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Inducing theta oscillations in the entorhinal hippocampal network in vitro

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Abstract

The hippocampal theta rhythm emerges as rhythmic and synchronized activities among the hippocampus and hippocampus-associated brain regions during active exploration, providing a potential means for inter-regional communication. However, after decades of research, the origins of the theta rhythm remain elusive, at least partly due to the difficulty in recording from all three essential regions for theta generation, namely the hippocampus itself, the septum, and the entorhinal cortex. For this reason, we established an in vitro theta model in a septo-entorhinal-hippocampal brain slice tri-culture system by pairing septal cholinergic inputs with hippocampal local activities. Our study shows that the local entorhinal cortical circuit may play an active and critical role in hippocampal theta rhythm generation. Our study also reveals a potential mechanism for theta rhythms to emerge as the functional results of dynamic interactions among the septum, hippocampus, and the entorhinal cortex, in the absence of clear pace makers.

Keywords

Entorhinal cortex; Hippocampus; Theta oscillation; Septum; Cholinergic transmission

Introduction

Hippocampal theta oscillations likely play critical roles in higher brain functions, including spatial and episodic memory formation (Buzsaki 2002, 2005; Buzsaki and Moser 2013; Hasselmo 2005; Winson 1978). At the circuitry level, they may coordinate activities among hippocampus-associated brain regions, including entorhinal cortex and neocortex (Battaglia et al. 2011). At the cellular level, they may provide temporal references for active place cells in the hippocampus and, thus, provide a potential mechanism for temporal coding of the relationships between locations for spatial memories or events for episodic memories (Burgess and O'Keefe 2011; Buzsaki 2005; Buzsaki and Moser 2013; Dragoi and Buzsaki 2006; Hasselmo 2005; Mizuseki et al. 2009). Besides the hippocampus itself, two other brain regions, the septum and the entorhinal cortex (EC), are considered essential for in vivo

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hippocampal theta generation (Buzsaki 2002; Stewart and Fox 1990); however, after decades of research, the origins of theta oscillations remain elusive.

Hippocampal inputs from the septum were originally thought to be the pacemakers of hippocampal theta oscillations, because septal lesion or inactivation abolishes hippocampal theta, and rhythmic activities can be recorded from septal neurons (Gogolak et al. 1968; Green and Arduini 1954; Petsche et al. 1968; Stewart and Fox 1990; Stumpf et al. 1962). Although numerous studies have proven that the septal inputs are essential for in vivo hippocampal theta generation, direct evidence to support the notion that hippocampal theta rhythm is directly paced by the *rhythmic* inputs from the septum is lacking (Stewart and Fox 1990). Studies in behaving animals have revealed that the activities of septal putative GABAergic and cholinergic neurons were too variable to account for the hippocampal theta rhythmicity (King et al. 1998), where cells were classified according to the spike waveforms from extracellular recordings and the classification was considered as indirect and not unambiguous by the authors and others (Dragoi et al. 1999). After the blockade of septal inputs to the hippocampus, the in vivo hippocampus was still able to generate theta oscillations with concurrent excitation (via carbachol or glutamate) and disinhibition (via picrotoxin) of the hippocampus (Colom et al. 1991; Heynen and Bilkey 1991). In vitro bath-applied cholinergic agonists (e.g., carbachol) can induce different types of oscillatory activities including theta-like oscillations in acute hippocampal slices, depending on the concentration of the agonist (Fellous and Sejnowski 2000; Fischer et al. 1999, 2002). Theta oscillations can even be observed in the isolated whole-hippocampus preparation in the absence of any external inputs or drug application (Goutagny et al. 2009). All of these studies suggest that the local hippocampal circuit is capable of generating theta-like oscillations without rhythmic septal inputs. Recent optogenetic studies have also shown that changing the frequency of septal cholinergic firing does not result in a significant change of hippocampal theta frequency (Dannenberg et al. 2015; Vandecasteele et al. 2014). Although septal GABAergic inputs could pace hippocampal activities (Dannenberg et al. 2015), it is difficult to directly prove that they are the pacemakers underlying theta rhythm.

Aside from the septum, another essential brain region for in vivo hippocampal theta generation is the EC (Bragin et al. 1995; Brankack et al. 1993; Kamondi et al. 1998; Mitchell and Ranck 1980; Stewart et al. 1992; Ylinen et al. 1995). While the septum is assumed to provide the major inhibitory inputs to the hippocampus, the EC is assumed to provide the major rhythmic excitatory inputs to the hippocampus through the perforant/temporoammonic (PP/TA) pathway to generate the largest theta current in stratum lacunosum-moleculare (slm) (Buzsaki 2002; Kamondi et al. 1998; Ylinen et al. 1995). Surprisingly, very little is known about the origin of EC theta rhythm. Theoretically, EC theta could originate in the EC local circuit or be entrained by either rhythmic septal inputs or rhythmic hippocampal inputs. To test these hypotheses, we established an in vitro theta model that incorporates the septum, EC, and the hippocampus. Our data here suggested that the EC local network may play an active and critical role in hippocampal theta generation.

