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Engrams and Brain Areas Crucial for Recent and Remote Contextual Memories

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Cell, Molecular and Developmental Biology

by

Li-Han Chou

September 2024

Thesis Committee:
Dr. Jun-Hyeong Cho, Chairperson
Dr. Iryna Ethell
Dr. Margarita Curras-Collazo

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The Thesis of Li-Han Chou is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE THESIS

Engrams and Brain Areas Crucial for Recent and Remote Contextual Memories

by

Li-Han Chou

Master of Science, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, September 2024
Dr. Jun-Hyeong Cho, Chairperson

Contextual memories are initially encoded in the hippocampus and gradually undergo maturation and stabilization in the neocortex during memory consolidation. Despite extensive research, the mechanisms underlying brain reorganization between the hippocampus and neocortex remain unclear. Here, we demonstrate that the retrieval of both recent and remote contextual fear memories in mice resulted from the reactivation of engram neurons in the medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and ventral hippocampus (VH). Additionally, we examined the role of the mPFC through recent to the consolidation of remote contextual memories using a novel virtual reality-based task. This approach allowed us to investigate the necessities of the mPFC in both aversive and reward contexts. Our findings reveal the inhibition of mPFC can lead to the failure of retrieval of both recent and remote contextual memories. Overall, our results identify the engram neurons involved in recent and remote contextual memories and

highlight the specific involvement of the mPFC in contextual memories across both recent and remote time points.

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Chapter 1 Introduction

1-1 Contextual memories and engram neurons

Contextual memories refer to the aspect of memory that involves the context or environment in which information is learned or experienced. It encompasses the ability to remember not only what happened (episodic memory) but also where and when it happened (contextual details). Contextual memories allow individuals to associate specific events, facts, or experiences with the circumstances surrounding them, providing a richer and more detailed recall. Impairments in contextual memories can occur in various neurological conditions, such as Alzheimer's disease, where individuals may struggle to remember where they are or the context of recent events. In addition, contextual memory deficits can be found in Post-Traumatic Stress Disorder (PTSD) patients, who often suffer from disrupted contextual memories following traumatic events and experience recurrent fear memories even in ordinary or safe situations. Therefore, contextual memories play a crucial role in navigating experience and linking events with their surrounding circumstances.

Contextual memory development involves four distinct stages: encoding, consolidation, retrieval and extinction, where engram neurons play a pivotal role. Engram neurons can be understood as the physical alterations that transpire in the nervous system in response to a learned experience, ultimately enabling the memory formation as well as retrieval of corresponding memory through reinstatement of engram ensembles. Typically, two or more of the following properties can be seen in the cells forming engram: (a) being

activated during memory encoding (b) demonstrating persistent biological changes in response to learning (c) being reactivated during memory recall (d) being necessary or sufficient for memory retrieval (Guskjolen & Cembrowski, 2023; Josselyn & Tonegawa, 2020; Tonegawa, Liu, et al., 2015; Tonegawa et al., 2018). During memory encoding, the neurons with higher intrinsic cellular excitability are preferentially recruited and have participation in initial learning. Specifically, the transcription factor cAMP Response Element-Binding protein (CREB), a common indicator for neuronal activity during memory encoding (Benito & Barco, 2010; Josselyn & Frankland, 2018), was observed to have enhancement in neurons biased toward engrams, whereas a reduction of CREB was found in neurons that are poorly activated (Han et al., 2007, 2009; Zhou et al., 2009). Intrinsic excitability can also affect synaptic plasticity, which indicates neurons naturally with higher excitability are often primed for subsequent synaptic plasticity (Abraham, 2008; Sehgal et al., 2013; Yousuf et al., 2020). Lines of evidence suggest that the hippocampus is a crucial component in the initial stages of learning (Bernier et al., 2017; Kandel et al., 2014; Kheirbek et al., 2013; Maren et al., 2013). In addition, manipulation of the hippocampus activity can lead to disruption or amplification of learning process (Toader et al., 2023). Furthermore, previous studies have shown that hippocampal engram neurons are required for the acquisition of contextual memory. For instance, CA3-CA1 engram circuit and ventral hippocampus (VH)–amygdala pathway are involved in initial contextual fear information encoding (Choi et al., 2018; Kim & Cho, 2020).

Long-term memory storage engages a broader network in the brain and leads to a reorganization and redistribution of circuits, known as system consolidation. According to

a standard theory, initially encoded contextual memories become less dependent on the hippocampus over time, with the neocortex stabilizing memories as their transitions from recent to remote stages (Frankland & Bontempi, 2005). An alternative model suggests that contextual memories are initially encoded in both the hippocampus and neocortex, with the hippocampal contribution either diminishing or remaining stable over time alongside cortical representation (Tse et al., 2007, 2011). Despite the coexistence of hippocampal- and medial prefrontal cortex (mPFC)-dependent memories, their phenomenological qualities differ based on which neuronal ensembles (hippocampal or neocortical components) are activated. Hippocampal-dependent memories are associated with contextual and episodic details, whereas mPFC-dependent memories support the development of gist-like qualities and schematic memory (Gilboa & Moscovitch, 2021; Nadel & Moscovitch, 1997; Richards et al., 2014; Sekeres et al., 2018; Winocur et al., 2010). Recent research has shown that hippocampal-dependent memories consolidate within hours (Kitamura et al., 2017), whereas mPFC-dependent memories require weeks for the strengthening of interhemispheric and local mPFC engram circuits during system consolidation (Kitamura et al., 2017; Lee et al., 2023). Notably, the dynamic interaction between hippocampal and mPFC engram neurons is pivotal in system consolidation. The indexing theory was proposed to formalize this interplay **(Figure 1-1)**, suggesting that the hippocampus indexes neuronal activity in the neocortex, facilitating neocortical reinstatement (Guskjolen & Cembrowski, 2023; Teyler & DiScenna, 1986; Teyler & Rudy, 2007). According to the theory, during memory consolidation, activating hippocampal representations leads to the reactivation of corresponding mPFC activity, enhancing local

synaptic connections and facilitating system consolidation. For retrieval, hippocampal activation can retrieve context-specific details by reinstating neocortical activity patterns present during initial encoding. In line with this theory, inhibiting hippocampal engram neurons during system consolidation results in impaired maturation of mPFC engram neurons and an inability to retrieve memories, affecting dendritic spine density, synaptic connectivity between engrams, and reactivation patterns during recall (Kitamura et al., 2017; Lee et al., 2023; Tanaka et al., 2014). Conversely, activating hippocampal engram neurons facilitates the reactivation of cortical neurons involved in encoding and retrieving specific memories (Guskjolen et al., 2018).

During memory retrieval, the neurons recruited in memory encoding are more likely to be reactivated. Evidence through time-dependent identification of immediate early genes (i.e., Arc and c-Fos) during memory encoding and retrieval shows that hippocampal/mPFC/ basolateral amygdala (BLA) engram neurons that were present during initial encoding are preferentially reengaged in response to retrieval of a context (Guzowski et al., 1999; Lee et al., 2023; Reijmers et al., 2007). Indeed, compelling evidence has indicated selectively silencing these engram neurons is sufficient to cause disruption of memory retrieval (DeNardo et al., 2019; Han et al., 2009; Kitamura et al., 2017; Park et al., 2016; Rashid et al., 2016). On the contrary, optogenetically or chemogenetically activating engram neurons across multiple neural regions can induce memory retrieval in the absence of contextual cues (Frankland et al., 2019; Garner et al., 2012; X. Liu et al., 2012; Tonegawa, Pignatelli, et al., 2015). Interestingly, reinstatement of cortical engram neurons

can be promoted via engram neurons in the hippocampus during memory retrieval (Tanaka et al., 2014), which corresponds to the indexing theory mentioned above.

Nonetheless, memory extinction can happen naturally without further reinforcement of the corresponding contextual memories, which might stem from inaccessibility to retrieval or unavailability of the memories resulting from degradation of engram neurons (Frankland et al., 2013). Accumulation of connectivity changes over time prevents the engram ensembles from recapitulating sufficient activity for memory retrieval. The underlying principle is synaptic modeling of engram neuron ensemble, which can be mediated by neurogenesis (Epp et al., 2016; Gao et al., 2018; Ishikawa et al., 2016), intracellular signaling (Y. Liu et al., 2016, 2018), NMDA-R and AMPA-R activity (Dong et al., 2015; Migues et al., 2010; Pv et al., 2019; Villarreal et al., 2002), as well as glial cell (microglia and astrocyte) regulation (Lee et al., 2021; Nguyen et al., 2020; Wang et al., 2020). However, extinction can also be recovered from artificial stimulation of engram neurons (Perusini et al., 2017; Roy et al., 2016), suggesting extinction caused by synaptic modeling often results from failed memory retrieval rather than complete erasure.

