Proximodistal heterogeneity in learning-promoted pathway-specific plasticity at dorsal CA1 synapses

(近遠位軸で変化する学習依存的な背側海馬CA1シナプスの可塑性)

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ABSTRACT

Contextual learning requires the delivery of AMPA receptors to CA1 synapses in the dorsal hippocampus. However, proximodistal heterogeneity of pathway-specific plasticity remains unclear. Here, we examined the proximodistal heterogeneity in learning-induced plasticity at the CA1 synapses with inputs from the entorhinal cortex layer III (ECIII) or from CA3. We subjected male rats to an inhibitory avoidance task and prepared acute hippocampal slices for whole-cell patch clamp experiments, where we stimulated ECIII-CA1 or CA3-CA1 input fibers to analyze evoked excitatory postsynaptic currents (EPSCs). Compared to untrained controls, trained rats exhibited higher AMPA/NMDA current ratios at proximal and intermediate, but not distal CA3-CA1 synapses, which suggested that region-specific plasticity occurred after learning. Moreover, trained rats exhibited higher AMPA/NMDA current ratios at intermediate and distal, but not proximal ECIII-CA1 synapses. These findings suggested the presence of proximodistal heterogeneity in pathway-specific postsynaptic plasticity. Regarding presynaptic plasticity, training slightly, but significantly increased the paired-pulse ratios of proximal and intermediate, but not distal CA3-CA1 synapses. Moreover, trained rats exhibited higher paired-pulse ratios at intermediate and distal, but not proximal ECIII-CA1 synapses, which suggested region-specific presynaptic plasticity. Finally, learning was clearly prevented by the bilateral microinjection of a plasticity blocker in the proximal or intermediate, but not distal CA1 subfields, which suggested functional heterogeneity along the proximodistal axis. Understanding region- and pathway-specific plasticity at dorsal CA1 synapses could aid in controlling encoded memory.

Key words: Contextual learning, AMPA receptor, glutamic acid, temporoammonic pathway, Schaffer's collateral pathway

INTRODUCTION

The hippocampal region, together with its adjacent parahippocampal region is critically involved in contextual memory formation encoding facts and events (Andersen et al., 2007). Being proved the existence of many memory-related cells such as place cells (O'Keefe and Dostrovsky, 1971), anxiety cell (Jimenez et al. 2018) etc., CA1 region is thought to be a critical region of memory processing. The entorhinal cortex (EC) conveys information received from other sensory cortices to the hippocampal CA1 region, both directly and indirectly. Direct temporoammonic projections originate from principal neurons in layer III (the TA pathway); in contrast, EC layer II neurons indirectly project through the dentate gyrus (DG) and CA3 (tri-synaptic pathway) regions (Steward and Scoville, 1976; Andersen et al., 2007). Both projections converge on single CA1 pyramidal neurons; in turn, CA1 dendrites form synapses with the TA pathway in the stratum lacunosum moleculare and with Schaffer's collaterals from CA3 in the stratum radiatum (Kajiwara et al., 2008).

Schaffer's collateral inputs play a critical role in contextual memory formation (Nakashiba et al., 2008) by establishing 13,059 - 28,697 synapses per single CA1 pyramidal neuron (Bezaire and Soltesz, 2013). Based on the results of an inhibitory avoidance (IA) task applied in rats as a hippocampus-dependent contextual learning paradigm (Izquierdo et al., 1998), we previously found that contextual learning delivered glutamate A1 subunit (GluA1)-containing AMPA receptors to CA3-CA1 synapses in the dorsal hippocampus (Mitsushima, et al., 2011, 2013).

Conversely, the TA pathway only forms up to 1,742 synapses per single CA1 pyramidal cell; much fewer connections than CA3-CA1 synapses (Bezaire and Soltesz, 2013). The TA pathway plays necessary physiological roles in temporal association memory (Suh et al., 2011), spatial learning (Brun et al., 2002, 2008), novelty detection (Vago and Kesner, 2008), and consolidation

of memory (Remondes and Schuman, 2004). Although many studies have shown that TA pathway synapses induced both long-term potentiation (LTP) and long-term depression (LTD) (Aksoy-Aksel and Manahan-Vaughan, 2013, 2015; Gonzalez et al., 2016), learning-induced synaptic plasticity has not been explored at these synapses.

Moreover, CA1 pyramidal cells show different intrinsic properties and functional significance along the proximodistal axis (Fig. 1; Igarashi et al., 2014). Proximal CA1 cells have higher spatial specificity and play a crucial role in spatial memory formation. In contrast, distal CA1 cells process non-spatial, object-related information, such as odor-based memory (Igarashi et al., 2014). The integration of object-related information with spatial and temporal contexts induces sequence coding activity, which is highest in the intermediate CA1 region (Ng et al., 2018). In addition, by controlling neuromodulators, topographic TA projections appear to allow the hippocampus to differentially encode new information along the proximodistal axis of CA1 (Ito and Schuman, 2012). Moreover, deep pyramidal neurons in the proximal CA1 region receive more inputs from the medial EC, and superficial neurons in the distal CA1 region receive more inputs from the lateral EC (Masurkar et al., 2017). Although many studies have shown that there are prominent functional differences along the proximodistal axis of the CA1 region (Henriksen et al., 2010; Knierim et al., 2014; Igarashi et al., 2014; Nakazawa et al., 2016; Oliva et al., 2016; Ng et al., 2018), there is no synaptic evidence for differences in learning-induced plasticity.

The present study aimed, first, to analyze learning-induced postsynaptic plasticity, in both the intermediate CA1 region and the proximal and distal CA1 regions, where previously unidentified patterns of learning-induced synaptic plasticity might occur. Hippocampus-dependent IA training can mimic the effect of LTP induction to Schaffer's collateral and also occlude subsequent LTP induction indicating that contextual memory formation induces LTP in CA3-CA1 synapses *in*

vivo (Whitlock et al., 2006). Many studies also have proved the role of GluA1-containing AMPA receptor in this learning process (Mitsushima, et al., 2011, 2013; Penn et al., 2017). However, there is still no evidence about pathway- and region-specific postsynaptic plasticity involved in this learning process.

Our second aim was to elucidate the presynaptic changes that occur after learning (Yang and Calakos, 2013). To that end, we analyzed the paired-pulse ratio (PPR), which can be used to detect learning-induced changes in the glutamate release probability. Contextual learning was shown to increase the phosphorylation of presynaptic protein called growth-associated protein-43 which is involved in long-term potentiation (LTP) and in hippocampus-dependent learning (Cammarota et al., 1997; Young et al., 2000; Young et al., 2002). Moreover, increase in phosphorylation of presynaptic protein, synapsin I, resulted in increased neurotransmitter docking, increased paired-pulse facilitation and enhanced hippocampus-dependent learning (Kushner et al., 2005). The inhibition of presynaptic synaptosomal nerve-associated protien-25 which involves in neurotransmitter release impaired long-term contextual fear memory and spatial memory (Hou et al., 2004). Although we previously found that learning induced a slight reduction in glutamate release probability at CA3-CA1 synapses (Mitsushima et al., 2013), the properties of region- or pathway-specific presynaptic plasticity are largely unknown.

