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Original Article

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Differential Encoding of Trace and Delay Fear Memory in the Entorhinal Cortex

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Trace fear conditioning is characterized by a stimulus-free trace interval (TI) between the conditioned stimulus (CS) and the unconditioned stimulus (US), which requires an array of brain structures to support the formation and storage of associative memory. The entorhinal cortex (EC) has been proposed to provide essential neural code for resolving temporal discontinuity in conjunction with the hippocampus. However, how the CS and TI are encoded at the neuronal level in the EC is not clear. In Exp. 1, we tested the effect of bilateral pre-training electrolytic lesions of EC on trace vs. delay fear conditioning using rats as subjects. We found that the lesions impaired the acquisition of trace but not delay fear conditioning confirming that EC is a critical brain area for trace fear memory formation. In Exp. 2, single-unit activities from EC were recorded during the pre-training baseline and post-training retention sessions following trace or delay conditioning. The recording results showed that a significant proportion of the EC neurons modulated their firing during TI after the trace conditioning, but not after the delay fear conditioning. Further analysis revealed that the majority of modulated units decreased the firing rate during the TI or the CS. Taken together, these results suggest that EC critically contributes to trace fear conditioning by modulating neuronal activity during the TI to facilitate the association between the CS and US across a temporal gap.

Key words: Fear, Memory, Conditioning, Entorhinal cortex

INTRODUCTION

Pavlovian fear conditioning is a model paradigm to investigate how the predictive relation between stimuli is learned and utilized. In typical fear conditioning, an initially neutral stimulus (conditioned stimulus, CS) such as a tone, is followed by a threat stimulus (unconditioned stimulus, US) such as a tactile pain and becomes a potent signal to elicit an array of defensive behaviors as well as sympathetic and endocrinal activation [1-3]. Temporal relationship between the two stimuli divides fear conditioning into two distinct categories: Delay conditioning is characterized by continuity between stimuli and trace conditioning by a stimulus-free gap, or trace interval (TI) between the CS and US. Delay and trace conditioning differ by several characteristics. Most notably, trace conditioning requires temporary memory storage and awareness of the stimulus contingency during the TI [4, 5], making the paradigm advantageous for investigating explicit memory with broad implications for human cognitive functions.

There are several brain regions that were found to be involved in trace fear conditioning, including the hippocampus [6-12], medial prefrontal cortex (mPFC) [8, 13-15], anterior cingulate cortex (ACC) [16, 17], perirhinal cortex [18, 19], and entorhinal cortex (EC) [20-23]. Among them, EC has been proposed to be a crucial structure to encode CS due to its anatomical and physiological features. The EC is interconnected with the amygdala and hippocampus by receiving strong inputs from the amygdala and

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relaying them to the hippocampus [24]. Especially, the inputs from the EC to the hippocampus have been shown to be necessary for temporal associative learning, including trace fear conditioning [20]. Another demonstration of the selective role of the EC in trace fear conditioning is that pre-training excitotoxic lesions of the EC showed impairment in trace fear but not in delay fear conditioning [21].

Despite the importance of the EC in trace conditioning, there is still a lack of understanding of how EC neurons encode trace fear conditioning while animals are behaving, especially focusing on the neural coding of the TI. To elucidate the key mnemonic process in trace fear memory, we investigated how trace fear conditioning changed neural activity in the EC by comparing pre- and post-training activity of EC neurons using *in vivo* single-unit recording. Learning-induced changes in firing rate (e.g., whether they are increased or decreased compared to before learning) during the CS and TI were categorized. In addition, EC neuronal activity during the delay conditioning was compared to the trace conditioning-related encoding. We aim to test the critical role of EC in trace fear conditioning and to elucidate how the TI is represented in the activity of EC neurons before and after learning. To confirm the critical role of EC, we first tested the effect of bilateral electrolytic lesions of EC on the acquisition of trace fear conditioning.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (210~315 g; Orient Bio, Gyeonggi-do, Korea) were housed individually in a climate-controlled vivarium and maintained on a 12-h reversed light/dark cycle (lights on at 9 p.m.) with *ad libitum* access to food and water. All experiments were conducted during the dark phase of the cycle in strict compliance with the guideline of "Care and Use of Laboratory Animals" from Korea University, Seoul, Korea.

Surgery

Animals were fully anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). For the behavioral experiment, a lesion electrode (0.3 mm in diameter, insulated with Epoxylite except for the 0.5 mm tip) was lowered until it reached the EC (two lesion sites bilaterally; AP: -6.8 mm, ML: ±5.0 mm, DV: -7.0 mm from dura; AP: -8.0 mm, ML: ±5.0 mm, DV: -5.0 mm from dura). Electrolytic lesions (n=26) were made with an adonal current (1.0 mA for 20 s at each site). The sham surgery was identical to lesion surgery except that no current was introduced (n=19). Following the procedure, the incision was sutured. For *in vivo* single-unit recording, the sixteen-channel electrode was implanted in the left EC (n=32, AP: -5.2 mm, ML: +6.3 mm, DV: -7.0 mm from dura). The electrode contained a bundle of sixteen 25 μm formvar-coated nichrome wires (A-M Systems) inside of polyimide tubing (0.0089 inner diameters, Small Parts) covered by a stainless steel cannula (25 G, Small Parts). The tip of the electrode was extended ~2.0 mm from the tubing. The impedance of the wire tips ranged 1~2 MΩ at 1 kHz. The electrode was secured by dental cement with eight anchoring surgical screws. Behavioral experiments and neural single-unit recording started after one week of recovery.

