Dependence of Generation of Hippocampal CA1 Slow Oscillations on Electrical Synapses

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Abstract Neuronal oscillations in the hippocampus are critical for many brain functions including learning and memory. The underlying mechanism of oscillation generation has been extensively investigated in terms of chemical synapses and ion channels. Recently, electrical synapses have also been indicated to play important roles, as reported in various brain areas *in vivo* and in brain slices. However, this issue remains to be further clarified, including in hippocampal networks. Here, using the completely isolated hippocampus, we investigated *in vitro* the effect of electrical synapses on slow CA1 oscillations (0.5 Hz–1.5 Hz) generated intrinsically by the hippocampus. We found that these oscillations were totally abolished by bath application of a general blocker of gap junctions (carbenoxolone) or a specific blocker of electrical synapses (mefloquine), as determined by whole-cell recordings in both CA1 pyramidal cells and fast-spiking cells. Our findings indicate that electrical synapses are required for the hippocampal generation of slow CA1 oscillations.

Keywords Electrical synapse · Hippocampus · Oscillation · CA1 · Mefloquine · Carbenoxolone

Introduction

Hippocampus-dependent brain functions rely on neuronal oscillations in hippocampal networks [1–4]. To understand the neural mechanism underlying the generation of oscillation, previous studies have extensively investigated the roles of various types of chemical synapses and ion channels [5–9]. More recently, growing attention has also been paid to the contribution of electrical synapses, i.e., Cx36 (connexin 36) gap junctions, which bridge neuronal membranes and allow electrical coupling and the transfer of small molecules between neurons [10]. Using a specific blocker of Cx36 gap junctions, the general blocker of gap junctions containing Cx36 and other connexins (e.g., Cx43 between glial cells), as well as Cx36-knockout mice, numerous studies have demonstrated the contribution of electrical synapses to neuronal oscillations in different brain regions including the hippocampus [11, 12]. In studies carried out in brain slices to assess neuronal oscillations induced by artificial stimulation (e.g., drug treatment), as well as in the brain *in vivo* to assess oscillations generated intrinsically by neural circuits, transmission at Cx36 gap junctions or Cx36-containing gap junctions has been commonly found to be capable of enhancing neuronal oscillations at relatively low frequencies, in particularly, theta (5 Hz–10 Hz) [13–16] and gamma (30 Hz–80 Hz) oscillations [17–21]. Moreover, it has been shown that network activity in the theta or gamma bands can be synchronized by transmission at Cx36 gap junctions or Cx36-containing gap junctions [12, 18, 21, 22]. In addition to theta and gamma

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oscillations, a few studies have also suggested an effect of Cx36 gap junctions on high-frequency oscillations (>100 Hz) [23–25]. Although electrical synapses have been indicated to be important for the generation of neuronal oscillations in various brain areas including the hippocampus, the functional role remains to be further investigated. A better understanding of this issue might be obtained from more direct pharmacological studies of the contribution of electrical synapses to network oscillations, given that in previous *in vivo* studies, electrical synapses or gap junctions may not have been totally blocked by drug treatment (e.g., with i.p. injection to avoid animal death) or be totally inactivated in Cx36-knockouts (due to possible compensatory factors), and in brain slices, neuronal oscillations cannot usually be generated by a local network and are induced by artificial stimulation (drug application [15, 16]).

In our previous report in the completely-isolated hippocampal formation *in vitro*, we showed that CA1 oscillations at a low frequency (0.5 Hz–1.5 Hz) can be intrinsically generated (i.e., with no artificial stimulation) [26]. In this study, by taking advantage of the completely isolated hippocampus, which allows more direct pharmacological manipulation (compared with *in vivo* experiments), we have investigated the effect of hippocampal electrical synapses on the generation of CA1 oscillations.

Materials and Methods

Electrophysiological Recording in the Isolated Hippocampus

All animal procedures were performed in accordance with the Animal Care and Use Committee of East China Normal University (reference number: AR201404015). As described previously [26], we used Sprague-Dawley rats from postnatal day 14 (P14) to P18. To isolate the hippocampal formation, animals were anaesthetized with pentobarbital (i.p., 80 mg/kg; unless otherwise specified), and after decapitation the brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF), which contained (in mmol/L) 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, and 11 glucose. One minute later, the brain was transferred to a cold plate, and the hemispheres were separated with a razor blade. The cerebellum, brain stem, and thalamus were moved aside to expose the hippocampal formation. The hippocampus was then completely isolated from the surrounding cortical issue with a small metal hook. This procedure was completed within 0.5 min–1 min. The entire hippocampus was then incubated in aCSF at room temperature for >1 h before electrophysiological recording.