Here, we stimulated each pathway to analyze learning-induced pre- and postsynaptic plasticity along the proximodistal CA1 axis. Understanding region- and pathway-specific plasticity at dorsal CA1 synapses might aid in controlling encoded memory in the hippocampus (Paw-Min-Thein-Oo et al., 2020).

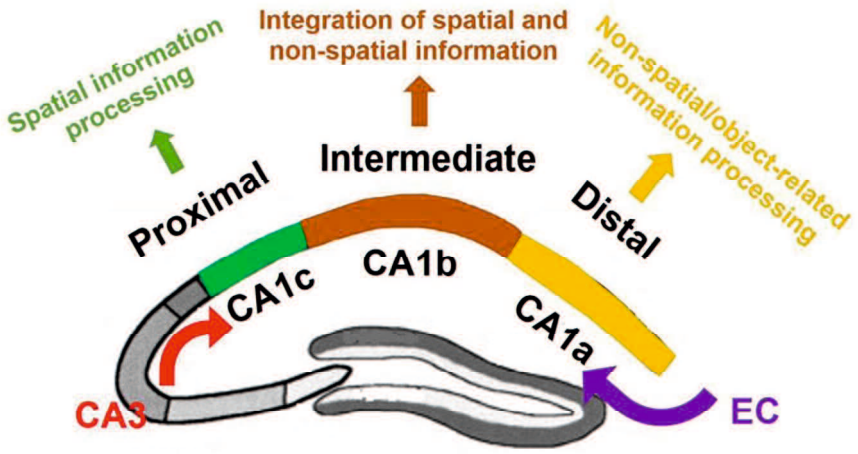

Figure 1. Schematic illustration showing CA1 sub-regions. Based on the original description of Lorente de No (1934), hippocampal CA1 region can be subdivided into CA1a, b and c which corresponds to distal, intermediate and proximal CA1 respectively. These sub-regions show significant functional heterogeneity.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (postnatal 4 weeks old) were obtained from Chiyoda Kaihatsu Co., Tokyo, Japan. Prior to the experiment, the rats were individually housed in plastic cages for a couple of days (40 × 25 × 25 cm) at a constant temperature (23 ± 1°C) under a 12-h light/dark cycle (lights on from 0800 to 2000) with *ad libitum* access to water and food (MF, Oriental Yeast Co. Ltd, Tokyo Japan). All animal housing and surgical procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University Graduate School of Medicine and performed in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Inhibitory avoidance (IA) task

Hippocampus-dependent IA training procedures were described previously (Mitsushima et al., 2011, 2013, Sakimoto et al., 2019). The IA training apparatus (length: 33 cm, width: 58 cm, height: 33 cm) consisted of a two-chambered box that contained a lighted safe side and a dark shock side, separated by a trap door (Fig. 2A). For training, rats were placed in the light side of the box, facing a corner opposite the door. After the trap door was opened, the rats could enter the dark box at will. The latency before entering the novel dark box was measured as a behavioral parameter (latency before IA learning). Four seconds after the animals entered the dark side, we closed the door and applied a scrambled electrical foot-shock (2 s, 1.6 mA) via electrified steel rods set into the floor of the box. The rats were kept in the dark compartment for 10 s before they were returned to their home cage. Untrained control rats were not moved from their home cages.

Thirty minutes after the procedure described above, the rats were placed on the light side as a memory retrieval test. The latency before entering the dark box was measured as an indicator of learning performance (latency after IA learning). The rats were sacrificed 30 min after the retrieval test. To evaluate plasticity before the retrieval test, some rats were sacrificed 30 min after the training without taking the retrieval test (Table 2).

Electrophysiological recordings

We previously published a detailed technical protocol for a patch clamp recording technique for use in brain slices that was used to analyze learning-induced synaptic plasticity (a short demonstration movie was included) (Kida et al., 2017, Sakimoto et al., 2019). With that

technique, we examined learning-induced synaptic plasticity in CA1 pyramidal neurons. Based on previous studies (Lorente de No, 1934; Henriksen et al., 2010; Oliva et al., 2016), we subdivided the CA1 area into distal, intermediate, and proximal CA1 regions.

After IA training, rats were anesthetized with pentobarbital, and acute brain slices were prepared (Mitsushima et al., 2011, 2013). Briefly, the brains were rapidly perfused with ice-cold dissection buffer (25.0 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7.0 mM $MgCl_2$, 25.0 mM glucose, 90 mM choline chloride, 11.6 mM ascorbic acid, 3.1 mM pyruvic acid) and gassed with 5% CO_2/95% O_2. Coronal sections that contained the CA1 area were cut 350 μm-thick (Leica vibratome, VT-1200) in dissection buffer and transferred to artificial cerebrospinal fluid (22–25°C, 114.6 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM glucose, 4 mM $MgCl_2$, 4 mM $CaCl_2$, pH 7.4, gassed with 5% CO_2/95% O_2). We obtained 3–4 slices from each rat. Based on the brain atlas by Paxinos and Watson (Paxinos and Watson, 1998), we selected 1–2 brain slices for patch clamp recordings (from 3.6 to 4.2 mm posterior to bregma). To isolate direct inputs to the TA pathway, we cut the indirect input routes from the EC that relayed via the trisynaptic pathway and subiculum (Fig. 2E).

With an infrared differential interference contrast microscope, we first identified the proximal boundary of the CA1 area (border between the CA1 and CA2 areas) and the distal boundary of the CA1 area (border between the CA1 and subiculum). The CA2 area was identified as a thicker pyramidal layer of wider and sparser cells. The subiculum was identified by the fasciola cinereum, which is a medially curved continuation of the hippocampus at the septal tip of the structure (Henriksen et al., 2010; Oliva et al., 2016). Based on the original description of Lorente de No (1934), we subdivided the CA1 area into CA1a, CA1b, and CA1c regions, which corresponded to the distal, intermediate, and proximal CA1 regions, respectively (Fig. 1).