Behavioral apparatus

Two distinct contexts (A and B) were used for the experiments. Context A was composed of a transparent Plexiglas chamber (30 cm×25 cm×20 cm) equipped with a grid floor (16 stainless steel rods, 5 mm in diameter) and blue house light. For context B, padding materials (Aspan, Orient Bio, Kyunggi-do, Korea) and red house light were added to the same transparent Plexiglas chamber as context A. In both context chambers, a speaker (8 cm×4 cm, 8 Ω) for presenting a tone and a DC fan for ventilation were attached to the wall. All experiments were conducted in a sound-attenuating cubicle (48 cm×55 cm×45 cm) so that the external noise could not disrupt the behavior experiments. A video camera for monitoring and recording the animal's behavior was fixed on the sidewall of the cubicle. The apparatus was cleaned between sessions with a 70% ethanol solution.

Behavioral procedure and analysis

Exp. 1

As illustrated in Fig. 1a, all animals were first acclimated to context A and B for 10 minutes each, followed by three CS presentations (10 s, 5 kHz, 80 dB, Coulbourn Instruments, Whitehall, PA) with 120 s interval between the CSs (*habituation*). The next day, the EC lesion and sham-lesion animals were randomly assigned to one of four groups receiving trace fear conditioning (*acquisition*, $Trace_{lesion}$, n=15, $Trace_{sham}$, n=10) or delay fear conditioning ($Delay_{lesion}$, n=11, $Delay_{sham}$, n=9). For the trace fear conditioning group, rats received seven pairings of the CS and US (0.5 s, 0.5 mA, delivered by a shock generator, Coulbourn Instruments, Whitehall, PA) with the 30 s TI in the context A. The inter-trial interval (ITI) varied randomly between 180 and 200 s. In the delay fear conditioning, rats received the seven pairings of CS and US in context A. Presentations of the CS and US were controlled by a multifunction I/O board (National Instruments, Austin, TX) and custom-written software based on LabView (National Instruments). Upon completion of the fear conditioning, the rats were returned to the