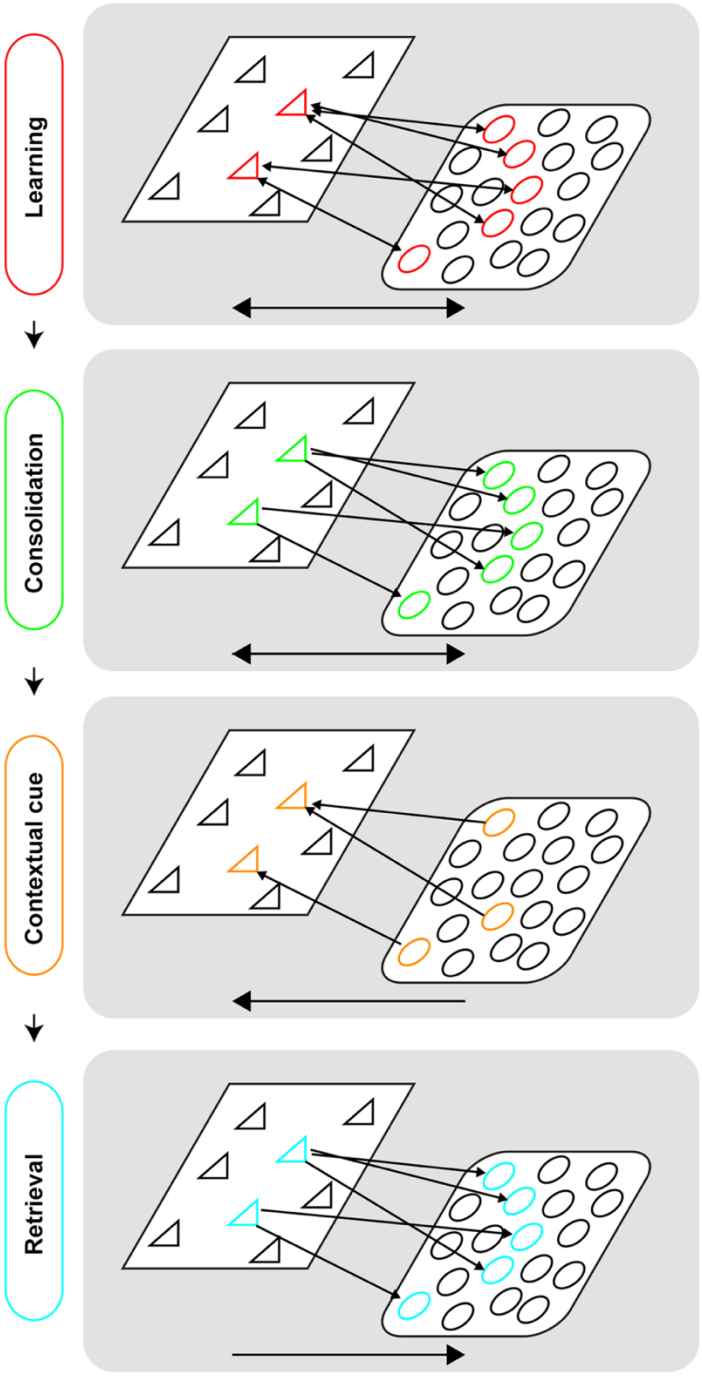


Figure 1-1. **Indexing theory for system consolidation of memory.**

(Guskjolen & Cembrowski, 2023)

1-2 Engram circuit for recent and remote contextual memory

System consolidation of memory has been considered a crucial process involved in long-term memory storage, strengthening labile memory into a more stable state. It is shown in previous studies that engram neurons are rapidly generated in the hippocampus, mPFC and BLA during contextual fear memory formation (Choi et al., 2018; Kim & Cho, 2020). Hippocampal engram neurons are important for acquisition and recent retrieval of contextual fear memories but gradually become less engaged with time. Instead, mPFC engram neurons mature over weeks with the input from hippocampal engram neurons and progressive strengthening within mPFC engram circuit, ultimately becoming a source of input to BLA engram neurons, which enables contextual fear memory retrieval at remote time points. However, BLA engram neurons remain active throughout the entire process from learning to remote recall. The engram neurons that are reactivated during retrieval are likely the same subpopulation generated in contextual fear learning due to a significant overlap of activated engram cells between memory encoding and recent/remote recall (Kitamura et al., 2017; Lee et al., 2023; Tonegawa et al., 2018). Notably, BLA engram neurons receive input from dorsal hippocampal engram through medial entorhinal cortex layer Va (MEC-Va) during recent recall and acquire direct input from mPFC engrams during retrieval at a remote time point. Additionally, mPFC-BLA pathway has been suggested as a critical role in remote but not recent contextual fear memory (Kitamura et al., 2017; *Ultrapotent Chemogenetics for Research and Potential Clinical Applications | Science*, n.d.). Indeed, the progressive strengthening of engram circuits between mPFC and

BLA, which can be directly and indirectly connected, was detected during system consolidation (Lee et al., 2023).

Despite extensive studies, the mechanism underlying the redistribution of memories remains unclear and requires further evidence to reconcile different theories and models. Recent research has highlighted brain areas that have received insufficient investigation yet play crucial roles in the interconnected circuits between the hippocampus and mPFC. For example, the retrosplenial cortex (RSC) has been identified as facilitating the transmission of signals from dorsal CA1 to mPFC engram neurons, thereby supporting the transfer of memory storage from the hippocampus to the neocortex (Lee et al., 2023). Optogenetic reactivation of RSC neurons following contextual fear conditioning has demonstrated its ability to induce system consolidation, reducing hippocampal dependence while enhancing contextual generalization (de Sousa et al., 2019). Moreover, anterior cingulate cortex (ACC) activation is associated with remote, but not recent, memory retrieval, indicating its role in maintaining long-term memory storage independent of the hippocampus (Frankland et al., 2004; Frankland & Bontempi, 2005; I et al., 2011; Kitamura et al., 2009). ACC-projecting dorsal CA1 engram neurons were also shown to increase through memory acquisition to remote recall (Refaeli et al., 2023). More recently, the anteromedial thalamus (AM) has been identified as a critical region for gating memory consolidation and promoting the stabilization of remote contextual memories. AM receives direct projections from the dorsal hippocampus (Wright et al., 2010) and sends direct outputs to ACC (Aggleton & Brown, 1999; Jankowski et al., 2013; Toader et al., 2023), making it a promising area for the transfer of contextual memories into long-term storage.

Inhibition of the AM-ACC circuit during learning can disrupt memory consolidation and remote recall, whereas its enhancement is sufficient to promote these processes (Toader et al., 2023). Despite the identification of additional brain regions involved in remote contextual memory, further research is needed to elucidate the specific contributions of engram neurons within these regions and to better understand how their coordinated activity facilitates the establishment and maintenance of long-term memory storage across distributed neural networks.

1-3 Summary and research aims

It has been shown that the hippocampal circuits are crucial for contextual memory formation, such as CA3-CA1 and ventral hippocampus-amygdala pathway (Choi et al., 2018; Kim & Cho, 2020). However, the hippocampus becomes less dependent during system consolidation of memory, in which the neocortex gradually matures and stabilizes as the permanent storage for contextual memories. In addition, BLA plays a crucial role in contextual fear memories, maintaining active through learning to remote memory retrieval (Kitamura et al., 2017; Tonegawa et al., 2018). While compelling evidence has shed light on the roles of the mPFC, BLA, and VH in recent and remote contextual memory, certain questions remain unanswered. For instance, it remains unclear whether the mPFC contributes to recent contextual memories or only long-term storage per se. Furthermore, the involvement of VH in remote contextual memories has not been thoroughly examined. Consequently, our objective is to investigate the specific brain regions involved in recent

and remote contextual memories and to elucidate their roles throughout the development of contextual memories.

Chapter 2 Methods

2-1 Animals

We obtained *Fos-iCreER*$^{T2(+/-)}$ × *ROSA-LSL-tdTomato*$^{(+/-)}$ mice through the mating of *Fos-iCreER*$^{T2(+/+)}$ (The Jackson Laboratory Stock, 030323, ME, USA) and Ai14 *ROSA-LSL-tdTomato*$^{(+/+)}$ mice (The Jackson Laboratory Stock, 007914, ME, USA). The mice were housed in home cages under a 12-hour light/dark cycle at a temperature of 23–25 °C, with food and water available ad libitum. The humidity level was maintained between 30 and 70%. The light cycle was set from 8 AM to 8 PM. Experiments were conducted using mice aged 8 to 12 weeks for both male and female individuals. All animal procedures were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Riverside.