The recording temperature in the submerged chamber was maintained at 28 °C–30 °C. Neurons were visualized under an Olympus microscope (DX50WI, Tokyo, Japan). Perforated whole-cell recordings with amphotericin B were made as described previously [26]. Patch pipettes with a 2.5 µm–3.0 µm tip were pulled from borosilicate glass tubing (Kimble Glass Inc., Queretaro, Mexico); they had a resistance of 3.0 MΩ–4.5 MΩ. The internal solution contained (in mmol/L) 136.5 K-gluconate, 17.5 KCl, 9.0 NaCl, 1.0 MgCl₂, 10.0 HEPES, 0.2 EGTA, and amphotericin B (0.3 mg/mL). A small amount (0.5 mg/mL–0.8 mg/mL) of glass beads (5 µm–15 µm in diameter; Polysciences, Inc., Warrington, UK) were included in the internal solution to maintain precipitate-free solution in the pipette tip [27]. The recording pipette was advanced into the hippocampus with a motor-driven manipulator (Siskiyou MMX7630, Siskiyou Corp., OR). Signals were acquired with patch-clamp amplifiers (Axopatch 700B, Axon Instruments, CA) and sampled at 5 kHz by a data acquisition card (Digidata 1440, Axon Instruments), with a 2 or 5 kHz low-pass filter. Liquid junction potentials (–13 mV) were corrected. Recordings with resting potentials between –65 mV and –75 mV were included for further analysis. The series resistance (not compensated for recording) was 48 ± 15 MΩ (mean $\pm$ SD). Unless otherwise specified, no holding currents were applied during current-clamp recording.

CA1 pyramidal cells (PCs) were recorded from the cell layer, and CA1 fast-spiking (FS) cells were recorded from the stratum oriens. To determine the cell type (PCs or FS cells), we evaluated the firing properties at depolarizing potentials (i.e., low firing rates with adaptation in PCs and sustained firing at high rates in FS cells) as well as spike half-widths (PCs, 3.2 ± 0.8 ms; FS cells, 1.7 ± 0.5 ms), as described in our previous report [26]. In further analysis of randomly-selected recordings, we found that putative CA1 PCs had a spike threshold (absolute value) of -53.6 ± 6.0 mV ($n = 35$) and spike amplitude (from spike threshold) of 54.2 ± 8.1 mV, both of which were significantly different from those found in putative FS cells ($n = 24$; spike threshold, -49.0 ± 5.4 mV, $P = 0.021$ unpaired *t*-test; spike amplitude, 41.0 ± 5.7 mV, $P = 0.041$).

Local field potentials (LFPs) were recorded in CA1 100 µm distal to the cell layer (in the stratum radiatum), using patch pipettes filled with aCSF, and signal acquisition was similar to whole-cell recording.

Electrophysiological Recording in Hippocampal Slices

Young rats (the same age as those for the isolated hippocampus) were initially anaesthetized with sodium pentobarbital (i.p., 80 mg/kg;). After decapitation, the

Fig. 1 CBX abolished V_m oscillations in CA1 PCs. **A** Two example PCs (from different hippocampal formations) showing V_m oscillations recorded in the absence and presence of CBX (action potentials were partially truncated; dashed lines, resting potentials). **B**, **C** Summary of all experiments as illustrated in **A** ($n = 15$), with V_m amplitudes normalized to the firing threshold of each cell and indicated by the gray scale (action potentials omitted in **B**; right, recordings 10–15 min after drug washout), and with time course plot (**C**) for the changes in oscillation frequencies (averaged across population data) induced by CBX (starting at time 0) and drug washout. **D** For the data shown in **B** and **C**. Left, firing probability of V_m oscillations (circles, individual data; bars, mean $\pm$ SEM); right, firing thresholds (from resting potential) (for data points at right: dots connected by lines, data from the same cell; dots with bars, mean $\pm$ SEM) recorded before and 10–15 min after CBX treatment. n.s., not significant.