Materials and methods

Animals and chemicals

Wild-type C57Bl6 mice and cholineacetyltransferase (ChAT)-Cre transgenic mice (of either sex) were originally purchased from Jackson Laboratory and bred at NIEHS. Mice were used for slice culture from post-natal day 6-8. The pups were housed with the dam under normal light/dark cycle. All procedures were approved and performed in compliance with NIEHS/NIH Humane Care and Use of Animals in Research protocols. Unless otherwise indicated, general chemicals were obtained from Sigma, and culture media were from Sigma or Invitrogen.

Co-culture slice preparation

Slice cultures were prepared as previously described (Gu et al. 2012), which was adapted from Stoppini et al. (Stoppini et al. 1991). Brain slices of 350 μm were cut with a vibratome (Leica, VT1000S). The detachable parts of the vibratome and surgical instruments for dissecting brains were all autoclaved. Briefly, mice (6–8 days old) were anaesthetized with isoflurane and decapitated. Brains were quickly removed into ice-cold cutting medium (MEM supplemented with HEPES 25 mM, 10-mM Tris-base, 10-mM glucose, and 3-mM MgCl_2 , pH 7.2). Horizontal entorhino-hippocampal slices [350- μm -thick, corresponding to Figure 143–150 of the Paxinos mouse brain atlas (Paxinos and Franklin, 2013)] and coronal septal slices were cut in cutting medium. As shown in Fig. 1a, the hippocampus, with EC attached, and medial septum tissues were then dissected out from the whole-brain slices and placed next to each other on 6-well polyester Transwell inserts (Corning) that were prefilled with 1.2-ml culture medium, which was prepared as a 2:1 mixture of Basal Medium Eagle (Sigma) and Earle's Balanced Salts Solution (Sigma) and supplemented with (in mM) 20 NaCl, 5 NaHCO_3 , 0.2 CaCl_2 , 1.7 MgSO_4 , 48 glucose, 26.7 HEPES, 10-ml/l penicillin–streptomycin (Invitrogen), insulin (1.32 mg/l) (Sigma), and 5 % horse serum (Invitrogen), with pH 7.2. The slices were stored in a CO_2 incubator at 34 °C and fed twice a week with a half change of media until being used for experiments. The electrophysiological experiments were done after 3–4 weeks of in vitro culturing.

Virus infection

All of the AAV viruses were packaged with serotype 9 helper at the Viral Vector Core facility at NIEHS. AAV serotype 9 helper plasmid was obtained from James Wilson at the University of Pennsylvania. The AAV vector with synapsin promoter (Addgene #26972) and ChR2 (Addgene #20297) plasmid were obtained from Karl Deisseroth at Stanford University (Witten et al. 2010). PmTurquoise2-N1 was a gift from Dorus Gadella (Addgene plasmid # 60561) (Goedhart et al. 2012) and was, subsequently, subcloned into an AAV vector under the synapsin promoter. The day after culturing, AAV viruses (5 nl) were microinjected into the desired areas with a Drummond “Nanoject” micro injector (Drummond Scientific). Protein expression can usually be detected approximately 1 week after virus infection.

Field-potential and whole-cell patch-clamp recordings

After 3 weeks of in vitro culturing, co-cultured slices were cut out from the inserts with the Transwell membrane attached and put into a submerged chamber, continuously perfused with 95 % O₂/5 % CO₂ balanced ACSF (in mM, 122 NaCl, 2.5 KCl, 1.3 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, 25 glucose) at a rate of 2 ml/min. The polyester membrane is transparent and does not interfere with electrophysiology experiments or confocal imaging. Field-potential or whole-cell patch-clamp recordings were performed under guidance of IR-DIC optics using a multiclamp 700B amplifier (Axon Instruments) with glass pipettes filled with either ACSF for field-potential recordings or an internal solution containing (in mM) 130 potassium gluconate (KGluc), 2 MgCl₂, 3 MgATP, 0.3 Na₂GTP, 10 KCl, 10 HEPES, and 1 EGTA, with pH ~7.2–7.3 and osmolarity ~280–290 mOsm for whole-cell patch-clamp recordings. Data were digitized with a Digi-data 1550, collected with Clampex and analyzed with Clampfit. Excitatory post-synaptic currents (EPSCs) were recorded at –60 mV under voltage clamp. Inhibitory postsynaptic currents (IPSCs) were recorded at 0 mV under voltage clamp. Excitatory post-synaptic potentials (EPSPs) were recorded at 0 pA under current clamp.

