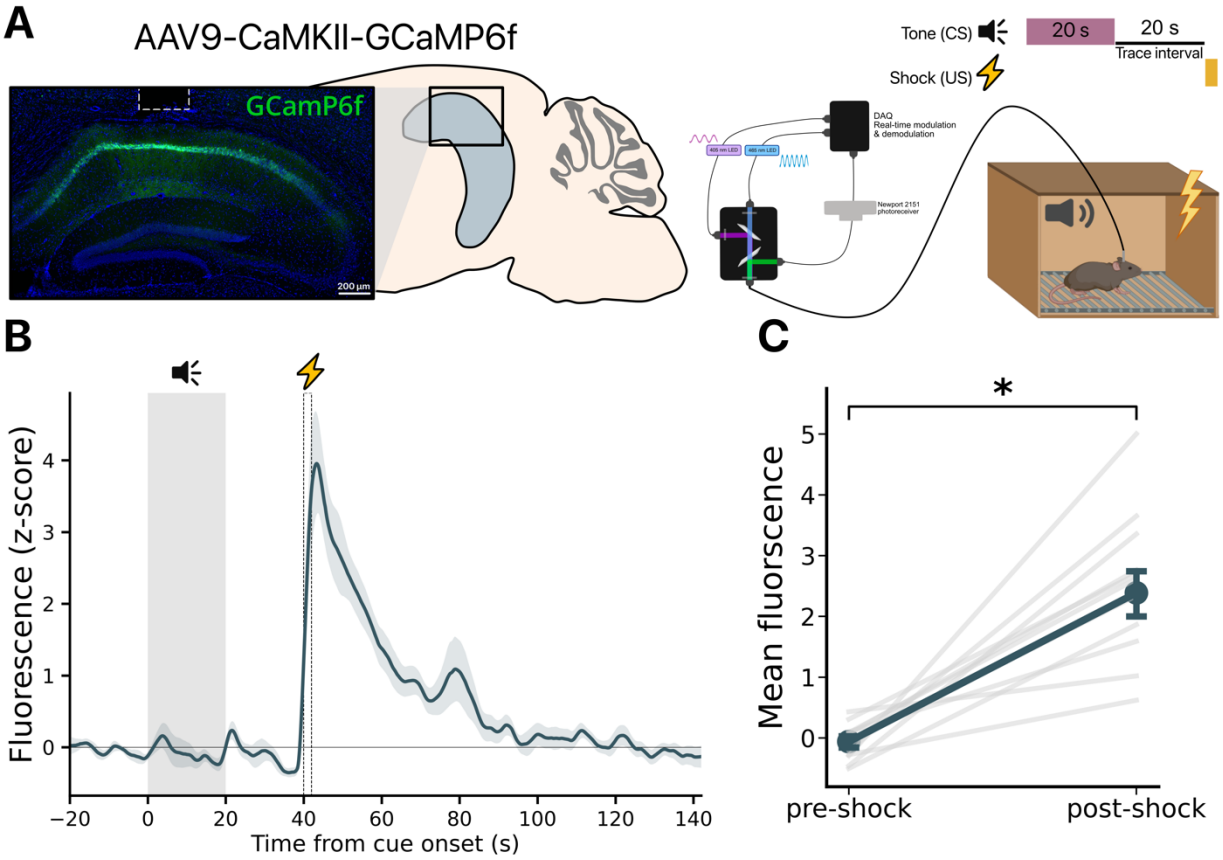

Figure 1. US-elicited increase in population-level GCaMP activity in CA1 during TFC.
(A) *Left*: Representative image of *post hoc* validation of GCaMP6s expression and optical fiber placement (white dotted lines) targeting CA1. *Right:* Schematic of the fiber photometry system used to measure bulk fluorescence 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.