brain was rapidly removed and placed in ice-cold aCSF (as used for the isolated hippocampus). Coronal slices (400 μ m thick) containing the dorsal hippocampus were cut on a vibratome (Leica, VT1000 S, Wetzlar, Germany), and were then incubated at room temperature for >1 h before electrophysiological recording. The temperature in the submerged recording chamber was maintained at 28 $^{\circ}$ C–30 $^{\circ}$ C, and perforated whole-cell recordings from CA1 PCs were conducted similar to that described for the isolated hippocampus. Bipolar tungsten electrodes were placed in the stratum radiatum for stimulation, which was produced by a pulse generator (Master-8; A.M.P.I., Jerusalem, Israel) through a stimulus isolator (ISO-Flex; A.M.P.I.). Inhibitory postsynaptic currents (IPSCs, recorded at -65 mV in voltage-clamp mode) were evoked at 0.07 Hz in the presence of bath-applied 6,7-dinitroquinoxaline-2,3-dione (DNQX, 40 μ mol/L) and D-2-amino-5-phosphonopentanoate (D-AP5, 50 μ mol/L) to prevent excitatory synaptic transmission.

Data Analysis and Statistics

Firing thresholds were defined as the membrane potential (V_m) value at which $dV/dt > 10$ V/s. Fourier power spectra of LFPs were calculated from randomly-selected 2-min recordings using MatLab (MathWorks Inc., MA). Unless otherwise specified, statistical significance was determined using the paired Student's *t*-test (for recordings before and after drug treatment), and average values are presented as the mean $\pm$ SEM.

Results

General Blocker of Cx36-Containing Gap Junctions Abolished CA1 Oscillations

As demonstrated in our recent report [26], spontaneous oscillations that were generated intrinsically at a low frequency (0.5 Hz–1.5 Hz) occur in CA1 neurons in the completely isolated hippocampal formation as used in the

Fig. 2 CBX abolished V_m oscillations in CA1 FS cells. **A–D** Data as in Fig. 1, but for FS cells ($n = 10$).

present study. In such an *in vitro* complete hippocampal formation, our whole-cell recordings show that such spontaneous CA1 oscillations consist primarily of membrane-potential (V_m) depolarizations, which are usually suprathreshold for generating neuronal firing, as shown in our previous study [26] and below in this study.

To examine the possible effect of electrical synapse-mediated activity on the intrinsically produced CA1 oscillations, we first recorded from CA1 PCs and used the general blocker of gap junctions carbenoxolone (CBX) to prevent transmission at electrical synapses (i.e., Cx36) as well as connexins between glial cells (e.g., Cx43) [28, 29]. We found in all CA1 PCs ($n = 15$; recorded in current-clamp mode without holding currents) that V_m oscillations were completely blocked when CBX (150 $\mu\text{mol/L}$) was applied in the bath (Fig. 1).

Our previous studies have shown that V_m oscillations of CA1 PCs are synchronized with CA1 interneurons and suggest an interneuron-based mechanism for the hippocampal generation of CA1 oscillations [26]. We then determined whether V_m oscillations of CA1 interneurons were also susceptible to the blocking effect of CBX. In another

group of experiments, current-clamp recordings were made from CA1 FS cells in the stratum oriens, in which we again found that V_m oscillations of all FS cells recorded disappeared during CBX treatment (Fig. 2), similar to the findings from PCs (Fig. 1). The absence of V_m oscillations in both CA1 PCs and FS cells after CBX application was consistent with our LFP recordings from CA1 (<100 μm distal to the cell layer), in which spontaneous LFP oscillations were totally blocked by CBX (Fig. 3).