Glass patch-recording pipettes (4–7 MΩ) were created with a horizontal puller (Model P97; Sutter Instrument, Novato, CA, USA) and filled with intracellular solution (115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA at pH 7.25). The recording chamber was perfused with artificial cerebrospinal fluid containing 0.1 mM picrotoxin and 4 μM 2-chloroadenosine at 22-25°C. To analyze the function of CA3-CA1 synapses, a bipolar tungsten stimulating electrode (Unique Medical Co., Ltd., Tokyo, Japan) was placed in the stratum radiatum layer of CA1, at ~200 – 300 μm lateral to the recorded cells. For ECIII-CA1 synapses, the stimulating electrode was placed in the stratum lacunosum moleculare layer of CA1, at ~200 – 300 μm medial to the recorded cells. We checked the latency of evoked EPSC responses after the stimulation, but the neurons did not show polysynaptic late responses. Moreover, we could not see any alive CA3 neurons in our slice angle of frontal section.

Whole-cell recordings were obtained from pyramidal neurons of the hippocampal CA1 layer with an Axopatch-1D amplifier (Axon Instruments Inc., Union City, CA, USA). Recordings were digitized with a Digidata 1440 AD board (Axon), recorded at 5 kHz, and analyzed offline with pCLAMP 10.4 software (Axon). The stimulus intensity was increased, until a synaptic response with an amplitude >10 pA was recorded. The electrically evoked EPSC amplitudes were measured from the peak of the postsynaptic current to the basal current level recorded immediately before electrical stimulation. For postsynaptic plasticity, we measured the ratio of AMPA receptor-mediated postsynaptic current to NMDA receptor-mediated current (AMPA/NMDA). This ratio was calculated as the ratio of the peak current at -60 mV to the current at +40 mV, at 150 ms after stimulus onset (40–60 traces were averaged for each holding potential). To visualize the AMPA/NMDA current ratio distribution, we plotted the cell-specific

ratios on the X-axis and their cumulative frequencies on the Y-axis. For presynaptic plasticity, 50–100 sweeps were recorded with paired stimuli applied at 100-ms intervals with 0.4 Hz frequency (1 trace per 2.5 sec), and the ratio of the second amplitude to the first amplitude was calculated as the PPR. To visualize the PPR distribution, we plotted the cell-specific ratios on the X-axis and their cumulative frequencies on the Y-axis.

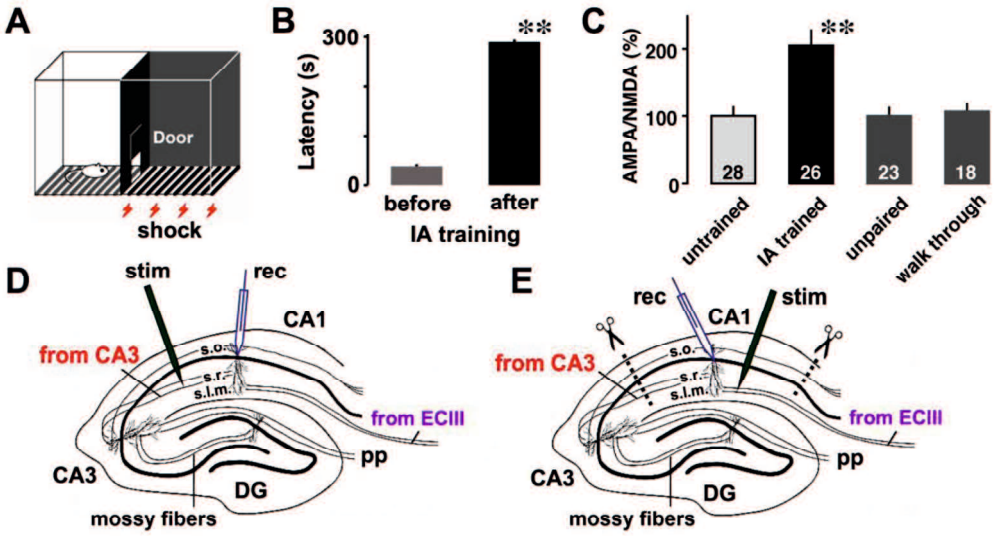

Figure 2. Inhibitory avoidance (IA) task and recording sites. (A) Schematic of the IA training apparatus. (B) Foot-shocks increased the latency for entering the dark box (N = 25). (C) AMPA/NMDA ratio of CA1 pyramidal neurons in untrained, IA trained, unpaired, and walk through rats. Unpaired rats received unpaired foot shock in their home cage. Walk through rats allowed to explore the shock cage for 1 min. (Mitsushima et al., 2011). (D, E) Diagrams show the locations of electrodes for patch-clamp analysis at (D) CA3-CA1 synapses and (E) ECIII-CA1 synapses. Error bars indicate + SEM. $^{**}P < 0.01$ *vs.* before or untrained.

Microinjection study

After rats were anesthetized with sodium pentobarbital (30–50 mg/kg, i.p.), a stainless-steel guide cannula (outer diameter, 0.51 mm) was implanted stereotaxically, just above of the target region in the dorsal hippocampus (Mitsushima et al., 2013, Sakimoto et al., 2019). After cannula implantation, a stylet was inserted into the guide cannula until the time for drug injection. The IA task was performed 1–3 days after the implantation.

On the day of the experiment, the stylet was replaced with a 1.0-mm longer injector (outer diameter 0.31 mm), without restraining animals in their home cages. According to the Paxinos and Watson brain atlas (Paxinos and Watson, 1998), we microinjected drugs into the following coordinates in the CA1 region: proximal (3.0 mm posterior to bregma, ±2.8 mm lateral to midline, 3.8 mm below the skull surface), intermediate (3.0 mm posterior to bregma, ±2.0 mm lateral to midline, 3.8 mm below the skull surface), and distal (3.0 mm posterior to bregma, ±1.2 mm lateral to midline, 3.8 mm below the skull surface). We measured the DV coordinates from the surface of the skull. Approximately 20 min before the IA learning procedure, we injected either an NMDA receptor antagonist (d-AP5, 3 μg/μl per side, Sigma Chemical Co., St. Louis, MO) or saline directly into the CA1 through fine, flexible silicone tubing (o.d.: 0.5 mm, Kaneka Medix Co. Osaka, Japan) without restraining the animals. Moreover, we confirmed that unilateral APV microinjection successfully blocked the learning-induced synaptic plasticity around the injected side, suggesting essential role of NMDA receptors on learning-induced synaptic plasticity. After the behavioral task, we injected the same volume (1 μl per side) of Luxol Fast Blue into the dorsal CA1 region to check the microinjected site.

Statistical analysis

We used the Mann-Whitney *U* test to analyze the AMPA/NMDA ratio and the PPR. We used the Wilcoxon signed-rank test to compare latencies before and after foot-shock. We used the chi-square test to evaluate the number of non-learners. A non-learner was defined as a rat that showed a shorter latency after the paired foot-shock than before the shock. We used the Kruskal-Wallis test to evaluate differences among three groups of rats: untrained, trained and sacrificed before the retrieval test, and trained and sacrificed after the retrieval test. This was followed by a *post-hoc* analysis with the Mann-Whitney *U* test. *P*-values <0.05 were considered significant.