2-2 Contextual fear conditioning and recall

Mice were individually housed in their home cages one day bfefore contextual fear conditioning (CFC). On the training day, mice were placed in Context A (dimension, 30 cm × 24 cm × 21 cm; stainless steel grid floor, white acrylic flat wall with black vertical stripes, white light illumination and benzaldehyde odor) within a fear conditioning chamber (Med Associates, Fairfax, VT, USA) between 9:30 AM and 10:30 AM. After a 3-minute habituation period, mice received the first unconditioned stimulus (electric foot shock, 0.5 mA, 2 s duration) and subsequently received four additional unconditioned

stimuli at 2-minute intervals. The temperature in the fear conditioning chamber was maintained at 23–25 °C.

Following CFC, mice were group-housed until two days prior to the recent and remote memory recall test. For the recall of contextual fear memories, mice were exposed to Context A for 5 minutes between 9:30 AM and 10:30 AM, preceded by 1 minute of acclimatization in Context A. Freezing behavior, defined as immobility lasting more than 2 seconds, was quantified as the percentage of time immobile. The movement of the mice in the fear conditioning chamber was recorded using a near-infrared camera and analyzed with EthoVision XT 11 software (Noldus, Leesburg, VA, USA).

2-3 Activity-dependent neuronal labeling

To open labeling window, mice received an intraperitoneal injection of 4-hydroxytamoxifen (4-OHT, 50–75 mg kg^{-1} of body weight; Sigma-Aldrich, H6278, St. Louis, MO, USA) 10 minutes after CFC under brief anesthesia with 5% isoflurane in the anesthesia chamber. The 4-OHT was prepared by dissolving it in DMSO (40 mg ml^{-1}), followed by further dissolution in saline containing 2% TWEEN 80 in a water bath at 40–42 °C, resulting in 1 mg ml^{-1} 4-OHT solution. To minimize background noise-induced neuronal labeling, mice were kept undisturbed in their home cages in a quiet environment for 8 hours before and after the 4-OHT injection.

The mice were divided into two groups: the home cage (HC) label group and the CFC label group. In the HC label group, neurons activated during the mice's time in their home cages were labeled with tdTomato through the injection of 4-OHT. CFC in Context A was

administered to the mice 2 days after the 4-OHT injection, and the recall of recent or remote contextual fear memories was tested in Context A 3 days or 28 days after CFC, respectively.

In the CFC label group, neurons active during CFC were labeled by 4-OHT injection immediately after the conditioning. Mice were tested for recent or remote contextual fear memories in Context A 3 days or 28 days after CFC, respectively.

2-4 c-Fos immunohistochemistry and analysis

Slices of the medial prefrontal cortex (mPFC), basolateral (BLA), and ventral hippocampus (VH), each 150 μm in thickness, were prepared using a vibratome and fixed 90 minutes after recent and remote memory recall tests. Following fixation in 4% paraformaldehyde (PFA) in PBS for an hour, the slices underwent permeabilization in PBS-T at room temperature for 2 days. Subsequently, the brain sections were blocked with PBS containing 5% goat serum at 4 °C for an hour. After a 10-minute wash with PBS-T, the slices were incubated with a polyclonal affinity-purified rabbit anti-c-Fos antibody (diluted 1:1,000 in PBS-T; 226003, Synaptic Systems, Göttingen Germany) at room temperature for 24 hours. Following three 10-minute washes with PBS-T, the slices were incubated with goat anti-rabbit IgG antibody-Alexa Fluor 647 (diluted 1:200 in PBS-T; A-21246, Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C for 24 hours. After three 10-minute washes with PBS-T, the slices were mounted on glass slides for confocal microscopic imaging.

For each mouse, z-series confocal microscopic images were captured for four representative fields (each covering 0.56 mm^2) of the mPFC, BLA, or VH. The images

were then z-stacked using ImageJ software. Manual counting of tdTomato (tdT^+) neurons and tdT^+/c-Fos^+ neurons was performed in the mPFC, BLA and VH. c-Fos^+ cells were quantified using Imaris 9 software (Bitplane). The proportion of c-Fos^+ neurons among all tdT^+ neurons in each mPFC, BLA, and VH field was calculated, and the proportions were averaged for each mouse.

2-5 Virtual reality system

The virtual reality system is customized and modified from a previously published paper (Toader et al., 2023). A 200-mm-diameter styrofoam ball was supported by a ball holder, which was connected to gas, allowing the ball to freely rotate forward and backward. Mice were head-fixed above the ball with headbars implanted on top of their heads during surgery. Virtual environments were created using the ViRMEnS1 MATLAB engine for virtual reality. These environments were displayed by back-projection onto white fabric positioned in front of the mouse's center. The screen occupied 220 degrees of the mouse's field of view, with the virtual scene projected onto it using a mini projector. Additionally, a National Instruments Data Acquisition (NIDAQ) device was employed to generate outputs, triggering various Arduinos that controlled tones, odors, air-puffs, and lick ports.

2-6 Virtual reality behavioral task

To optimize mice's behavior, mice were deprived of water for two days, followed by water restriction with approximately 1g hydrogel to maintain 85-88% of their body weight throughout the whole process of virtual reality behavioral task. After the body weight steadily stayed around 85%, mice initially received pre-training to learn licking and

traveling in the virtual environment. The pre-training context consisted of a linear track from position 20 to 270, which contained a start zone (empty, position 20-233) and an outcome zone (circles, position 233-270). ~15 μl water was delivered with a tone sound (started at position 233) while the mice entered the outcome zone. In the beginning, the distance from starting point to outcome zone was relatively short and the track moved automatically with a resting speed. Throughout the training process, the distance and speed were gradually increased and decreased respectively to improve mice's behavior.

Once the mice were able to travel the full distance and display anticipated licking right before the tone, mice were introduced to a new linear track to measure the baseline of water licking rate. At the beginning of baseline measurement, mice were exposed to one of two different contexts where Context A contained vertical white lines and Context B was composed of horizontal white lines. After completing the start zone and entering the cue zone, the mice received distinct olfactory and auditory cues in Context A (mint odor, high-frequency sound) and Context B (lemon odor, white noise). ~4 μl water was released with a tone sound (started at position 245) once the mice successfully reached the outcome zone and licked the water port in both contexts.

Subsequently, mice were introduced to the training which contained the same contexts as baseline measurement. However, the outcome zone in Context A in training was associated with ~4 ul sucrose as reward while the one in Context B was related to air-puff delivery as punishment. The mice were trained to predict reward and punishment as they ran down the linear track along with the presence of visual, olfactory and auditory cues for

3 days. Their recent and remote retrieval tests were therefore conducted on the following day and 28 days later respectively. During the retrieval sessions, only visual, olfactory and auditory cues were presented but sucrose and air-puff was absent in the contexts.

Retraining and Reversal training. Mice continued to receive retraining and reversal training after the completion of training. Mice were retrained for four days in the same setting as training, but underwent reversal training in a distinct environment, in which sucrose reward was swapped to Context B while air-puff delivery was changed to Context A. Reversal training lasted for four days, and two retrieval tests were performed on the next day after the final retraining and the last reversal training respectively.

Behavioral analysis. Mice abilities to learn and recall contextual information was assessed by the calculation of discrimination index (DI) in all behavioral tasks. DI was calculated as the following equation:

$$\text{DI} = \frac{\text{mean lick rate in reward context} - \text{mean lick rate in aversive context}}{\text{mean lick rate in reward context} + \text{ mean lick rate in aversive contex}}$$

A DI of 1 or -1 indicates perfect performance in discriminating the contexts during the behavioral tests, while a DI of 0 reflects random performance. For all sessions, we calculated the anticipatory lick rates in the position window of 135-145 (with the tone starting at 145) using the following equation:

$$\text{Lick rate} = \sum_{i=1}^{n} \frac{lick_i}{t_i}$$

2-7 Chemogenetic inhibition of mPFC

Mice were anesthetized by placing them in a transparent chamber filled with 5% isoflurane prior to surgery. Throughout the surgical procedure, anesthesia was maintained at 1.5% isoflurane administered via nostrils using a precision vaporizer. Subsequently, mice were immobilized in a stereotaxic frame equipped with nonrupture ear bars (David Kopf Instruments, Tujunga, CA, USA), and ophthalmic ointment was applied to prevent eye dryness. Hair of scalp was shaved and trimmed area was sterilized with 70% ethanol swabs. A midline scalp incision was made for the access to the skull, followed by the application of hydrogen peroxide on the skull to allow the apparent outline of bregma. Bilateral craniotomies were made using a microdrill which led to a 0.5 mm hole through the skull. Glass capillaries loaded with virus-containing solution were inserted into targeted brain region and the virus-containing solution was delivered at a rate of 0.1 µl min^{-1} using a 10 µl Hamilton microsyringe and a syringe pump.