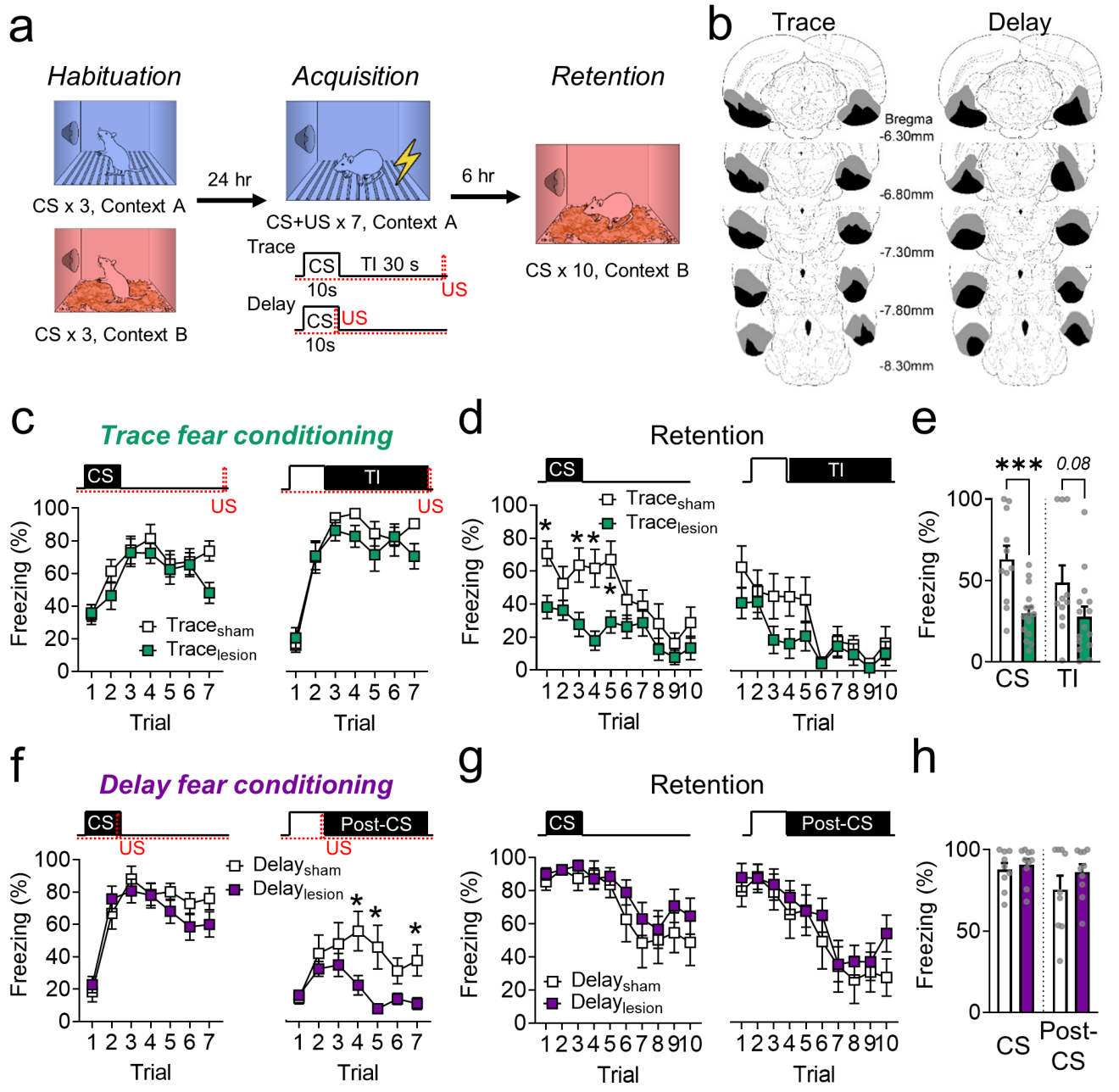

Fig. 1. C lesion on trace vs. delay fear conditioning in Exp. 1. (a) Behavioral procedures for trace and delay fear conditioning. (b) Histological reconstructions of the lesion sites in the EC (black, the smallest lesions; grey, the largest lesions). (c-e) The effects of bilateral EC lesion on trace fear conditioning. (c) Differences in freezing responses to the CS (left) and TI (right) between $Trace_{sham}$ (n=11, white square) and $Trace_{lesion}$ (n=15, green square) during the acquisition session (7 trials). (d) Differences in freezing responses to the CS (left) and TI (right) during the retention session (10 trials). (e) The mean freezing level of the first five trials during the retention session of trace fear conditioning. (f~h) The effects of bilateral EC lesion on delay fear conditioning. (f) Differences in freezing responses to the CS (left) and post-CS (right) between $Delay_{sham}$ (n=9, white square) and $Delay_{lesion}$ (n=10, purple square) during the acquisition session. (g) Differences in freezing responses to the CS (left) and post-CS (right) during the retention session. (h) The mean freezing level of the first five trials during the retention session of delay fear conditioning. Data for c, d, f, and g were assessed by a repeated measures of ANOVA followed by Bonferroni post-hoc test (*p<0.05), and data for e and h were assessed by independent *t*-test (***p<0.01). All data are represented as mean±standard error of the mean (SEM).

home cage. Six hours after the fear conditioning, animals were brought back to context B and received 10 CS-only trials without the US (*retention*).

The behavioral index of fear learning was assessed by freezing (defined as the absence of movement except for respiration [25]). Two experimenters who were blind to the experimental condition

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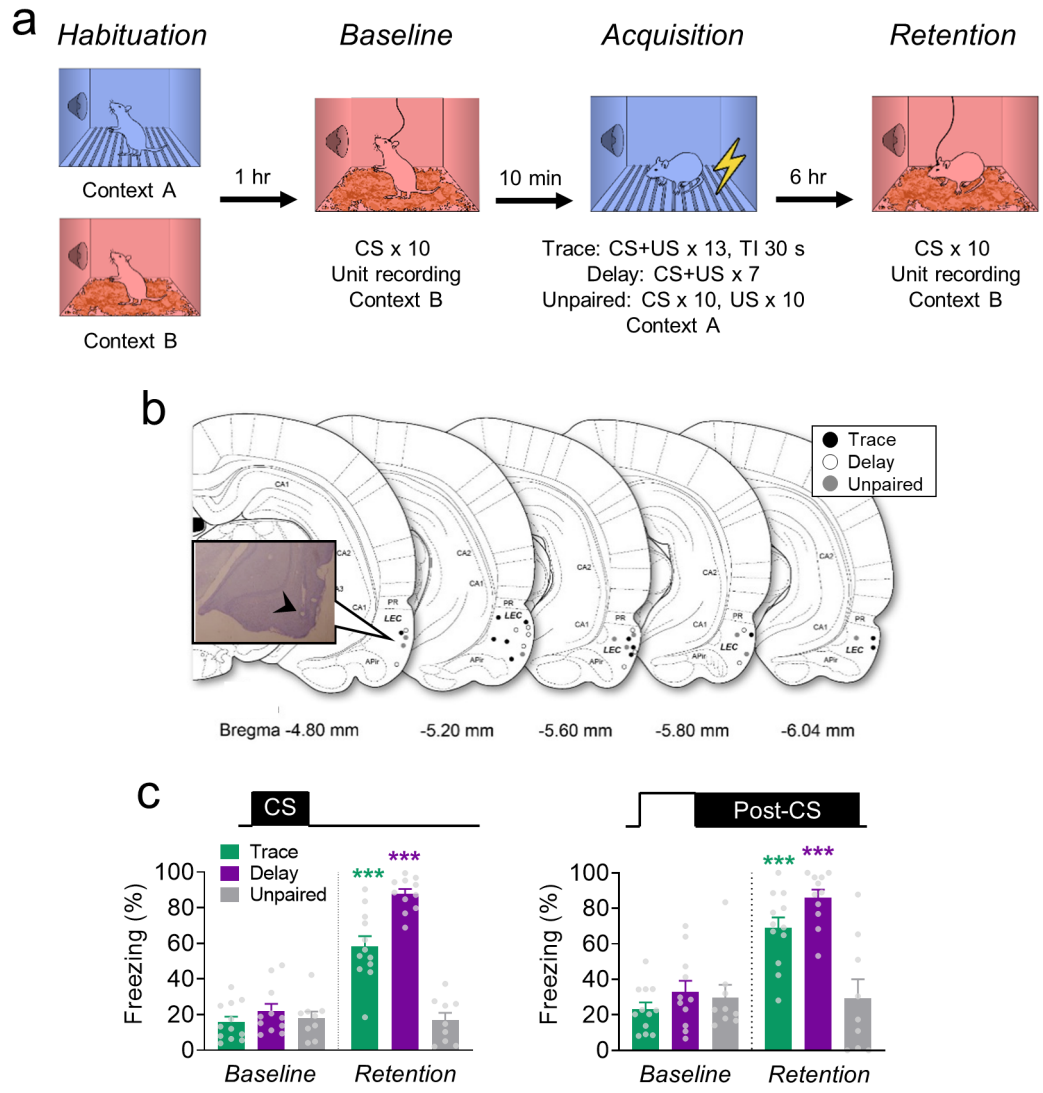