In the above experiments from PCs and FS cells, we further found that after drug washout, the V_m oscillations of CA1 neurons gradually re-emerged, suggesting the capacity of the hippocampus to regenerate CA1 oscillations in the absence of external stimulation. However, the frequency of these re-emerging oscillations did not recover to the baseline level (before drug application), even 20 min after drug washout (Figs. 1C, 2C), possibly because the regeneration required a longer period for recovery, and/or CBX was difficult to thoroughly wash out, as previously reported [30]. Nonetheless, the partially-regenerated V_m oscillations were usually suprathreshold, and both the firing probabilities of V_m oscillations and firing thresholds were

Fig. 3 No CA1 oscillations occurred in LFP recordings during CBX or mefloquine treatment. **A, B** Example LFP recordings showing the absence of CA1 oscillations during CBX (**A**; inset, LFP at higher temporal resolution) or mefloquine application (**B**). **C** Fourier power spectra of LFPs recorded before and after CBX or mefloquine application, calculated from data shown in **A** and **B**.

unchanged, as compared with the recordings before CBX treatment (Figs. 1D, 2D).

Selective Blocker of Cx36 Electrical Synapses Abolished CA1 Oscillations

The findings with CBX treatment suggested that the CA1 oscillation in PCs (Fig. 1) and FS cells (Fig. 2) required transmission at Cx36-containing gap junctions. In the subsequent experiments, we used a selective Cx36 blocker (mefloquine) [31] to assess more directly the contribution of electrical synapses to CA1 oscillations. In such experiments, we also found that bath application of mefloquine (25 $\mu\text{mol/L}$) totally blocked the V_m oscillations of CA1 neurons in all recordings from PCs (Fig. 4) and FS cells (Fig. 5), as well as in LFP recordings in CA1 (Fig. 3). These results provided more direct evidence for the essential role of electrical synapses in the hippocampal generation of CA1 oscillations.

In addition, similar to the findings with CBX treatment, V_m oscillations regenerated after mefloquine washout (Figs. 4, 5), and the frequency did not recover to the baseline level within 20 min after washout. Also, such partially-regenerated V_m oscillations (in both PCs and FS

cells) were usually suprathreshold and had a firing probability and firing threshold similar to those recorded before drug application (Figs. 4D, 5D).

Synchronization of Partially-Regenerated CA1 V_m Oscillations After Drug Washout

We have reported that CA1 V_m oscillations in the completely isolated hippocampus are strongly synchronized among neurons [26]. In the present study, the recordings after drug washout showed a gradual re-emergence of CA1 V_m oscillations, although the frequency (within 20 min after washout) was significantly lower than that before drug application. We next asked whether the partially-regenerated V_m oscillations were also synchronized between CA1 neurons. To answer this, we made paired whole-cell recordings from CA1 neurons (mainly PCs, because the probability of achieving paired recordings from FS cells was low, and we had already shown that CA1 V_m oscillations are highly synchronized between PCs and FS cells [26]), and found that after drug (CBX or mefloquine) treatment, the regenerated CA1 V_m oscillations were highly synchronized between various neurons within 15 min after washout, similar to that before drug

Fig. 4 Mefloquine abolished V_m oscillations in CA1 PCs. **A–D** Data are displayed as in Fig. 1, for recordings from CA1 PCs but with mefloquine treatment ($n = 11$).

application (Fig. 6). These results indicated that the gradually-regenerating CA1 oscillations were synchronized in CA1 networks, and this synchronization could in turn be responsible for further regenerating CA1 oscillations.

No Effect of CBX or Mefloquine on Inhibitory Synaptic Transmission

Our previous report in the completely isolated hippocampus has shown that the intrinsically generated CA1 oscillations are largely dependent on GABAergic synaptic transmission in the hippocampus [26]. In this study, with the findings of the blocking effects of CBX and mefloquine on CA1 oscillations, we further determined whether such drug treatment influenced GABAergic synaptic transmission in CA1 neurons. We therefore made perforated whole-cell recordings from CA1 PCs in hippocampal slices to measure the inhibitory postsynaptic currents (IPSCs) evoked by Schaffer collateral stimulation (recordings at -65 mV in voltage-clamp mode, with $40 \mu\text{mol/L}$ DNQX and $50 \mu\text{mol/L}$ D-AP5 in the bath solution). We found no change in the stimulation-evoked IPSCs of CA1 PCs after applying CBX or mefloquine (Fig. 7).

Discussion

In the completely isolated hippocampus, we investigated the role of electrical synapses in the hippocampal generation of low-frequency CA1 oscillations. We found that the V_m oscillations of both CA1 PCs and FS cells were totally abolished when Cx36 and astrocyte connexins were blocked by CBX, or when Cx36 was specifically blocked by mefloquine. These findings suggest an electrical synapse-based mechanism for the hippocampal generation of CA1 neuronal oscillations.