Injection of 1 µl AAV-pSyn-hM4Di-mCherry (Addgene, #44362-AAV5, Watertown, MA, USA) was performed bilaterally into mPFC (mPFC/PL) (1.9 mm rostral to bregma, 0.4 mm lateral to the midline, and 1.2 mm ventral to the pial surface) in mice a month before pre-training for chemogenetic inhibition. Mice from the same cohorts were injected with 1 µl AAV-pSyn-mCherry (Addgene, #114472-AAV5, Watertown, MA, USA) bilaterally as control groups. For training sessions, mice were habituated to handling and injection by intraperitoneal injection of 0.3 ml saline 30-45 minutes prior to the training during training day 1-3. On the recent and remote recall sessions, both groups of mice

received an intraperitoneal injection of clozapine N-oxide hydrochloride (CNO; 3 mg kg^{-1} of body weight, dissolved in saline; Sigma-Aldrich, SML2304, St. Louis, MO, USA) and were tested 30-45 minutes after injection. Both groups of mice underwent a second remote recall one day after the initial session, receiving a 0.01 ml g^{-1} saline injection 30-45 minutes before retrieval.

2-8 Statistical Analysis

All the quantification graphs in this study were generated with GraphPad Prism 10.0 (GraphPad software) and data are presented as the means ± SEM. For statistical comparisons, we used Welch's t-test, and $p < 0.05$ was considered statistically significant.

Chapter 3 Results

3-1 Identification of engram neurons associated with recent contextual memories in mPFC, BLA and VH.

First of all, we wanted to identify the involvement of engram neurons in medial prefrontal cortex (mPFC), basolateral amygdala (BLA) and ventral hippocampus (VH) in recent contextual memories. We used *Fos-iCreER^{T2}* × *ROSA-LSL-tdTomato* knock-in mice, which were divided into contextual fear conditioning (CFC) or home cage (HC) label groups to label active neurons during either CFC or rest periods in home cages. Mice in the CFC label group were singly housed for two days before conditioning, where they learned to associate a neutral context with aversive stimuli (i.e., shock). Following conditioning, these mice were injected with 4-hydroxytamoxifen (4-OHT) to activate iCreER^{T2} driven by the Fos promoter, leading to tdTomato (tdT) expression. In contrast, mice in the HC label group received 4-OHT while in their home cages and underwent CFC two days later. Both groups underwent a contextual fear memory recall test three days after conditioning, where mice were exposed to the same context as during CFC but without shock. Subsequently, they were sacrificed for brain slice preparation, followed by fixation with paraformaldehyde (PFA) and c-Fos immunohistochemistry **(Figure 3-1. A-B)**.

Mice from both groups exhibited robust freezing behavior during recent recall, indicating successful memory retrieval without significant differences between groups

(Figure 3-1. C). Consistent with behavioral results, there were no significant differences in c-Fos cell density observed in the mPFC, BLA, and VH between the CFC and HC label groups. However, tdT expression was notably higher in the mPFC, BLA, and VH of the CFC label group, suggesting increased activation of neurons during fear conditioning compared to rest periods in home cages **(Figure 3-1. D-I)**.

Furthermore, we analyzed the proportion of Fos^+ cells among tdT^+ cells to assess the reactivation of engram neurons in both groups. Our findings reveal a greater proportion of Fos^+ cells among tdT^+ cells in the mPFC, BLA and VH within CFC label group, indicating a higher likelihood of reactivation of neurons active in conditioning at a recent time point. These results corroborate previous research identifying increased activities of BLA-projecting VH neurons in the retrieval of recent contextual memories (Kim & Cho, 2020) and suggests an additional role for the mPFC in this process **(Figure 3-1. D-I)**.

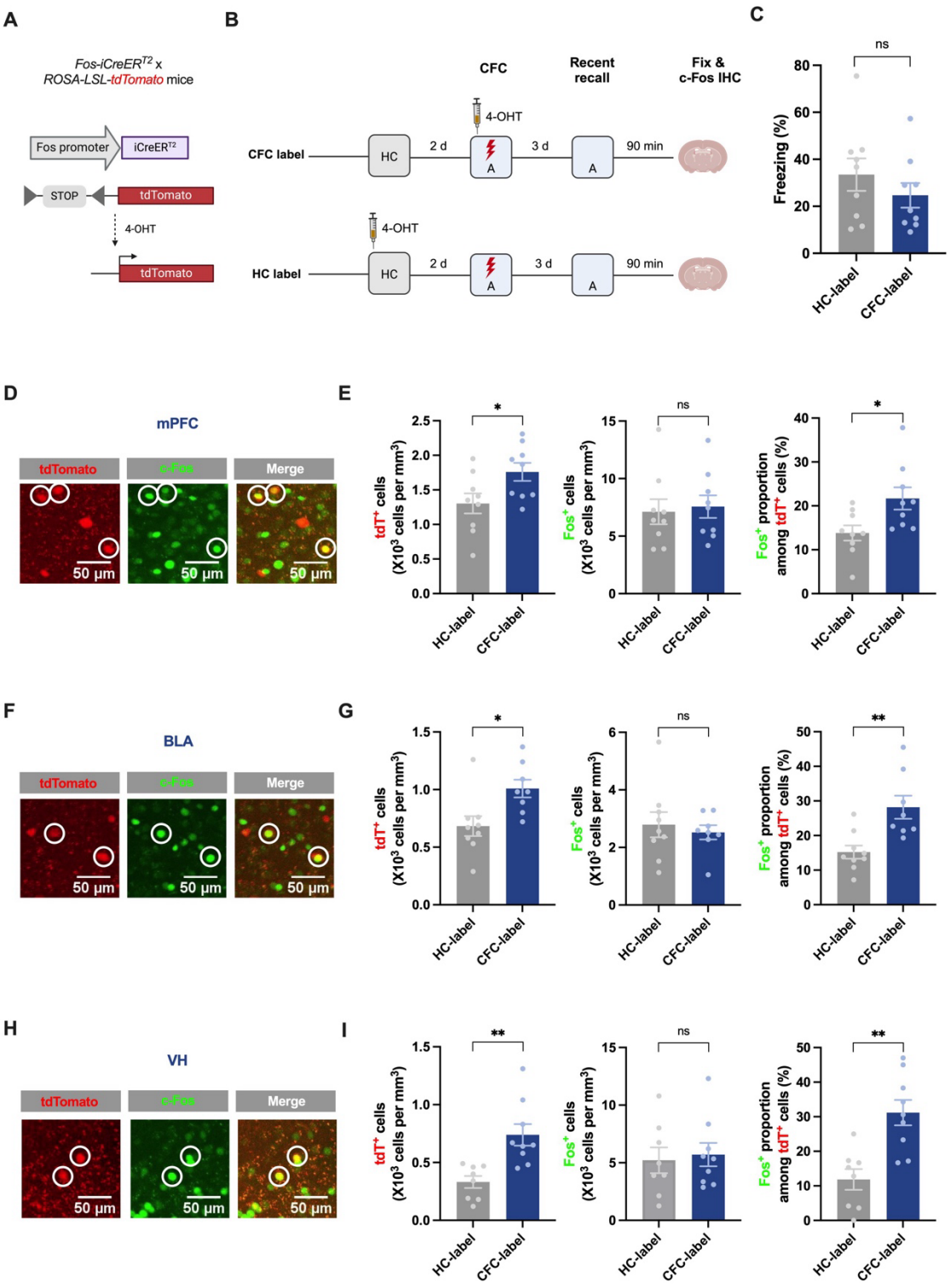

A
Fos-iCreER^T2 x
ROSA-LSL-tdTomato mice
Fos promoter
iCreER^T2
STOP
tdTomato
4-OHT
tdTomato
B
CFC
Recent recall
Fix & c-Fos IHC
4-OHT
CFC label
HC
2 d
A
3 d
A
90 min
4-OHT
HC label
HC
2 d
A
3 d
A
90 min
C
ns
Freezing (%)
HC-label
CFC-label
D
mPFC
tdTomato
c-Fos
Merge
50 μm
E
*
tdT+ cells (X10^3 cells per mm^3)
ns
Fos+ cells (X10^3 cells per mm^3)
*
Fos+ proportion among tdT+ cells (%)
HC-label
CFC-label
F
BLA
tdTomato
c-Fos
Merge
50 μm
G
*
tdT+ cells (X10^3 cells per mm^3)
ns
Fos+ cells (X10^3 cells per mm^3)
**
Fos+ proportion among tdT+ cells (%)
HC-label
CFC-label
H
VH
tdTomato
c-Fos
Merge
50 μm
I
**
tdT+ cells (X10^3 cells per mm^3)
ns
Fos+ cells (X10^3 cells per mm^3)
**
Fos+ proportion among tdT+ cells (%)
HC-label
CFC-label