Fig. 2. Behavioral results from Exp.2. (a) Training and recording procedures. (b) Histological reconstructions of the recording sites in the EC (Trace, black; Delay, white; Unpaired, grey) and a photomicrograph of electrode tips in the EC. (c) The mean freezing levels of Trace (n=12, green; green asterisks=baseline vs. retention of Trace), Delay (n=11, purple; purple asterisks=baseline vs. retention of Delay), and Unpaired (n=9, grey) to the CS (left) and post-CS (right) during the baseline and retention sessions. Each circle represents individual data. Data for c were assessed by paired *t*-test (***p<0.01). All data are represented as mean±SEM.

scored freezing responses from the recorded video during the CS and TI (or 30 s after the CS). A custom-written program was used to keep track of scoring and to compute total freezing time.

Exp. 2

The procedure for the recording experiment is shown in Fig. 2a. All rats were habituated to context A and B for 10 minutes each and were placed back in their home cages (*habituation*). During the baseline session, 10 CSs were delivered without the US in context B, with the ITI ranged between 120~150 s. Ten minutes later, rats were randomly assigned to one of the three groups (Trace, n=13; Delay, n=11; Unpaired, n=8) for the *acquisition* session in context A. Rats in the trace fear conditioning group received 13 pairings of the CS and US with 30 s TI. The ITIs varied between 210~240 s. In the Delay group, rats received 7 pairings of the CS and US, co-terminating. Rats in the Unpaired group received 10 CSs and 10 USs in a pseudorandom order without overlapping one another. Due to discrepant freezing levels between the trace and delay conditioning protocols (freezing is greater for delay conditioning if all the conditioning parameters are identical), we employed different numbers of trials for the two conditioning groups [26]. This way, the level of freezing was comparable for the two conditioning groups, at around 75% during the TI (Trace) and the CS (Delay) (Fig. 2c). Six hours later, all rats received 10 CS-only

trials in context B (*retention*).

In vivo single-unit recording, spike sorting and data analysis

For the *in vivo* single-unit recording, neural activity was recorded during the baseline and retention sessions to investigate neural changes after the trace or delay fear conditioning. Signals from the electrodes were amplified (×10,000), band-pass filtered (300~6,000 Hz), and digitized at 32 kHz by a dedicated recording system (Neuralynx, ERP-27, Bozeman, MT). The recorded neural activity was clustered automatically using the KlustaKwik (written by K. D. Harris, Rutgers University, Newark, New Jersey), and further corrections were conducted by the MClust 3.3 spike sorting program (written by A. D. Redish, University of Minnesota, Minneapolis, Minnesota). A custom-written MATLAB code (version 6.5) was used for generating perievent time histograms (PETH) and analyzing numerical data similar to the protocol used in our previous study [27].

Sorted unit activity was binned into 100-ms bins for further analyses and normalized to the pre-CS period (20 s) using a z-score transformation. Significant firing rate change was confirmed by when the mean firing rate during the CS/TI was statistically different from the firing rate of the pre-CS period. Neural responses were categorized into one of the four patterns: CS-increased, CS-decreased (both a sustained change in firing rate during the CS presentation), TI (post-CS)-increased, and TI (post-CS)-decreased (both a sustained change in firing rate during the TI or post-CS presentation).

Histology

Histological verification of the lesion site was performed after all the experimental procedures were completed (Fig. 1b, 2b). Animals were fully anesthetized with an overdose of pentobarbital sodium (120 mg/kg, i.p.) and perfused intracardially with a 0.9% saline and 10% paraformaldehyde solution. For the recording experiment, small lesions were made (5 of 16 wires, 100 μA, 8 s each) before the perfusion to check the locations of the electrode tips. The brains were removed and stored in a 30% sucrose solution overnight. Coronal slices (50 μm thick) cut by a tabletop microtome (Leica SM2000R, Leica Microsystems, Richmond Hill, Ontario, Canada) were mounted on gelatin-coated slides and stained with a 2% potassium ferrocyanide solution and cresyl violet. Stained slides were cover-slipped with Permount (Fisher Scientific, Hampton, NH) and examined under a microscope. Reconstructions of the lesion sites/recording tips were made on the rat brain atlas [28].