RESULTS

Inhibitory Avoidance (IA) Task

Rats were subjected to an IA task to determine the location, if any, at which contextual memory occurred in the three CA1 subfields of the dorsal hippocampus (Fig. 2A; Izquierdo et al., 1998; Mitsushima et al., 2011, 2013). In this learning paradigm, rats were allowed to cross from the light side to the dark side of the box. In the dark side, an electric foot-shock (1.6 mA, 2 s) was delivered. As contextual learning performance, we measured the latency to enter the dark side before giving the shock, and compared it with the latency at 30 min after the IA task. With paired foot-shock, the latency was consistently longer after training than before training (Z_{48} = -4.372, P < 0.001, Fig. 2B).

Postsynaptic Plasticity

To determine the extent of pathway- and region-specific learning-induced postsynaptic plasticity at the CA3-CA1 and ECIII-CA1 synapses, we measured currents mediated by AMPA receptors

and NMDA receptors at these synapses. Since the synaptic change was not observed in unpaired or walk through controls (Mitsushima et al., 2011; Mitsushima et al., 2013), the ratios of these current amplitudes were compared between the untrained and trained rat groups.

As shown in Figure 3A, we recorded at proximal, intermediate, and distal CA3-CA1 synapses. We found that IA training significantly increased the AMPA/NMDA ratio at proximal CA3-CA1 synapses (U_{55} = 234.5, P = 0.006, Fig. 3B: left) and intermediate CA3-CA1 synapses (U_{89} = 635, P = 0.001, Fig. 3B: middle). However, at distal CA3-CA1 synapses, the increase was not significant (U_{67} = 446.5, P = 0.077, Fig. 3B: right). These results suggested that contextual learning postsynaptically strengthened CA3-CA1 synapses in the proximal and intermediate regions.

Similarly, we analyzed the AMPA/NMDA ratios recorded in proximal, intermediate, and distal ECIII-CA1 synapses (Fig. 3D). IA training significantly increased the ratio at intermediate ECIII-CA1 synapses (U_{104} = 841, P < 0.001, Fig. 3E: middle) and distal ECIII-CA1 synapses (U_{60} = 231.5, P = 0.002, Fig. 3E: right). However, at proximal ECIII-CA1 synapses, the difference was not significant (U_{83} = 678.5, P = 0.058, Fig. 3E: left). These results suggested that contextual learning postsynaptically strengthened ECIII-CA1 synapses in the intermediate and distal regions. Cumulative distributions of cell-specific AMPA/NMDA ratios are shown in Figures 3C (CA3-CA1 synapses) and 3F (ECIII-CA1 synapses) (Paw-Min-Thein-Oo et al., 2020).

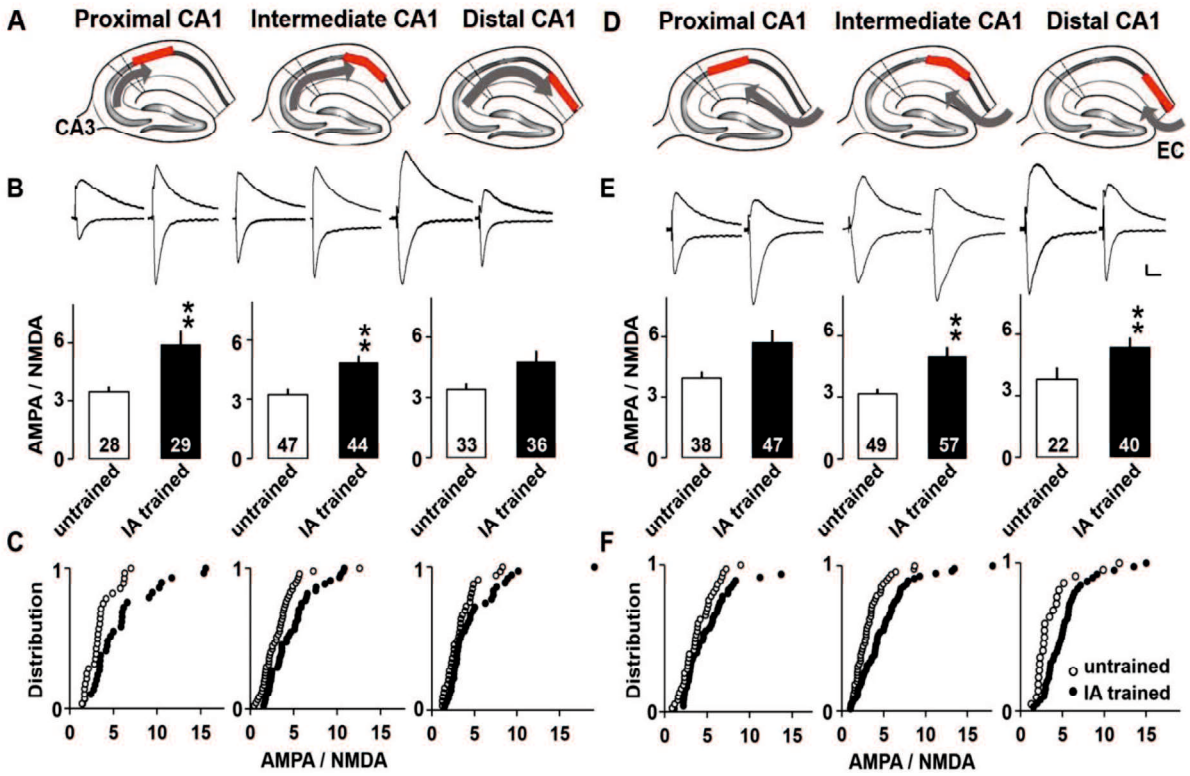

Figure 3. Postsynaptic plasticity at CA3-CA1 and ECIII-CA1 synapses. (A) The proximal, intermediate, and distal CA1 subfields (red areas) of the dorsal hippocampus where we recorded the AMPA/NMDA ratios of CA3-CA1 synapses. (B) IA training significantly increased the mean AMPA/NMDA ratios at CA3-CA1 synapses in the proximal and intermediate regions. The upper insets show representative traces. The number of cells in each group is shown at the bottom of each bar. (C) Cumulative distribution of AMPA/NMDA ratios in untrained and trained rats. (D) The proximal, intermediate, and distal CA1 subfields (red areas) where we recorded the AMPA/NMDA ratios of ECIII-CA1 synapses. (E) IA training significantly increased the mean AMPA/NMDA ratios at ECIII-CA1 synapses in the intermediate and distal regions. The upper insets show representative traces. The number of cells in each group is shown at the bottom of each bar. (F) Cumulative

distribution of AMPA/NMDA ratios in untrained and trained rats. Vertical and horizontal scale bars represent 20 pA and 40 ms, respectively. Error bars indicate + SEM. $^{**}P < 0.01$ *vs.* untrained.