Figure 3-1. **mPFC, BLA and VH engram neurons for recent contextual memories were identified with activity-dependent labeling.**

(A) Mouse line used for the experiment. **(B)** Timeline of behavioral test. **(C)** Freezing levels during recent memory retrieval. ns, not significant. **(D)** Confocal images showing neurons with expression of tdTomato (red) or c-Fos (green) in mPFC. Representing neurons are indicated by circles. **(E)** Comparison of cell density of tdT+ cells (left), Fos+ neurons (middle), and Fos+ proportion among tdT+ neurons (right) in mPFC between CFC-label and HC-label groups. Unpaired *t* test (HC-label group: 9 mice, CFC-label groups: 8 mice). ns, not significant. *$p < 0.05$, data are mean ± SEM. **(F)** Confocal images showing neurons with expression of tdTomato (red) or c-Fos (green) in BLA. Representing neurons are indicated by circles. **(G)** Comparison of cell density of tdT+ cells (left), Fos+ neurons (middle), and Fos+ proportion among tdT+ neurons (right) in BLA between CFC-label and HC-label groups. Unpaired *t* test (HC-label group: 9 mice, CFC-label groups: 9 mice). ns, not significant. *$p < 0.05$, **$p < 0.01$, data are mean ± SEM. **(H)** Confocal images showing neurons with expression of tdTomato (red) or c-Fos (green) in VH. Representing neurons are indicated by circles. **(I)** Comparison of cell density of tdT+ cells (left), Fos+ neurons (middle), and Fos+ proportion among tdT+ neurons (right) in VH between CFC-label and HC-label groups. Unpaired *t* test (HC-label group: 8 mice, CFC-label groups: 9 mice). ns, not significant. **$p < 0.01$, data are mean ± SEM. HC-label: Home Cage label group. CFC-label: Contextual Fear Conditioning label group. mPFC: medial prefrontal cortex. BLA: basolateral amygdala. VH: ventral hippocampus.

3-2 Identification of engram neurons associated with remote contextual memories in mPFC, BLA and VH.

We next investigated whether engram neurons in the mPFC, BLA, and VH are involved in remote contextual memories as well. To do this, we repeated the procedure mentioned above with a new cohort of mice but conducted the remote recall four weeks after fear conditioning **(Figure 3-2. A)**. The results show similar freezing behavior between groups, which was reflected by comparable c-Fos expression **(Figure 3-2. B-D)**. In addition, a higher density of tdT^+ cells in the CFC label group also mirrored our earlier findings, suggesting stronger activation during fear conditioning in mPFC, BLA and VH **(Figure 3-2. C-H)**. Importantly, the CFC label group exhibited a significantly higher proportion of c-Fos^+ cells among tdT^+ cells in all these areas, indicating that neurons activated during fear learning were more likely to be reactivated during remote recall **(Figure 3-2. C-H)**. In this experiment, we successfully replicated the roles of the mPFC and BLA in remote contextual memories from previous report of our lab (Lee et al., 2023). Moreover, we made a new discovery via identifying a novel involvement of the VH in remote contextual memories.

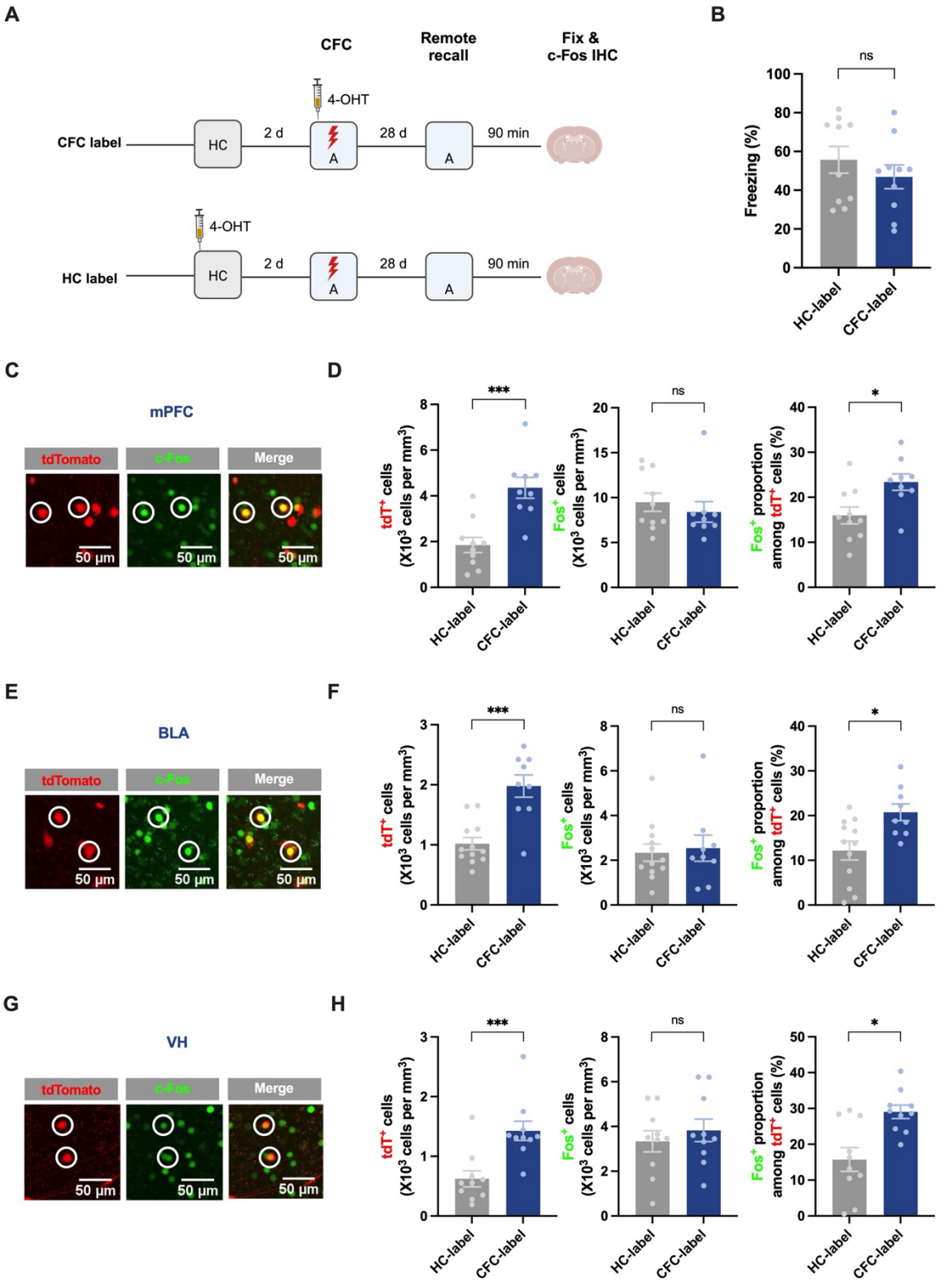

A
CFC
Remote recall
Fix & c-Fos IHC
4-OHT
CFC label
HC
2 d
A
28 d
A
90 min
4-OHT
HC label
HC
2 d
A
28 d
A
90 min
B
ns
Freezing (%)
HC-label
CFC-label
C
mPFC
tdTomato
c-Fos
Merge
50 μm
D
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tdT+ cells (X10³ cells per mm³)
Fos+ cells (X10³ cells per mm³)
Fos+ proportion among tdT+ cells (%)
HC-label
CFC-label
E
BLA
F
G
VH
H

Figure 3-2. **mPFC, BLA and VH engram neurons for remote contextual memory were identified with activity-dependent labeling.**

(A) Timeline of behavioral test. **(B)** Freezing levels during remote memory retrieval. ns, not significant. **(C)** Confocal images showing neurons with expression of tdTomato (red) or c-Fos (green) in mPFC. Representing neurons are indicated by circles. **(D)** Comparison of cell density of tdT+ cells (left), Fos+ neurons (middle), and Fos+ proportion among tdT+ neurons (right) in mPFC between CFC-label and HC-label groups. Unpaired *t* test (HC-label group: 10 mice, CFC-label groups: 9 mice). ns, not significant. *p < 0.05, ***p < 0.001, data are mean ± SEM. **(E)** Confocal images showing neurons with expression of tdTomato (red) or c-Fos (green) in BLA. Representing neurons are indicated by circles. **(F)** Comparison of cell density of tdT+ cells (left), Fos+ neurons (middle), and Fos+ proportion among tdT+ neurons (right) in BLA between CFC-label and HC-label groups. Unpaired *t* test (HC-label group: 10 mice, CFC-label groups: 9 mice). ns, not significant. *p < 0.05, ***p < 0.001, data are mean ± SEM. **(G)** Confocal images showing neurons with expression of tdTomato (red) or c-Fos (green) in VH. Representing neurons are indicated by circles. **(H)** Comparison of cell density of tdT+ cells (left), Fos+ neurons (middle), and Fos+ proportion among tdT+ neurons (right) in VH between CFC-label and HC-label groups. Unpaired *t* test (HC-label group: 8 mice, CFC-label groups: 10 mice). ns, not significant. *p < 0.05, ***p < 0.001, data are mean ± SEM. CFC-label: Contextual Fear Conditioning label group. HC-label: Home Cage label group. mPFC: medial prefrontal cortex. BLA: basolateral amygdala. VH: ventral hippocampus.