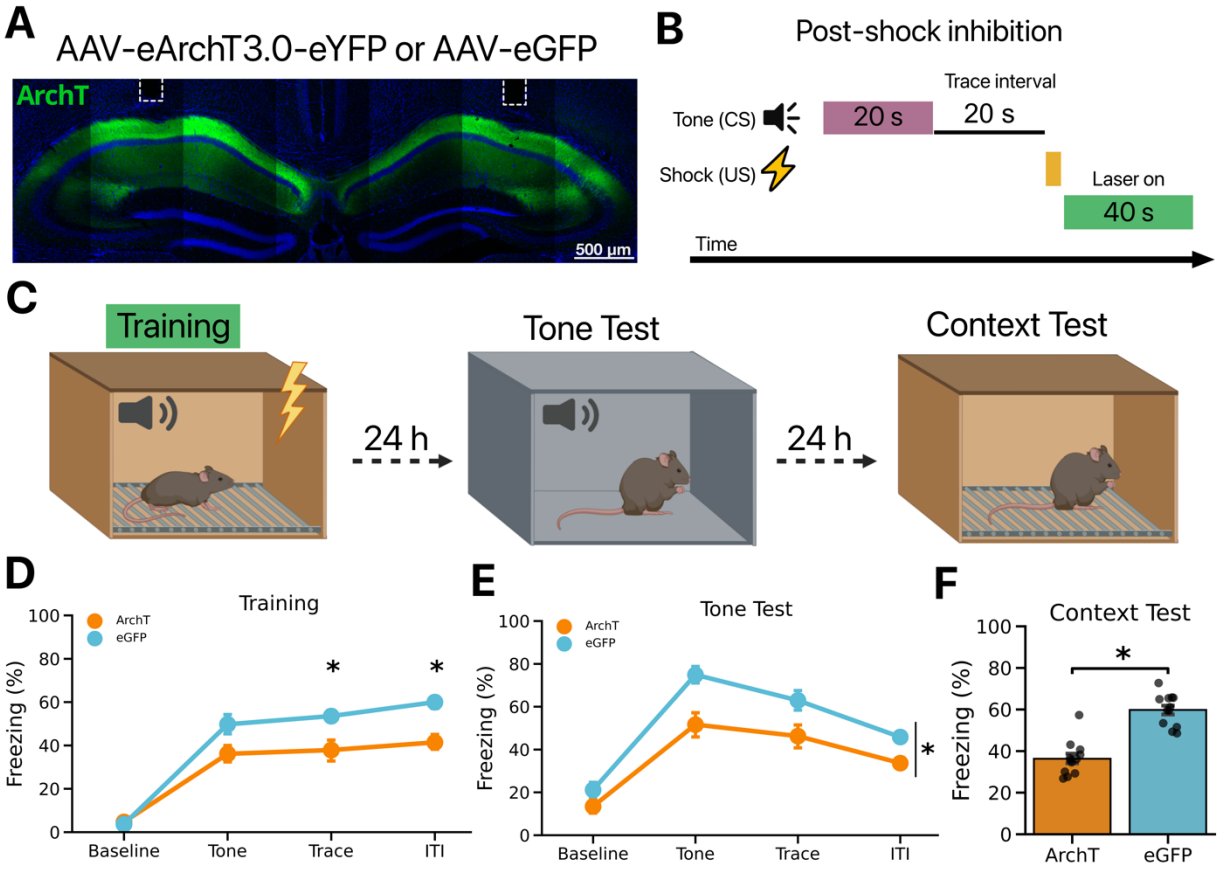

Figure 2. Effects of CA1 inhibition after the footshock on memory.
(A) Representative image of *post hoc* validation of AAV expression and optical fiber placement (white dotted lines) targeting CA1.
(B) Experimental design to silence CA1 after the footshock during learning.
(C) On the first day mice 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.