Bilateral AP5 microinjections

In the IA task, rats mostly showed long latencies in the light side after learning about the aversive context. In contrast, they showed shorter latencies in the absence of aversive stimuli (Sakimoto et al., 2019). To determine the role of postsynaptic plasticity, AP5 was bilaterally microinjected into the three CA1 regions (Table 1). After AP5 injections into the proximal or intermediate region, training failed to increase the latency, and the number of non-learners reflected the level of chance (4 of 8 rats). Non-learners were defined as rats with shorter latencies after the aversive foot-shock than before the foot-shock. However, after AP5 injections into the distal CA1 region, the paired foot-shock test consistently increased the latency (8 of 8 rats). In saline control groups, the paired foot-shock test mostly increased the latency (23 of 24 rats). These results suggested that postsynaptic plasticity in the proximal and intermediate CA1 regions played a functional role in IA learning (Paw-Min-Thein-Oo et al., 2020).

Table 1. IA task performance in rats injected with saline or AP5

		IA latency (s)		Non-learner
Group	CA1 area	Before shock	After shock	(n/total N)
	Proximal	38.0 ± 8.3	269.9 ± 30.1*	0 / 8
Saline	Intermediate	19.3 ± 9.1	247.0 ± 38.1*	1 / 8
	Distal	30.1 ± 6.0	245.8 ± 32.7*	0 / 8

	Proximal	35.4 ± 12.9	85.4 ± 36.5	4 / 8 #
AP5	Intermediate	22.8 ± 6.8	65.0 ± 35.5	4 / 8 #
	Distal	48.0 ± 12.9	88.9 ± 32.0*	0 / 8

* $P < 0.01$ *vs.* before, # $P < 0.01$ *vs.* saline group

Presynaptic Plasticity

We also investigated the role of presynaptic plasticity at CA3-CA1 and ECIII-CA1 synapses during IA training-induced contextual memory formation. We applied paired stimuli at 100-ms intervals, recorded paired-pulse responses, and calculated the PPRs to determine glutamate release probability at these excitatory synapses.

As shown in Figure 4A, we recorded at proximal, intermediate, and distal CA3-CA1 synapses. We found that IA training significantly increased the PPRs at proximal CA3-CA1 synapses (U_{73} = 429.5, P = 0.004, Fig. 4B: left) and intermediate CA3-CA1 synapses (U_{70} = 394, P = 0.004, Fig. 4B: middle), but not at distal CA3-CA1 synapses (U_{62} = 370.5, P = 0.058, Fig. 4B: right). These results suggested that contextual learning also induced presynaptic plasticity by reducing the glutamate release probability at CA3-CA1 synapses in proximal and intermediate regions.

Similarly, we evaluated the PPRs in proximal, intermediate, and distal ECIII-CA1 synapses (Fig. 4D). IA training significantly increased the PPRs at intermediate ECIII-CA1 synapses (U_{124} = 1540.5, P = 0.032, Fig. 4E: middle) and distal ECIII-CA1 synapses (U_{69} = 395, P = 0.009, Fig. 4E: right), but not at proximal ECIII-CA1 synapses (U_{62} = 379.5, P = 0.104, Fig. 4E: left). Thus, IA training significantly reduced the glutamate release probability at ECIII-CA1 synapses in intermediate and distal CA1 regions. The cumulative distributions of cell-specific PPRs are

shown in Figures 4C (CA3-CA1 synapses) and 4F (ECIII-CA1 synapses) (Paw-Min-Thein-Oo et al., 2020).

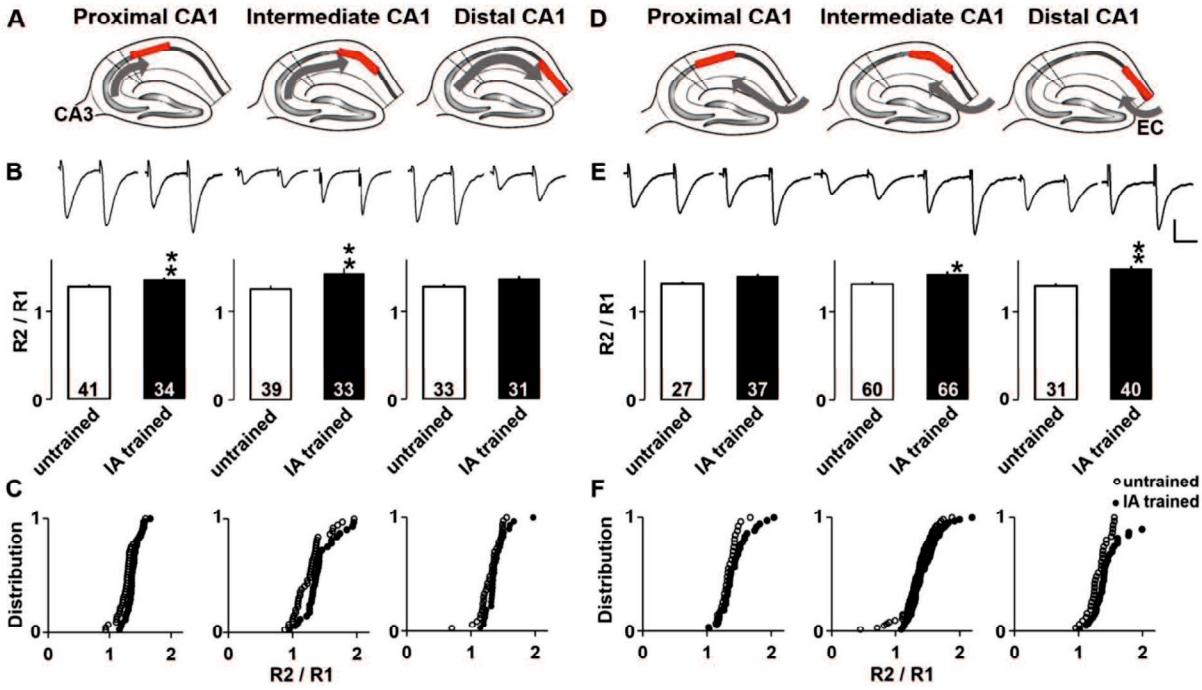

Figure 4. Presynaptic plasticity at CA3-CA1 and ECIII-CA1 synapses. (A) The proximal, intermediate, and distal CA1 subfields (red areas) of the dorsal hippocampus where we recorded PPRs of ECIII-CA1 synapses. (B) IA training significantly increased the PPRs (R2/R1) at CA3-CA1 synapses in the proximal and intermediate regions. A higher ratio suggests a lower release probability of glutamate. The upper insets show representative traces. The number of cells in each group is shown at the bottom of each bar. (C) Cumulative distributions of PPRs in untrained and trained rats. (D) The proximal, intermediate, and distal CA1 subfields (red areas) where we recorded PPRs of ECIII-CA1 synapses. (E) IA training significantly increased the PPRs at ECIII-CA1 synapses in the intermediate and distal regions. A higher ratio suggests a lower release probability of glutamate. The upper insets show representative traces. The number of cells in each group