3-3 Establishment of virtual reality-based contextual memory task

To further explore recent and remote contextual memories in a more complicated setting, we introduced a virtual reality task where mice were head-fixed and navigated a styrofoam ball within virtual environments displayed on a monitor. The virtual contexts included a linear track with three zones: start, cue, and outcome. During trials, mice began in the start zone with visual cues and received olfactory and auditory cues upon entering the cue zone. These cues indicated either Context A (vertical lines, mint odor, high-frequency sound) or Context B (horizontal lines, lemon odor, white noise) **(Figure 3-3-1. A-B)**. We calculated anticipatory lick rates and normalized them as a Discrimination Index (DI) to assess learning behavior. After initial pre-training in a distinct context without cues for one to two weeks, mice were exposed to both Context A and B, where both were associated with water rewards to establish baseline lick rates. As expected, mice exhibited comparable lick rates and a near-zero DI, indicating no initial preference for either context design **(Figure 3-3-1. C-E)**.

Subsequently, mice underwent three days of training (T1-3), where Context A predicted sucrose reward and Context B predicted aversive air-puff in the outcome zone. Recent (R1) and remote memory recall (R2) occurred one day after the last training and 28 days later, respectively, with no reinforcement in either context. One day of retraining (T4) followed recent recall to mitigate memory extinction during remote recall. Our findings demonstrate that mice effectively discriminated between the two contexts throughout training, displaying higher lick rates in Context A and lower rates in Context B **(Figure 3-3-1. C-D)**. The mice showed strong significance of lick rates between

Context A and B in both recent (R1) and remote recall (R2) **(Figure 3-3-1. F-G)**. In addition, the DI remained high as well **(Figure 3-3-1. H)**, revealing that the mice retained the ability to retrieve contextual memories at both recent and remote time points.

Next, we conducted reversal training with the same group of mice to assess the flexibility of their learning. We retrained the mice for four days to prevent memory extinction following remote recall (ReT1-4), and then placed the mice under Context A and B with reversed the unconditioned stimuli (i.e., switch sucrose reward to Context B and air-puff punishment to Context A) for four days (Rev1-4). Two recall sessions were performed one day after the last retraining (R3) and the final reversal training (R4). Our results show a significant shift in the mice's preferences from retraining to reversal training **(Figure 3-3-2. A-B)**, which was also reflected in recent recall (R3 and R4) **(Figure 3-3-2. C-D)**. Overall, the findings suggest that the mice exhibited considerable adaptability in learning environments with varying paired stimuli.

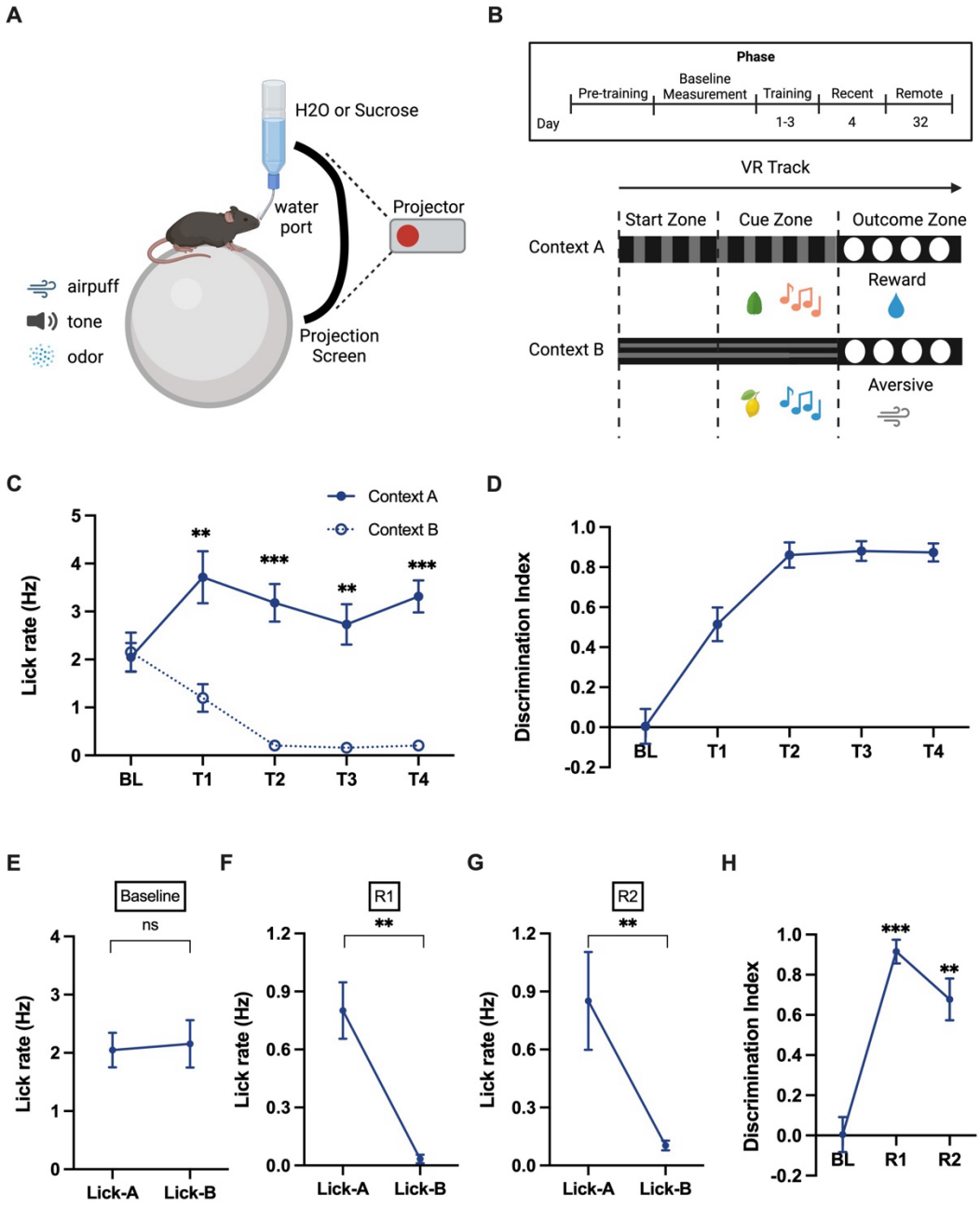


Figure 3-3-1. **Wild-type mice performance in virtual reality-based contextual memory task.**

(A-B) Schematic demonstrating experimental setup for virtual reality-based contextual memory task. **(C)** Anticipatory lick rates (Hz) in Context A (reward) and B (aversive) from baseline measurement (BL) to training (T1-3) and retraining (T4). Paired *t* test (n=6). **p < 0.01, ***p < 0.001, data are mean ± SEM. **(D)** Discrimination index quantified from (C). **(E)** Anticipatory lick rates (Hz) in Context A and B during baseline measurement. **(F)** Anticipatory lick rates (Hz) in Context A and B during recent recall. Paired *t* test (n=6). **p < 0.01, data are mean ± SEM. **(G)** Anticipatory lick rates (Hz) in Context A and B during remote recall. Paired *t* test (n=6). **p < 0.01, data are mean ± SEM. **(H)** Discrimination index quantified from (E-G). Paired *t* test (n=6). **p < 0.01, ***p < 0.001, data are mean ± SEM.