Three major types of neuronal oscillation occur in the *in vivo* hippocampus, as commonly demonstrated by LFP recording [11, 32, 33]: theta rhythms (~ 5 Hz–10 Hz, also suggested to have a broader range of 3 Hz–12 Hz), gamma rhythms (~ 30 Hz–80 Hz), and sharp wave–ripples (~ 110 Hz–250 Hz). In the completely isolated hippocampus, we found spontaneous CA1 oscillations (in both LFPs and V_m changes) at 0.5 Hz–1.5 Hz, considerably slower than those found *in vivo* (e.g., compared with theta bands). The clearly slowed CA1 oscillations recorded in our experiments may be attributed to a largely impaired capacity of the *in vitro* hippocampus to produce network activity, for example, due to the absence of extra-

Fig. 5 Mefloquine abolished V_m oscillations in CA1 FS cells. **A–D** Data are displayed as in Fig. 4, but for recordings from FS cells with mefloquine treatment ($n = 9$).

hippocampal inputs as well as other *in vivo* machinery or conditions for oscillation generation. However, these *in vitro* CA1 oscillations may be analogous to theta oscillations, because V_m changes occurring at theta bands measured by *in vivo* whole-cell recording from CA1 PCs have also been found to consist primarily of large-amplitude V_m depolarizations (often able to generate neuronal firing) [34–36], which are similar to the slow CA1 oscillations shown in this study (Fig. 1).

No other CA1 oscillations (e.g., at higher frequencies comparable to gamma or ripple bands) were found in the isolated hippocampus, as indicated by the power spectra of CA1 LFP activity. On the other hand, our analysis of whole-cell recording data detected oscillatory spike activity that occurred at a periodicity similar to that of V_m changes (Fig. S1), consistent with the high probability of oscillatory V_m depolarizations for generating neuronal firing (Figs. 1D, 2D, 4D, 5D).

In the hippocampus and other brain areas, the effects of electrical synapses on the generation of neuronal oscillations have been investigated in both *in vivo* brains and slices. However, this issue remains to be further clarified, for example, given that in *in vivo* experiments drug

treatment may not be sufficient to completely block electrical synapses or gap junctions, and in *in vitro* experiments neuronal oscillations are usually produced by artificial stimulation but not intrinsically by a neural network [15, 16]. In our experiments in the completely isolated hippocampus, we have shown the presence of slow (0.5 Hz–1.5 Hz) V_m oscillations that were generated intrinsically in CA1 neurons. In addition, such a slow CA1 oscillation occurred when animals were anaesthetized with different drugs, such as pentobarbital and ketamine (Fig. S2A), and even occurred in hippocampus isolated from adult animals (Fig. S2B). We adopted the complete hippocampus isolated from young rats to evaluate the contribution of electrical synapses to hippocampal neuronal oscillations. With drug treatment in such *in vitro* recordings, surprisingly we found that the slow CA1 oscillations (in V_m changes, LFPs, and neuronal firing) were totally abolished by blocking electrical synapses or gap junctions with mefloquine or carbenoxolone. These results indicate that electrical synapses are necessary rather than partially responsible for generating these slow oscillations, a neuronal mechanism of neuronal oscillations for which little previous evidence is available.

Fig. 6 Partially-regenerated CA1 oscillations after drug washout were highly synchronized. **A, B** Paired whole-cell recordings from CA1 neurons showing highly synchronized V_m oscillations before and within 10–15 min after CBX (A) or mefloquine treatment (B). **C** Cross-correlograms between V_m oscillations recorded before (left) and within 10–15 min after (right) CBX treatment ($n = 4$ pairs) or mefloquine treatment ($n = 4$ pairs) (grey, results of all individual neuronal pairs; black, results averaged across all data from both CBX and mefloquine experiments). **D** Summary of the peak values of cross-correlograms for the data shown in C (circles, CBX; squares, mefloquine). In the data shown in C and D, 1 PC and 1 FS cell were recorded in two of the neuronal pairs, and all other paired recordings were from PCs.