is shown at the bottom of each bar. (F) Cumulative distributions of PPRs in untrained and trained rats. Vertical and horizontal scale bars represent 50 pA and 50 ms, respectively. Error bars indicate + SEM. *$P < 0.05$, **$P < 0.01$ *vs.* untrained.

Learning-induced synaptic plasticity before and after the retrieval test

To evaluate the potential influence of the memory retrieval test, we compared the data measured before and after the test to data from untrained controls (Fig. 5). Compared to controls, IA training clearly increased the AMPA/NMDA ratios, even before the test (CA3-CA1, $\chi^2_2 = 42.562$, $P < 0.001$, Fig. 5B left. ECIII-CA1, $\chi^2_2 = 24.579$, $P < 0.001$, Fig. 5B right). In contrast, IA training increased the PPRs only after the test (CA3-CA1, $\chi^2_2 = 9.081$, $P = 0.011$, Fig. 5E left. ECIII-CA1, $\chi^2_2 = 15.3$, $P < 0.001$, Fig. 5E right) (Paw-Min-Thein-Oo et al., 2020).

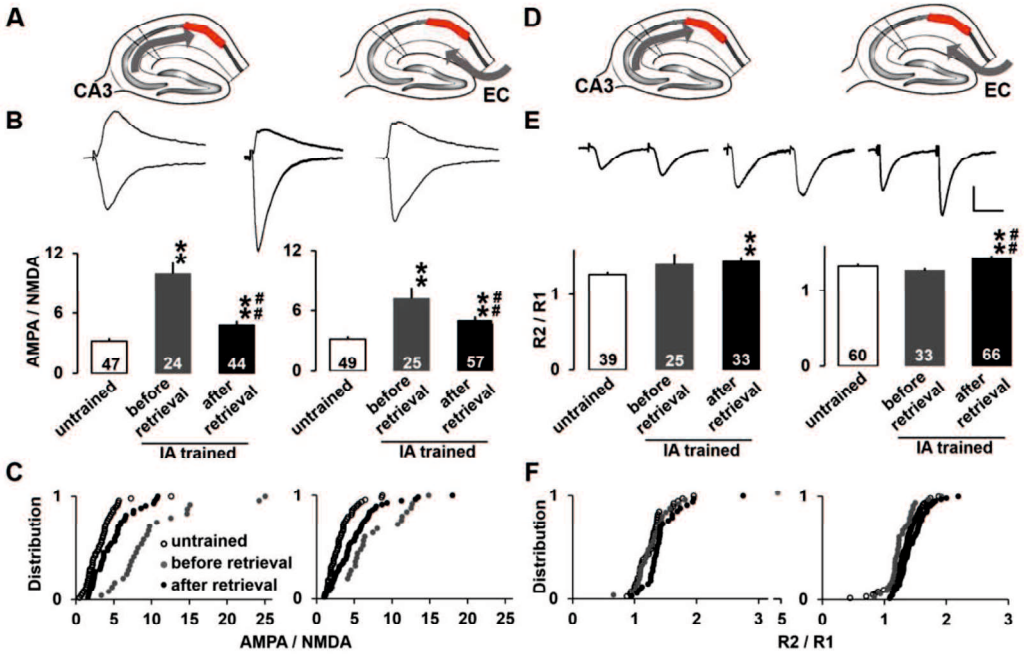

Figure 5. Synaptic plasticity before and after the memory retrieval test. (A) Schema of CA3-CA1 (*left*) and ECIII-CA1 (*right*) inputs in the intermediate CA1 region (arrows

point to the region). (B) IA training increased the mean AMPA/NMDA ratios, both before and after the retrieval test. The upper insets show representative traces at ECIII-CA1 synapses. The number of cells in each group is shown at the bottom of each bar. (C) Cumulative distribution of the AMPA/NMDA ratios. (D) Schema of CA3-CA1 (*left*) and ECIII-CA1 (*right*) inputs in the intermediate CA1 region (arrows point to the region). (E) IA training increased the PPR only after the retrieval test. The upper insets show representative traces at ECIII-CA1 synapses. The number of cells in each group is shown at the bottom of each bar. (F) Cumulative distribution of PPRs. Vertical and horizontal scale bars represent 50 pA and 50 ms, respectively. Error bars indicate + SEM. **P* < 0.05, ***P* < 0.01 *vs.* untrained, ## *P*<0.01 *vs.* before the test.

Table 2. Numbers of animals, slices, and cells used for patch clamp studies

Figures	area	training	animals	slices	cells
	Proximal	–	3	7	28
	Proximal	+	5	10	29
3B	Intermediate	–	7	12	47
	Intermediate	+	9	16	44
	Distal	–	3	6	33
	Distal	+	4	8	36
	Proximal	–	4	8	38
	Proximal	+	5	10	47
3E	Intermediate	–	10	18	49
	Intermediate	+	11	19	57
	Distal	–	3	6	22
	Distal	+	5	9	40
	Proximal	–	5	11	41
	Proximal	+	4	7	34
4B	Intermediate	–	8	14	39
	Intermediate	+	5	9	33
	Distal	–	5	10	33
	Distal	+	4	8	31

	Proximal	–	4	9	27
	Proximal	+	5	10	37
4E	Intermediate	–	10	18	60
	Intermediate	+	11	20	66
	Distal	–	3	6	31
	Distal	+	5	10	40
	Intermediate	–	7	12	47
5B left	Intermediate	+ w/o test	4	8	24
	Intermediate	+	9	16	44
	Intermediate	–	10	18	49
5B right	Intermediate	+ w/o test	3	7	25
	Intermediate	+	11	19	57
	Intermediate	–	8	14	39
5E left	Intermediate	+ w/o test	4	7	25
	Intermediate	+	5	9	33
	Intermediate	–	10	18	60
5E right	Intermediate	+ w/o test	3	7	33
	Intermediate	+	11	20	66

DISCUSSION

In the present study, we found that sub-region specific learning-induced both pre-/post-synaptic plasticity in both CA3-CA1 (Fig. 3B & 4B) and ECIII-CA1 synapses (Fig. 3E & 4E). Learning induced pre-/post-synaptic plasticity in proximal and intermediate regions at the CA3-CA1 synapses, whereas the plasticity was obvious in intermediate and distal regions at ECIII-CA1 synapses. These results suggest proximodistal heterogeneity of pathway-specific plasticity after contextual learning.