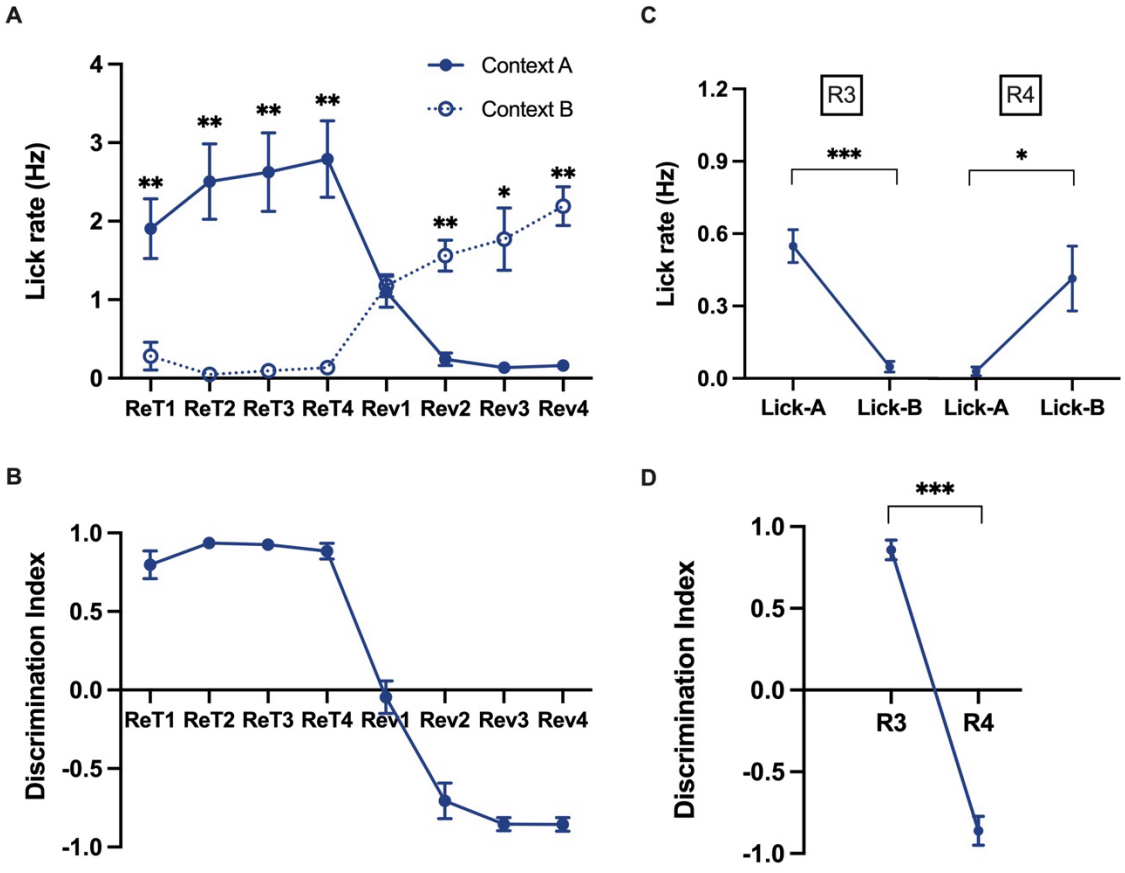


Figure 3-3-2. **Wild-type mice performance in virtual reality-based contextual memory task.**

(A) Anticipatory lick rates (Hz) in Context A and B during retraining (ReT1-4) and reversal training (Rev1-4). Paired *t* test (n=5). ns, not significant. *p < 0.05, **p < 0.01, data are mean ± SEM. **(B)** Discrimination index quantified from (A). **(C)** Anticipatory lick rates (Hz) in Context A and B during recent recall after retraining and reversal training. Paired *t* test (n=5). *p < 0.05, ***p < 0.001, data are mean ± SEM. **(D)** Discrimination index quantified from (C). Paired *t* test (n=5). ***p < 0.001, data are mean ± SEM.

3-4 mPFC is required for contextual memory retrieval at recent and remote time points.

To assess the necessity of the mPFC in recent and remote memory recall, we bilaterally expressed hM4Di in the mPFC of mice and subjected them to virtual reality contextual memory tasks three weeks after surgery. Both experimental groups underwent identical training procedures as wild-type mice but received clozapine-N-oxide (CNO) injections during recent (R1-CNO) and remote recall sessions (R2-CNO). Throughout the training phase, both groups exhibited consistent learning abilities **(Figure 3-4-1. A-B)**. However, mice with chemogenetic inhibition exhibited significantly reduced lick rates in Context A compared to control mice expressing mCherry during both recent and remote recall **(Figure 3-4-1. D)**. This finding suggests that global inhibition of the mPFC can markedly impair both recent and remote contextual memory retrieval. **(Figure 3-4-1. D)**.

To ensure that CNO did not induce non-specific effects on motivated behavior and motor function, we conducted a second remote recall test with saline injection in both groups of mice (R2-SAL) the day after the initial remote recall (R2-CNO) without retraining. The results demonstrate consistent maintenance of significant differences in lick rates between Contexts A and B **(Figure 3-4-1. D)**. Importantly, there were no significant differences in terms of lick rates in Context A between the two remote recall sessions (R2-CNO and R2-SAL) within the mCherry group, suggesting that CNO did not adversely affect licking behavior **(Figure 3-4-1. E-F)**. To further verify this finding, we calculated the lick rates in Context A during training under different combination of injections **(Figure 3-4-2. A)**. The ratio of lick rates in Context A between CNO and saline injections

was calculated and showed no significant difference between groups, confirming that CNO injection did not prevent the motivated performance **(Figure 3-4-2. B)**. Finally, we assessed running speed during two remote recall sessions and found similar speeds between the hM4Di group and controls in both contexts, indicating that CNO had no effect on motor function either **(Figure 3-4-2. C-D)**.

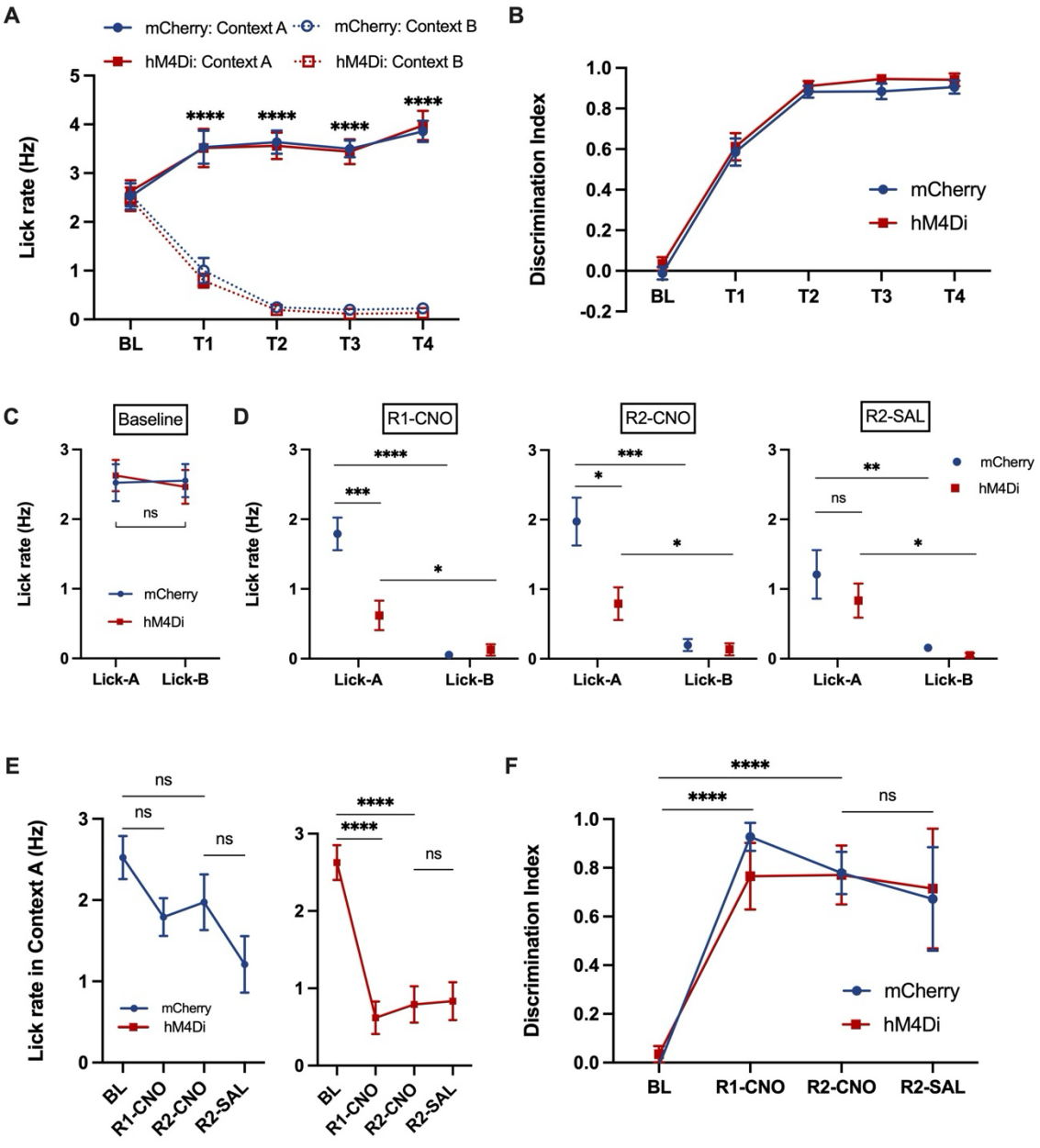


Figure 3-4-1. **Both recent and remote contextual memory recalls are mPFC-dependent.**