Fig. 7 No change in IPSCs after CBX or mefloquine treatment. **A** Two examples of CA1 PCs recording in hippocampal slices (with bath application of 40 $\mu\text{mol/L}$ DNQX and 50 $\mu\text{mol/L}$ D-AP5) showing the average IPSCs evoked by Schaffer collateral stimulation in the absence (gray dashed lines) and presence (black solid lines) of CBX (left) or mefloquine (right). **B, C** Summary of all experiments as in A ($n = 5/\text{group}$), with IPSC amplitude before drug application plotted against the values after drug application (B), and IPSC amplitudes (normalized to the values before drug application in individual data) averaged over population data (C).

Mefloquine has been reported to be a specific blocker of Cx36 gap junctions (i.e., electrical synapses between neurons). On the other hand, there are some non-specific

blocking effects of mefloquine on certain ion channels, such as L-type Ca^{2+} channels, delayed rectifier channels, and ATP-sensitive K^{+} channels, but the IC_{50} values for

blocking these channels are 3- to 15-fold higher than those required for Cx36 blockade [31, 37–39]. In our study, a dose of 25 $\mu\text{mol/L}$ mefloquine was used to block Cx36; this has been reported to exert only weak effects on ion channels [31]. As for chemical synapses, although we found no effect on GABAergic synaptic transmission (Fig. 7), previous reports have indicated that this concentration can result in small changes of excitatory synaptic transmission in brain slices as a slight ($< 5\%$) reduction of evoked field excitatory postsynaptic potentials [31]. However, although some weak non-specific effects may be exerted on excitatory chemical synapses as well as some ion channels in our recordings with the use of 25 $\mu\text{mol/L}$ mefloquine, our other findings suggest that such effects may not be able to cause the disappearance of CA1 oscillations. First, in a recent report in the completely isolated hippocampus [26], we have examined the dependence of CA1 V_m oscillations at the level of hippocampal network excitation by partially blocking AMPA receptors with a low dose of DNQX, and showed that CA1 V_m oscillations are still generated even with a 35%–45% reduction of AMPA receptor-mediated EPSPs. Second, in an unpublished study in this *in vitro* hippocampal formation, we also found little change in CA1 V_m oscillations when hyperpolarization-activated (I_h) channels are blocked. Moreover, the disappearance of CA1 oscillations in the presence of CBX, a drug that blocks transmission at Cx36 and other gap junctions [28, 40] but without the same non-specific actions of mefloquine on chemical synapses and ion channels, further reduces the possibility that the blocking effects of mefloquine on CA1 oscillations resulted from its non-specific effects. Nonetheless, the specificity of the contribution of electrical synapses to neuronal oscillations remains to be further examined, for example, using Cx36-knockout mice.

One explanation of the dependence of the CA1 oscillation on Cx36 electrical synapses is the requirement of electrical coupling between interneurons for oscillation generation. It is known that Cx36 is highly expressed in interneurons [21, 41, 42]. In addition, both *in vivo* and *in vitro* studies have demonstrated that interneurons in a local network are critical for producing neuronal oscillations [5, 6, 43–46]. In the completely isolated hippocampus, we have also found that GABAergic transmission is required for the intrinsically-generated CA1 oscillations [26]. Thus, we propose that electrical transmission at Cx36 electrical synapses between interneurons may be essential for the hippocampal generation of neuronal oscillations.

Also, we suggest that Cx36 between hippocampal PCs is also likely involved in the hippocampal generation of CA1 oscillations, given the presence of Cx36 in PCs reported in more recent studies [41, 42, 47]. In addition, the interaction of activity in PCs and interneurons has been reported to be

critical for producing neuronal oscillations, including in the hippocampal circuit [43, 48, 49].

Previous studies *in vivo* and in brain slices have demonstrated the contribution of electrical synapses to oscillation generation in various brain regions including the hippocampus, particularly with respect to theta and gamma oscillations [11, 12]. In this study, we further addressed this issue by taking advantage of the *in vitro* complete hippocampus, in which neuronal oscillations can be intrinsically generated. Our pharmacological and electrophysiological experiments indicate the requirement of electrical synapses for the hippocampus to generate a slow (0.5 Hz–1.5 Hz) V_m oscillation in both CA1 PCs and CA1 FS neurons. These findings provide new insights into the hippocampal mechanism for oscillation generation on the basis of electrical synapses, as well as hippocampus-dependent functions such as learning and memory.

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