Postsynaptic CA3-CA1 plasticity

It is well known that high frequency stimulation of Schaffer's collateral induced LTP at hippocampal CA1 pyramidal neurons (Bliss et al., 1993, Morris 2003), and IA training can mimic the effect of this stimulation to Schaffer's collateral and also occlude subsequent LTP induction indicating that contextual memory formation induces LTP in CA3-CA1 synapses *in vivo* (Whitlock et al., 2006). Selective inhibition of synaptic output from CA3 by using tetanus toxin-based method in transgenic mice has shown that the tri-synaptic input to CA1 is required for rapid one-trial contextual learning, pattern completion-based memory recall, and spatial tuning of CA1 cells (Nakashiba et al., 2008). By combining *in vivo* gene delivery and *in vitro* patch-clamp recordings, we previously demonstrated that contextual learning depends on synaptic delivery of GluA1-containing AMPA receptors at dorsal CA1 synapses (Mitsushima et al., 2011). Moreover, AMPA receptor immobilization approaches further showed that interference of surface diffusion of AMPA receptors markedly impairs learning and synaptic potentiation of Schaffer collaterals and commissural inputs to the CA1 area (Penn et al., 2017). Here we confirmed learning-induced postsynaptic plasticity at CA3-CA1 synapses and its heterogeneity along the proximodistal axis (Fig. 3B). Moreover, our fluctuation analysis showed that the number of AMPA receptor channels increased without changing the cation current per channel (Sakimoto et al., 2019).

Postsynaptic ECIII-CA1 plasticity

As LTP has been successfully elicited in ECIII-CA1 synapses both *in vitro* (Remondes and Schuman, 2003) and *in vivo* (Gonzalez et al., 2016), direct input from EC via TA pathway is believed to play an important role in memory formation process. In spite of making relatively less number of synapses than Schaffer's collateral (Bezaire and Soltesz, 2013), previous studies

have shown some functional roles of temporoammonic projection such as comparisons of ECIII-CA1 inputs conveying current experience with previously stored associations relayed through tri-synaptic pathway (Vago and Kesner, 2008) and the functional interaction between these two pathways like modulation of tri-synaptic pathway inputs by TA pathway (Buzsáki, 2002; Remondes and Schuman, 2002). Some studies even suggested that coincident input from both TA and tri-synaptic pathway generates distal dendritic plateau potential which can promote action potential output mode as burst firing (Takahashi et al., 2009). TA pathway also plays an important role in memory consolidation (Remondes and Schuman, 2004). Chronic stress induces a selective decrease in AMPA receptor-mediated synaptic excitation at ECIII-CA1 synapses, and impaired long-term memory consolidation in Morris water maze test (Kallarackal et al., 2013). Although specific inactivation of ECIII-CA1 synapses in transgenic mice suggested a role for the TA pathway in temporal association memory (Suh et al., 2011), it remained unknown whether training could induce synaptic plasticity. This report was the first to show that learning induced an increase in the AMPA/NMDA ratio, which suggested that postsynaptic plasticity occurred at ECIII-CA1 synapses along the proximodistal axis.

Proximodistal heterogeneity of postsynaptic plasticity

We found proximodistal heterogeneity in plasticity at both CA3-CA1 and ECIII-CA1 synapses. Region-specific plasticity was observed along the proximodistal axis of CA3-CA1 synapses, which conveys integrated information related to both spatial and non-spatial components (Igarashi et al., 2014). The CA3-CA1 inputs showed postsynaptic plasticity in the proximal/intermediate regions, but not the distal region, where object-related information is

mostly processed. These results suggested that the proximal/intermediate CA1 regions played a role in processing integrated information for IA learning.

The TA pathway from the medial EC might convey spatial information to the proximal CA1 region, but the TA pathway from the lateral EC appears to transmit non-spatial, object-related information to the distal CA1 (Witter et al., 1989; Tamamaki and Nojyo, 1995; Igarashi et al., 2014; Knierim et al., 2014). Moreover, there is some interesting controversial issues concerning with role of this direct EC input in spatial memory formation. Although Moser's group suggested that direct ECIII-CA1 input alone is sufficient for establishing and maintaining fundamental properties of CA1 place cells (Brun et al., 2002) and this input is required for accuracy of location-specific firing pattern of CA1 place cells (Brun et al., 2008), Nakashiba et al. in 2008 showed that this direct input is sufficient only for incremental spatial learning but spatial tuning and spatial information required CA3-CA1 input. In our study, we found that IA training significantly increased the AMPA/NMDA ratio at the intermediate/distal, but not the proximal ECIII-CA1 synapses. Although the detailed inputs from medial or lateral EC show distinct heterogeneity in deep *vs* superficial CA1 neurons (Masurkar et al., 2017), TA inputs from the lateral EC might be important in processing non-spatial object-related information for IA learning.

In our experiment, IA training induced postsynaptic plasticity at CA3-CA1 synapses, but not at ECIII-CA1 synapses, in the proximal CA1 region, where spatial information is predominantly processed. In contrast, the training strengthened the ECIII-CA1, but not the CA3-CA1 synapses in the distal CA1 region, where non-spatial, object-related information processing predominantly occurs. However, unlike those two regions, the intermediate CA1 region showed postsynaptic plasticity at both CA3-CA1 and ECIII-CA1 synapses after IA training. The intermediate CA1

region receives TA pathways from both the lateral and medial EC; therefore, many pyramidal neurons seem to integrate spatial, temporal, or object-related information (Sequence cell: Ng et al., 2018). Consequently, it is possible that synaptic plasticity in this region might be necessary for establishing contextual memory by assembling information from Schaffer's collaterals and two TA pathways (Paw-Min-Thein-Oo et al., 2020).

To analyze the functional role of postsynaptic plasticity further, we bilaterally microinjected AP5 into the three CA1 regions. Bilateral microinjections into the proximal or intermediate CA1 regions clearly prevented learning. In contrast, injections into the distal CA1 region attenuated, but did not completely prevent learning. The distal CA1 region might play an important role in non-spatial object-related memory, because in a previous study, bilateral inactivation of distal CA1 neurons clearly impaired performance in a novel object recognition task (Burke et al., 2011; Ito and Schuman, 2012). Taken together, the present and previous results suggest a functional heterogeneity along the proximodistal axis.