(A) Anticipatory lick rates (Hz) in Context A and B through baseline measurement (BL) to training (T1-4) between mCherry and hM4Di groups. ****p < 0.0001 for between Context A and B for mCherry and hM4Di groups, Paired *t* test (mCherry group: 11 mice, hM4Di group:11 mice), data are mean ± SEM. **(B)** Discrimination index quantified from (A). **(C)** Anticipatory lick rates (Hz) in Context A and B during baseline measurement between mCherry and hM4Di groups. Unpaired *t* test (mCherry group: 11 mice, hM4Di group:11 mice). ns, not significant, data are mean ± SEM. **(D)** Anticipatory lick rates (Hz) in Context A and B during recent recall with CNO injection (R1-CNO, left), remote recall with CNO injection (R2-CNO, middle), and remote recall with saline injection (R2-SAL, right) between mCherry and hM4Di groups. Paired *t* test for comparison in groups and unpaired *t* test for comparison between groups (mCherry group: 11 mice, hM4Di group:11 mice). ns, not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, data are mean ± SEM. **(E)** Anticipatory lick rates (Hz) in Context A through baseline measurement (BL) to recent (R1-CNO) and remote recall (R2-CNO, R2-SAL) between mCherry and hM4Di groups. Paired *t* test (mCherry group: 11 mice, hM4Di group:11 mice). ns, not significant. ****p < 0.0001, data are mean ± SEM. **(F)** Discrimination index quantified from (E). Paired *t* test (mCherry group: 11 mice, hM4Di group:11 mice). ns, not significant. ****p < 0.0001, data are mean ± SEM.

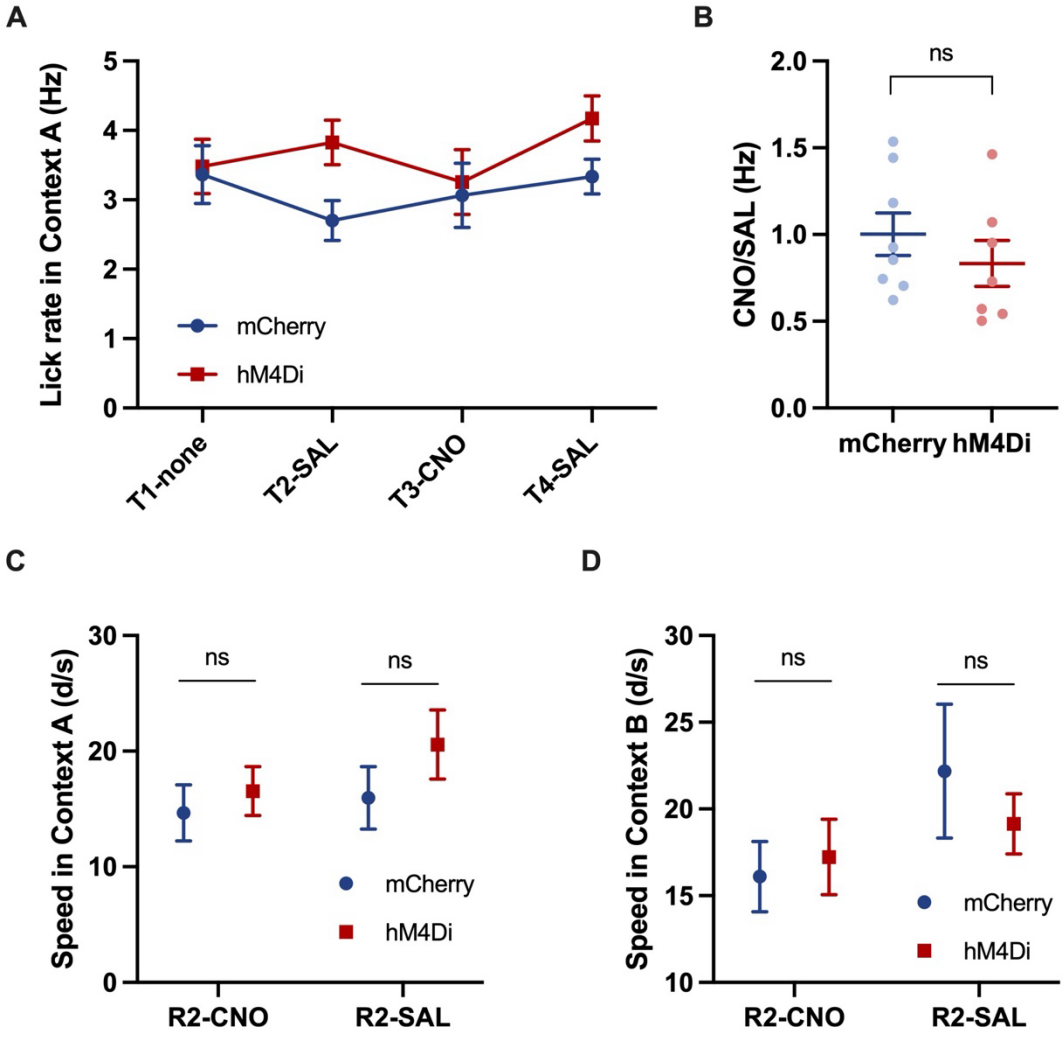


Figure 3-4-2. **CNO has no non-specific effect on motivated behavior and motor function.**

(A) Anticipatory lick rates (Hz) in Context A during training with different injections. **(B)** Ratio of lick rates in Context A quantified from (A). Unpaired *t* test (mCherry group: 8 mice, hM4Di group: 7 mice). ns, not significant. **(C)** Speed in Context A during two remote recall sessions. Unpaired *t* test (mCherry group: 10 mice, hM4Di group: 10 mice). ns, not

significant. **(D)** Speed in Context B during two remote recall sessions. Unpaired *t* test (mCherry group: 10 mice, hM4Di group: 10 mice). ns, not significant.

Chapter 4 Discussion

In this study, we employed contextual fear conditioning—a widely accepted method for studying contextual memories—to identify engram neurons in the mPFC, BLA, and VH. Reactivation of these neurons contributes to both recent and remote contextual memory retrieval. Our findings replicated prior research on the reactivation of engram neurons in the VH for recent contextual memories, as well as the involvement of mPFC and BLA engram neurons in remote contextual memories (Kim & Cho, 2020; Lee et al., 2023; Xu et al., 2016). Additionally, we uncovered a novel role of mPFC in recent contextual memories and VH in remote contextual memories. Furthermore, we introduced a novel approach—a virtual reality-based behavioral task—to explore the roles of mPFC, BLA, and VH more comprehensively. This advanced setup enabled us to test mice across a broader range of contexts easily designed within the virtual environment. We designed two distinct contexts associated with reward and punishment, allowing us to investigate both aversive and reward contextual memories in mice. Our study demonstrated that wild-type mice could learn how to discriminate between these contexts within three days and successfully retrieve contextual memories at both recent and remote time points. They also exhibited flexibility in learning when we switched the unconditioned stimuli between contexts, maintaining their contextual memories during recent recall. Subsequently, we globally inhibited mPFC using surgical expression of hM4Di to assess its role in recent and remote contextual memories. Overall, our results highlight the involvement of mPFC in contextual memories across both recent and remote time points, aligning with its known role in system

consolidation of memory. This contrasts with previous research that predominantly associates mPFC with aversive contextual memories.

However, our study only investigated the implication of mPFC itself, not its specific engram neurons, in recent and remote contextual memories. To deepen our understanding of the neocortical engram circuit's participation in contextual memories, future experiments should selectively inhibit mPFC engrams in the virtual reality-based behavioral task. Based on established roles of mPFC engram neurons in system consolidation and our findings, we hypothesize their necessity for recent and remote contextual memories, particularly in reward contexts. Moreover, we have yet to explore the involvement of other brain regions such as BLA and VH in this context. Given the crucial role of the BLA in contextual fear memories, inhibiting the BLA may result in impaired recall of recent and remote aversive contextual memories. Additionally, brain areas proposed to contribute to remote contextual memories, such as ACC, RSC, and AM, warrant further investigation. Previous research in similar virtual reality settings has implicated ACC and AM in long-term memory storage, yet their engram neurons remain unelucidated. In conclusion, further investigation into the specific engram neurons of the mPFC and their interaction with other brain regions is essential to fully clarify their roles in recent and remote contextual memories, particularly in varied environmental contexts.

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