Statistical analysis

To compare freezing responses during the CS and TI, all behavioral data were transformed to a percent of total CS or TI duration. The statistical significance was determined with repeated measures ANOVA (*posthoc*: Bonferroni's comparison), independent *t*-test and paired *t*-test. Firing rate changes throughout the trials between pre-CS and CS/TI were tested with paired *t*-test or Kruskal-Wallis test when a non-parametric test is necessary. Proportional changes by learning were examined by the Chi-square test. All statistical analyses were performed by SPSS and GraphPad Prism (version 8).

RESULTS

Exp.1: electrolytic lesions of EC disrupted the trace but not delay fear conditioning

During the acquisition of trace conditioning, a repeated measures ANOVA revealed that there was a significant effect of conditioning trials (F (6,144)=8.316, p<0.01), but no significant effect of group (F (1,24)=1.671, p=0.2085) nor interaction (Group x Trial, F (6,144)=0.9781, p=0.4423) on freezing to the CS (Fig. 1c, left). Likewise, there was a significant effect of conditioning trials (F (4.251,102.0)=30.71, p<0.01), but no significant effect of group (F (1,24)=1.255, p=0.2736) nor interaction (F (6,144)=1.076, p=0.3798; a repeated measures ANOVA) on freezing to the TI (Fig. 1c, right). These results indicate that both groups acquired the conditioned response at a similar rate.

During the retention test, however, there were significant effects of conditioning trials (F (1,24)=8.956, p<0.01), group (F (3.472,83.33)=9.163, p<0.01), and interaction (F (9,216)=2.000, p<0.05; Fig. 1d, left, a repeated measures ANOVA) on the CS. During the TI, there was a significant effect of trials (F (3.229,77.50)=10.24, p<0.01) but no significant effect of group (F (1,24)=2.660, p=0.1159) nor interaction (F (9,216)=1.094, p=0.3686; Fig. 1d, right, a repeated measure ANOVA).

The reduction of freezing to the CS in $Trace_{lesion}$ was more evident during the first five trials of the retention session as revealed by the independent *t*-test (Fig. 1e; t (24)=3.952, p<0.01). A similar trend was observed during the TI albeit not statistically significant (t (24)=1.822, p=0.0809, independent *t*-test). These results indicate that electrolytic EC lesions produced a deficit in fear memory formation following trace conditioning without affecting freezing response to the US.

For the delay conditioning group, a repeated measures ANOVA revealed that there was a significant effect of conditioning trials (F (4.081,69.38)=28.02, p<0.01), but no significant effect of group (F (1,17)=0.5235, p=0.4792) nor interaction (Group x Trial, F

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Table 1. The number of CS-, TI (post-CS)-, CS+TI-, and non-responsive cells from Trace, Delay, and Unpaired during the baseline and retention sessions and the number of increased and decreased cells during the CS and TI (post-CS) from the three groups. *p<0.05, **p<0.01

Response pattern	Trace (63)		Delay (36)		Unpaired (61)	
	Baseline	Retention	Baseline	Retention	Baseline	Retention
CS-responsive	7	3	5	5	8	6
TI-responsive	5	11	5	3	11	10
CS+TI-responsive	8	15	2	8	9	7
Non-responsive	43	34	24	20	33	38
CS	Baseline	Retention	Baseline	Retention	Baseline	Retention
Total responsive	15 (24%)	18 (29%)	7 (19%)	13 (36%)**	17 (28%)	13 (21%)
Increased	5	2	5	1	10	7
Decreased	10	16	2	12	7	6
TI (post-CS)	Baseline	Retention	Baseline	Retention	Baseline	Retention
Total responsive	13 (21%)	26 (41%)*	7 (19%)	13(29%)	20 (33%)	16 (26%)
Increased	4	5	1	1	6	4
Decreased	9	21	6	12	14	12

(6,102)=1.501, p=0.1852) on the freezing to the CS during the acquisition. However, during the post-CS period (30 s after the CS), significant effects of conditioning trials (F (3.696,62.83)=5.802, p<0.01), group (F (1,17)=5.486, p<0.05) and interaction (Group x Trial, F (6,102)=2.976, p<0.05) were confirmed by a repeated measure of ANOVA (Fig. 1f).

During the retention test, there was a significant effect of conditioning trials (F (3.215,54.65)=9.116, p<0.01, Fig. 1g, left), but no significant effect of group (F (1,17)=1.049, p=0.3200) nor interaction (Group x Trial, F (9,153)=0.4132, p=0.9266) to the CS. Similar results were found for the post-CS period of the retention test: the effect of conditioning trials (F (3.507,59.62)=11.62, p<0.01) but not the effect of group (F (1,17)=1.669, p=0.2137) nor interaction (Group x Trial, F (9,153)=0.3601, p=0.9521, Fig. 1g, right, a repeated measures ANOVA) was significant. Likewise, there was no significant difference between $Delay_{sham}$ and $Delay_{lesion}$ when the first five trials were compared (independent t-test: t (17)=0.5774, p=0.5712 for the CS; t (17)=1.139, p=0.2703 for the post-CS, Fig. 1h). Taken together, these results suggest a selective role for the EC in trace fear conditioning, specifically in the encoding and storage of associative memory.