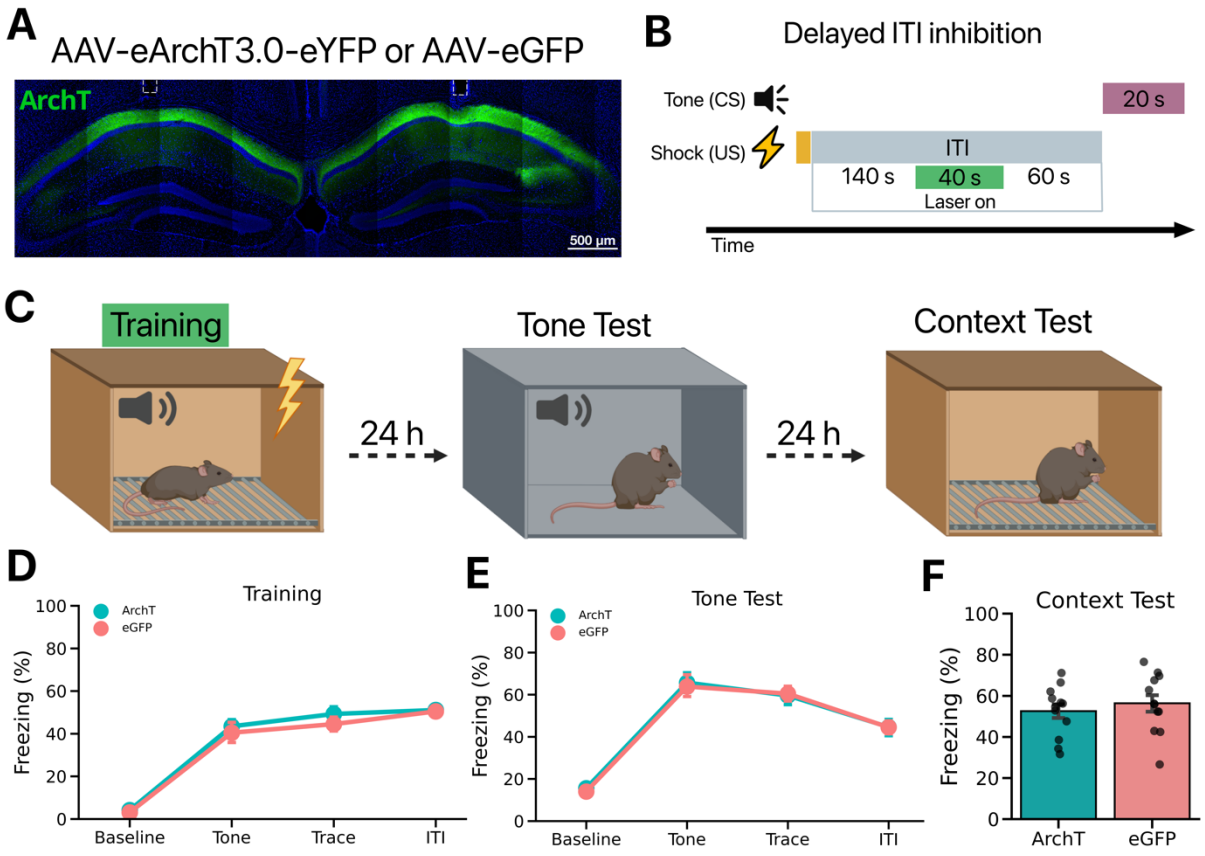

Figure 3. Effects of delayed ITI inhibition of CA1 on TFC memory.
(A) Representative image of *post hoc* validation of AAV expression and optical fiber placement (white dotted lines) targeting CA1.
(B) Experimental design to silence CA1 during the ITI. On the first day mice underwent TFC while laser 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.

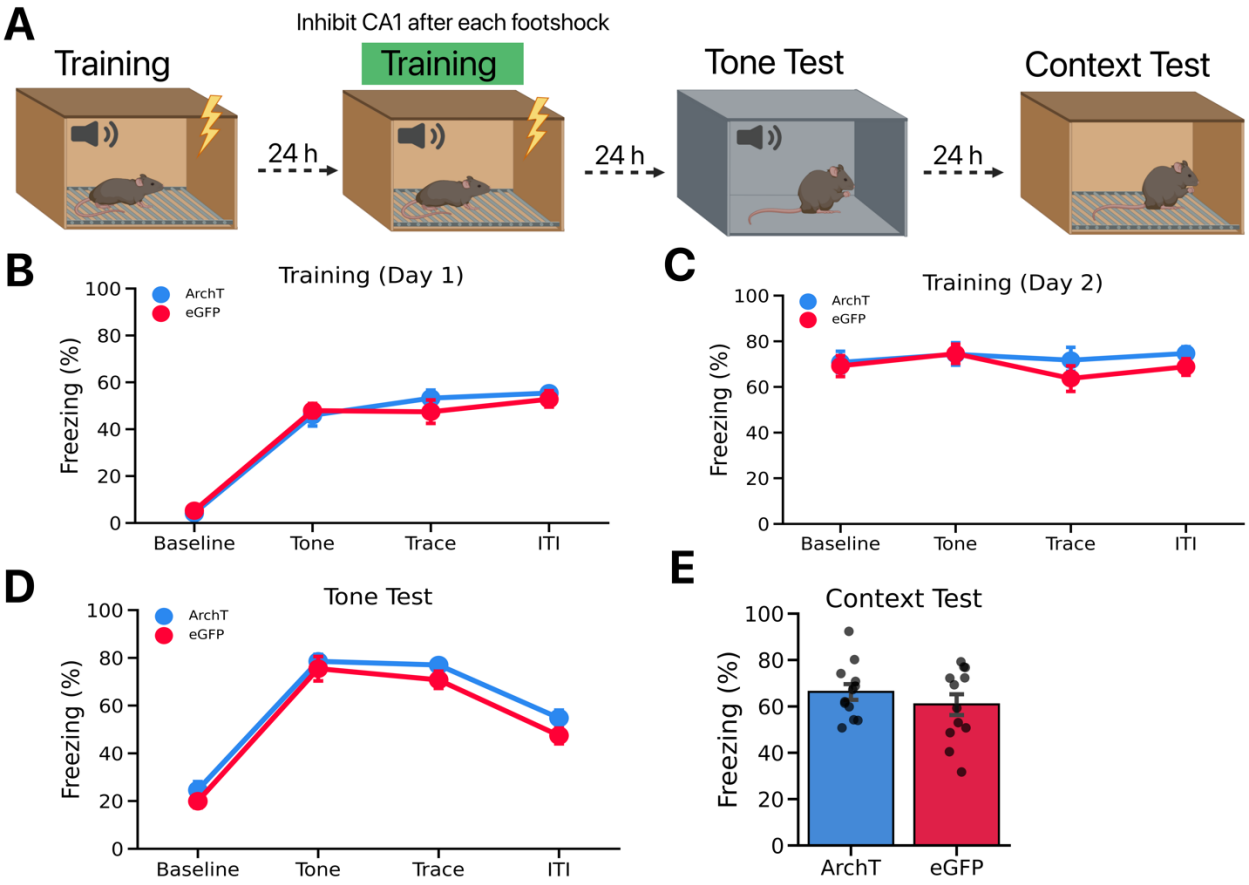

Figure 4. Effects of delayed ITI CA1 inhibition on TFC memory.
(A) Experimental design to silence CA1 after the footshock on the second training day. Both groups received TFC training on the first day without any laser stimulation. On the second training day, mice underwent trace fear conditioning while 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).

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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