Presynaptic CA3-CA1 plasticity

Although most studies suggested that LTP induction increases glutamate release probability (Zakharenko et al., 2002; Emptage et al., 2003; Bayazitov et al., 2007; Enoki et al., 2009; Ahmed and Siegelbaum, 2009; Yang and Calakos, 2013), that may depend on the pattern of LTP induction protocol and the basal release probability level (Schulz et al, 1994). As to the issue, we recently revealed temporal dynamics of the glutamate release probability after IA training: the release transiently increase at 5 min after the training, and then slightly but significantly decreased 60 min after the training (Sakimoto et al., 2019). The longer-term effect of decrease may be due to the feedback system via presynaptic NMDA receptors (Padamsey et al. 2017).

Here we analyzed the PPR 60 min after the training and found proximodistal heterogeneity of learning-induced decrease in glutamate release probability at CA3-CA1 synapses We consistently confirmed the late changes in PPR after training (Fig. 4B, Mitsushima et al., 2013, Sakimoto et al., 2019).

Presynaptic ECIII-CA1 plasticity

Although in mice, ECIII-CA1 synapses showed greater PPRs than CA3-CA1 synapses (Ahmed and Siegelbaum, 2009), in rats, ECIII-CA1 and CA3-CA1 synapses showed similar PPRs (Speed and Dobrunz, 2009; Aksoy-Aksel and Manahan-Vaughan, 2013). We consistently confirmed similar PPRs for both inputs along the proximodistal axis. Moreover, it was previously completely unknown whether presynaptic plasticity occurred after LTP induction or learning. Here, we found that IA training slightly, but significantly enhanced the PPR at ECIII-CA1 synapses, which suggested that a late reduction occurred in the glutamate release probability at 60 min after the training. Like CA3-CA1 synapses, presynaptic NMDA receptor feedback might be responsible for the late reduction in ECIII-CA1 synapse activity (Padamsey et al., 2017).

Proximodistal heterogeneity in presynaptic plasticity

Inputs from the lateral or medial EC showed distinct proximodistal heterogeneity in the presynaptic properties of ECIII-CA1 synapses (Ito and Schuman, 2012). Moreover, a previous study showed that novel-place exposure primarily induced c-fos expression in the distal half of CA1 neurons, which suggested that functional proximodistal heterogeneity was present in that region (Ito and Schuman, 2012). Here, we demonstrated region-specific presynaptic plasticity at

both CA3-CA1 and ECIII-CA1 synapses. IA training significantly induced presynaptic changes at proximal/intermediate, but not distal CA3-CA1 synapses; conversely, it induced presynaptic changes at intermediate/distal, but not proximal ECIII-CA1 synapses. These results provided evidence of pathway-specific presynaptic plasticity along the proximodistal axis. However, postsynaptic receptor desensitization or lateral diffusion might have influenced the PPR (Yang and Calakos, 2013). A recently described optogenetic approach would be necessary to investigate spine-specific presynaptic plasticity after contextual learning. One study showed that the probability of glutamate release from a CA3-engram to a CA1-engram cell was significantly greater than the probability at other types of synapses (Choi et al., 2018).

Changes in release probability can be achieved by various possible mechanisms such as modifications of the release machinery, alterations in calcium influx through voltage-gated calcium channels, or changes in intrinsic membrane excitability of the presynaptic terminal (Yang and Calakos, 2013). cAMP/PKA cascade may play a critical role in pre-synaptic changes at hippocampal mossy fiber-CA3 synapses (Weisskopf et al., 1994; Huang et al., 1994; Castillo et al., 1997; Villacres et al., 1998). Various molecular targets were also proposed to be involved in alteration of release probability such as RIM1α (Calakos et al., 2004), Synaptotagmin1 (Schoch et al., 2002), Rab3 (Wang et al., 1997) and voltage-gated calcium channles (VGCCs) (Kaeser et al.; 2011; Han et al., 2011). One possible explanation for our result, i.e., learning-induced decrease in glutamate release probability is depletion of glutamate vesicle being used up during LTP formation in memory formation, as vesicle refilling speed at central glutamatergic synapses is not fast enough to fill up vesicles replenished through fast recycling pathways (< 1 sec) (Hori et al., 2012).

Synaptic plasticity before and after the retrieval test

Although the pathway-specific pre/post-synaptic plasticity was still unknown in different stages of memory processing, encoding rather than retrieval test is important to induce post-synaptic plasticity (Fig. 5). Further analysis of the temporal dynamics of plasticity revealed that the postsynaptic change was peaked around 10 min after the encoding, but gradually declined (Sakimoto et al., 2019). In contrast, PPR was increased only after the retrieval test, suggesting slower effect on presynaptic plasticity. Detailed temporal dynamics of the presynaptic changes suggested that the presynaptic glutamate release acutely increased at 5 min after the training but decreased at 60 min (Sakimoto et al., 2019).

Unlike consolidation at system level, which takes place several days to weeks after learning, synaptic consolidation can undergo within first few hours after learning (Albo and Gräff, 2018). Although the causal relationship is still unclear, both pre- and postsynaptic plasticity may be necessary for synaptic consolidation of contextual memory, whereas post-synaptic plasticity alone may play an important role in the acquisition of contextual memory (Paw-Min-Thein-Oo et al., 2020).

Conclusion

In the present study, we detected pathway- and region-specific synaptic plasticity after contextual learning. The pathway-specific plasticity appeared to be strongest near the anatomical origin of the pathway; i.e., either the proximal CA1 region, for Schaffer's pathway, or the distal CA1 region, for the TA pathway. We showed that training promoted plasticity at CA3-CA1 synapses in proximal/intermediate CA1 regions, which are responsible for processing spatial information during rapid, one-trial contextual learning. In contrast, the training clearly altered

ECIII-CA1 synapses in the distal/intermediate CA1 regions, which are responsible for processing non-spatial/object-related memories of the context. For both CA3-CA1 and ECIII-CA1 synapses, the learning induced synaptic plasticity in the intermediate CA1 region. This finding suggested that the intermediate region might be important for integrating spatial and non-spatial information from a given context (Paw-Min-Thein-Oo et al., 2020).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CA - cornu amonis
EC - entorhinal cortex
ECIII - layer III of entorhinal cortex
EPSC - excitatory postsynaptic current
D-AP5 - D-2-amino-5-phosphonopentanoic acid
DG - Dentate gyrus
GluA1 - Glutamate A1 subunit
IA - inhibitory avoidance
LTP - long-term potentiation
LTD - long-term depression

NMDA - N-methyl-D-aspartate

PPR - paired-pulse ratio

TA - temporoammonic pathway

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