Exp. 2: distinct modulations of the EC neurons during trace vs. delay fear conditioning

To examine how the EC encodes trace fear conditioning distinctively compared to other types of fear learning, three groups of rats underwent trace, delay, or unpaired fear conditioning (Trace, Delay, or Unpaired). Similar to the behavioral results from Exp. 1, trace and delay fear conditioning induced freezing to the CS (Trace: t (11)=7.419, p<0.01; Delay: t (10)=11.48, p<0.01, Fig. 2c, paired t-test) and also during the TI (post-CS in the case of delay conditioning; Trace: t (11)=7.250, p<0.01; Delay: t (10)=6.037, p<0.01, Fig. 2c, paired t-test). On the contrary, unpaired conditioning was not effective in eliciting freezing to the CS (CS: t (8)=0.2555, p=0.8048; post-CS: t (8)=0.05114, p=0.9605, Fig. 2c, paired t-test).

Next, single-unit activities from EC neurons were analyzed. During the baseline session, EC neurons from the three groups of rats showed a comparable level of firing. Specifically, the overall baseline firing rates were not statistically different among the three groups (Trace=1.43±0.95 Hz from 63 cells; Delay=1.53±1.19 Hz from 36 cells; Unpaired=1.76±1.12 Hz from 61 cells, F (2.157)=0.02762, p=0.9728, one-way ANOVA), and the selective firing rates during just the CS or TI (post-CS) were also not different between the three groups (CS: H=4.488, p=0.1060; TI: H=2.787, p=0.2483, Kruskal-Wallis test).

To identify the mode of modulation, firing rates during the CS and TI (post-CS) were compared to that during the pre-CS period (20 s prior to the CS onset). Table 1 shows the number of responsive and non-responsive cells during trace, delay and unpaired conditioning. In Trace, proportions of CS-, TI-, CS+TI- and non-responsive cells during the retention session changed compared to the baseline session: an increased number of cells responded to the TI and the CS+TI (13 cells→26 cells; χ^2 (3, n=63)=7.032, p=0.0709, Chi-Square Test). The increased number of CS+post-CS-responsive cells during the retention session was also found in Delay (not statistically significant, 2 cells→8 cells; χ^2 (3, n=36)=4.464, p=0.2156, Chi-square test) but not in Unpaired (χ^2 (3, n=61)=0.9354, p=0.2156, Chi-square test). Interestingly, significant changes in the proportion of responsive cells were found in cells that showed decreased firing rates during the CS or TI (Fig. 3a, b). After trace fear conditioning, significantly more cells showed TI-modulated responses, especially by decreasing their firing rates during the TI (χ^2 (2, n=63)=6.854, p<0.05, Chi-square test). On the other hand, delay fear conditioning led to more cells decreasing

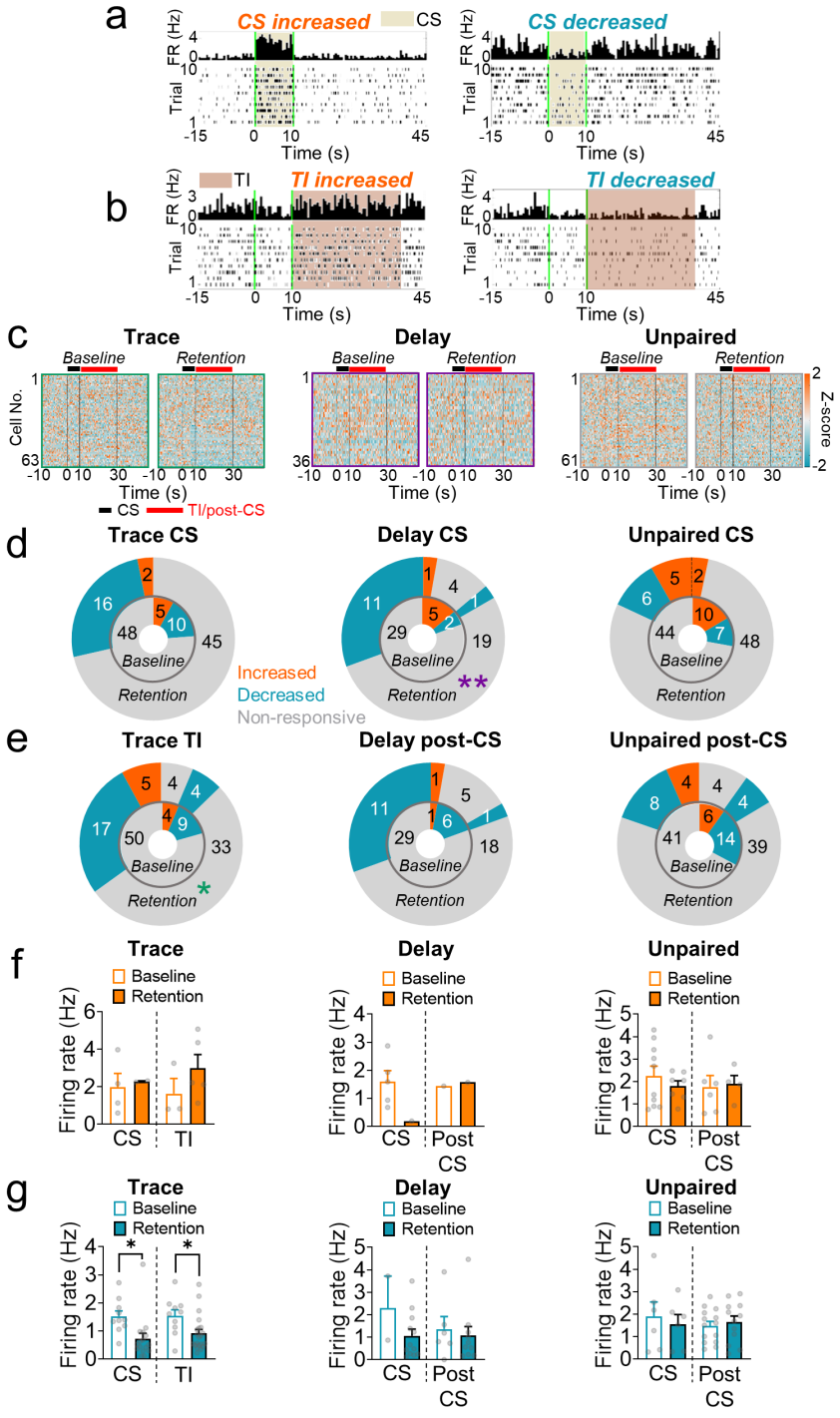

Fig. 3. Activity of EC units during the CS and TI (post-CS) from Trace, Delay, and Unpaired. (a) The peri-event histograms (PETHs) and raster plots of representative EC cells that showed increased firing rates during the CS (left) and decreased firing rates during the CS (right). (b) The PETHs and raster plots of representative EC cells that showed increased firing rates during the TI (left) and decreased firing rates during the TI (right). (c) Z-scored heatmaps of cells during baseline and retention from Trace (left), Delay (middle), and Unpaired (right). Each row indicates firing rates of individual cells throughout the baseline and retention periods. (d) The proportion of CS-responsive cells during the baseline (inner donut chart) and the retention (outer donut chart) sessions from Trace (left), Delay (middle), and Unpaired (right). (e) The proportion of TI (post-CS)-responsive cells during the baseline (inner donut chart) and the retention (outer donut chart) sessions from Trace (left), Delay (middle), and Unpaired (right). (f) The mean firing rates of the CS-increased cells from the baseline vs. retention sessions and the mean firing rates of the TI (post-CS)-increased cells from the baseline vs. retention sessions (left, Trace; middle, Delay; right, Unpaired). (g) The mean firing rates of the CS-decreased cells from the baseline vs. retention sessions and the mean firing rates of the TI (post-CS)-decreased cells from the baseline vs. retention sessions (left, Trace; middle, Delay; right, Unpaired). Data for e and f were assessed by paired *t*-test (*p<0.05), and each circle represents individual cell data. All data are represented as mean±SEM.

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their firing rates during the CS (χ^2 (2, n=36)=10.50, p<0.01, Chi-square test). None of these trends was found in Unpaired (χ^2 (2, n=61)=0.7802, p=0.6770, Chi-square test).

In addition to the proportions of responsive cells, we investigated how the neuronal responses are modulated throughout the acquisition of CR by tracking the activity during the baseline and retention sessions (Fig. 3c). Z-scored heatmaps between baseline and retention sessions illustrate learning-induced firing changes during the CS and TI in all three groups. There was a considerable shift in the neural response before and after conditioning. For example, cells showed CS- or TI (post-CS)-modulated firing before learning, but after learning, a different set of cells became CS- or TI (post-CS)-responsive. This trend was found in all three groups, yet a significant proportion was changed during the CS in Delay (Fig. 3d, χ^2 (2, n=36)=10.50, p<0.01, Chi-square test) and during the TI in Trace (Fig. 3e, χ^2 (2, n=63)=6.854, p<0.05, Chi-square test). These results indicate that more cells were engaged during the CS after delay fear conditioning, and more cells were engaged during the TI after trace fear conditioning. These also coincide with the behavioral changes where rats showed more freezing responses during the CS after delay fear conditioning and more freezing responses during the TI after trace fear conditioning (Fig. 2c).

To examine the conditioning-induced modulation further, we compared the firing rates of CS and TI (post-CS)-responsive cells during the retention session with those from the baseline session. Independent *t*-test revealed that there was no significant increase of overall firing rates of CS-increased cells from the baseline vs. retention sessions in all three groups (Trace: t (4)=0.2799, p=0.7934; Delay: t (4)=1.487, p=0.2111; Unpaired: t (15)=0.8182, p=0.4261, Fig. 3f). Likewise, there was no significant increase of overall firing rates of TI/post-CS-increased cells from the baseline vs. retention sessions in all three groups (Trace: t (6)=1.167, p=0.2875; Delay: only one cell each; Unpaired: t (8)=0.1905, p=0.8537, Fig. 3f, independent *t*-test). These results suggest that the excitability of the EC neurons did not change indiscriminately.

Instead, CS- and TI-decreased cells in Trace during the retention session showed significantly lower firing rates compared to those from the baseline session (CS: t (24)=2.789, p<0.05; TI: t (29)=2.526, p<0.05, Fig. 3g, independent *t*-test), while there was no such change in Delay and Unpaired (Delay: CS, t (12)=1.397, p=0.1876, post-CS, t (14)=0.4403, p=0.6664; Unpaired: CS, t (10)=0.4362, p=0.6720, post-CS, t (24)=0.5314, p=0.6000, Fig. 3g, independent *t*-test). These results indicate that trace fear conditioning not only recruited more cells to become responsive to the CS and TI but also increased the degree of modulation to those events.

DISCUSSION

In the current study, we found that EC is not only critical for trace fear conditioning but also maintains significant modulation of neural activity during the stimulus-free trace interval. Bilateral lesions of the EC attenuated conditioned freezing during the retention session following trace conditioning. The same lesion had no effect on the fear memory of delay conditioning. The preferential role of the EC in trace fear learning has been reported by multiple studies using various techniques [21, 22, 29], and the underlying mechanisms are thought to be through monosynaptic connections with the hippocampus [30]. The entorhinal-hippocampal circuit has been suggested as a primary substrate for temporal associative learning and memory by bridging the temporally discontinuous stimuli [20, 31, 32].

A possible mechanism for 'filling the gap' between the CS and US would require maintaining neuronal activity representing the CS during the TI without the overt presence of the CS. This, 'eligibility trace' is essential for plasticity in various theoretical models as it provides a temporal window for associating the US with the CS trace [33, 34]. Indeed, several studies have shown that EC neurons display sustained activity during the delay phase of delayed match or nonmatching to sample tasks [35, 36]. Moreover, slice electrophysiology studies found 'persistent firing' neurons in the EC [37-39]. A model proposed by Kitamura et al. explains the distinctive neural circuits for delay vs. trace fear conditioning, which involves persistent firing neurons in the EC and feed-forward inhibition by the EC for the fine-tuned memory process of the hippocampus [31, 32].

In line with the previous models at various conceptual and mechanistic levels, the current study found a learning-related modulation of neural dynamics within the EC during the trace interval. The number of TI-responsive neurons were increased following trace fear conditioning. This includes CS+TI-responsive neurons, where more than half of the TI-responsive cells were also CS-modulated (n=15, 58%). Surprisingly, EC neurons also responded to the CS following delay fear conditioning, which would suggest that EC neurons might be responsive to multiple learning-relevant stimuli even though they are critically involved only in trace conditioning. Considering the results from the lesion experiment, CS-responsive neurons in EC might play a secondary role in delay fear conditioning as the critical locus of delay fear memory is in other areas [1].

The most unexpected finding was that a majority of EC cells responded to the CS and/or TI with decrease in firing rates. Based on previous studies [21, 37], we hypothesized that the EC neurons would show persistent activity during the TI to maintain the as-

sociability of the trace CS with the temporally distant US. In an *in vitro* recording study, a strong correlation between the strength of the trace conditioning and the modulation of neuronal activity in the EC was found. Only the neurons from rats with successful acquisition of trace conditioning showed increased ability to fire persistently [40]. The contrasting firing characteristics of EC neurons between the *in vitro* and the *in vivo* (current study) studies might also be due to the differences in the recording sites [37, 38] as the previous studies have mainly focused on the deep layers of the EC (layer V). In the current study, most of the electrodes were located within the superficial layers (between layer II and III), based on the histological examination (28 out of 33 animals), leaving the question of whether the patterns of modulation during TI differ by the layers unanswered.

If the critical contribution of the EC to trace fear conditioning is mediated by increased depression of the neuronal activity during TI, it is reasonable to speculate that an additional brain circuit might be required to support the temporally discontinuous association between the CS and US. One such candidate is the hippocampus, which has been implicated as a critical structure for trace fear conditioning [21, 37, 40]. Based on a finding that a long-range inhibitory projection (LRIP) from the EC fine-tunes hippocampal-dependent memory by promoting the integration of multisensory inputs, a recent study proposed that the EC-hipppocampal modulation would mainly involve disinhibition [41]. Specifically, selective inhibition of the long-range inhibitory projection to the hippocampal CA1 interneurons resulted in increased fear response to the conditioning context and overgeneralized fear to a novel context. Conceptually, the decreased firing of EC neurons during the CS/TI as found in the current study proposes that the mnemonic contribution of the EC to trace conditioning is completed within the integrated EC-hippocampal circuit via LRIP.

Alternatively, the decreased firing rate might be a result of reduced movement as the rats showed increased freezing after trace conditioning. For example, neurons in the anterior cingulate cortex (ACC) modulate the firing rate in relation to the speed of movement such that ACC neurons increase firing rates when animals terminate freezing behaviors [16]. Therefore, the down-regulation of EC neuronal activity in the current study could partially originate from the EC (EC→ACC). It is equally plausible to assume that the decreased activity of EC neurons may be the outcome of the decreased activity of ACC neurons (ACC→EC) because the EC and ACC are reciprocally connected direct projections [42, 43]. A distinct population of cells in the medial EC that respond positively to running speed (i.e., speed cells) supports the relationship between the firing rate and running speed [44, 45]. However, the current study recorded from the lateral EC, not from the medial EC, and functional dissociation between the medial and lateral EC has been widely reported (spatial vs. temporal) [46]. In addition, we found no correlation between movement speed and EC neuronal activity during the baseline session, providing little support for the motoric account of the current results.

To sum up, the current study suggests a new role for the EC in trace fear conditioning by uncovering a unique pattern of neural activity, a learning-related decrease in firing rate, during the CS and TI. Although several previous studies unequivocally argued for the critical role of the EC in trace fear conditioning as the current results also have confirmed, the findings from the recording experiment shed a new light on a circuit-level understanding of the mnemonic processing responsible for temporal associative learning. Further studies are warranted to identify the precise role of the EC in trace fear conditioning via its functional connections with multiple brain regions such as the hippocampus and prefrontal cortex.

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