Synaptic responses were evoked by electrically stimulating the Schaffer collateral (SC) pathway in the CA1 stratum radiatum layer with a Grass Stimulator (Glass Instruments) coupled with current isolators. The stimulation intensity was usually 1–10 μ A for 0.1 ms. To selectively activate septal cholinergic inputs optogenetically, light-sensitive ChR2 was selectively expressed in cholinergic neurons by microinjecting a Cre-inducible AAV containing a double floxed inverted ChR2 (fused with mCherry for visualization) to the septal tissue from ChAT-Cre transgenic mice (Gu et al. 2012; Witten et al. 2010). Cholinergic terminals expressing ChR2 and mCherry were visualized with 543-nm light and activated with 488-nm light (20 ms) through a 40 $\times$ objective with an Andor spinning disk confocal microscope (Andor technology). The cholinergic projections could also be activated through a traditional stimulating electrode near the juncture of septum, EC, and the hippocampus, as shown in Fig. 1c.

Confocal microscopy

To image the fluorescent protein expression patterns, the cultured slices were cut out from the inserts with the Transwell membrane attached, rinsed with cold PBS, and fixed in 4 % PFA at 4 °C for 30 min. After thoroughly rinsed of PFA, the slices were briefly dried and mounted on coverslips and imaged under a confocal microscope with a Zeiss LSM710 operation system. Tile scans under a 20 $\times$ objective were generally collected to obtain high-resolution images of the whole slices. The images shown in Fig. 1b were z-stacked with approximately 50- μ m thickness.

Statistics

For field-potential recordings, the theta power and theta phase were analyzed with NeuroExplorer. The peak power from individual recordings was used to compare the theta power between groups of treatments. The peak power for an individual recording was obtained by averaging peaks from five consecutive traces immediately before and 10 min after pairing, unless otherwise specified. The theta phase analysis always used CA1 slm

theta as the reference and the troughs as 0°. For whole-cell recordings, the amplitudes of SC stimulation-induced synaptic responses were analyzed with Clampfit, and graphs were drawn with Excel. The amplitudes were normalized to the mean of the 10 min baseline recording before the cholinergic pairing protocol. Percent changes were calculated by comparing with the average of 10 min baseline recording. Values were presented as mean $\pm$ sem. Two-tailed Student *t* tests with equal variance or unequal variance where appropriate was performed to compare changes from baseline treatment or control treatment, unless otherwise specifically stated.

Results

An in vitro theta model: septo-entorhinal-hippocampal slice co-culture

Although theta-like oscillations can be induced in isolated hippocampal preparations in vitro, it is well known that septal and EC inputs are essential for hippocampal theta generation in vivo. To better understand the dynamic interactions among these three brain regions during theta generation, we established a septo-entorhinal-hippocampal slice co-culture system where a piece of medial septum brain slice was placed next to the hippocampal-entorhinal slice to form a tri-culture that includes the three major brain sub-regions for in vivo theta generation (Fig. 1). This tri-culture system allowed us to directly observe or manipulate any or all three regions to understand the dynamic interactions among them during theta oscillations. After culturing the slices on transwells for 2 weeks, we examined the major inter-regional projections among these three regions in the tri-culture system. First, we looked at septal cholinergic projections to the hippocampus and EC (Fig. 1b, left panel). We injected AAV viruses containing Cre-inducible double floxed inverted mCherry into the septal tissue from ChAT-Cre transgenic mice (Gu et al. 2012; Witten et al. 2010). Although somatic mCherry expression was restricted to the septal cholinergic neurons (Fig. 1b, left upper panel), the axonal projections could also be seen throughout the hippocampus and the EC (Fig. 1b, left lower panel). We then examined EC projections to the hippocampus (Fig. 1b, middle panel). AAV viruses containing the fluorescent protein Turquoise2 under the synapsin promoter were injected into EC. Somatic expression of Turquoise2 was restricted to EC (Fig. 1b, middle upper panel), while the axonal projections could be seen in the hippocampus through the perforant and temporoammonic pathways (Fig. 1b, middle lower panel). Finally, we examined the hippocampal projections to the EC (Fig. 1b, right panel). AAV viruses containing the fluorescent protein Turquoise2 under the synapsin promoter were injected into the hippocampal CA1 subregion. While the somatic expression of Turquoise2 was restricted to CA1 subregion (Fig. 1b, right upper panel), the axonal projections could be seen in the EC deeper layers. Thus, the inter-regional connections among septum, hippocampus, and EC are well preserved in this in vitro tri-culture system, with projection patterns resembling those of in vivo projections connecting these brain regions (Gu and Yakel 2011; Kohara et al. 2014; Naber et al. 2001; Witter 1993). Similar projection patterns were observed across all the slices we examined (nine slices from three batches for each projection pathway), suggesting that the angle of slicing was not very strict, and part of the projections may be regenerated in the culture preparation.

Timed septal cholinergic inputs promote theta generation

In our tri-culture system, we found that activation of the septal cholinergic inputs or stimulation of hippocampal activity alone could not induce theta. However, co-activating these two pathways within a short time window could readily induce hippocampal theta oscillations (Fig. 2). Septal cholinergic inputs were stimulated either through activating ChR2 that was specifically expressed in cholinergic neurons or through a stimulating electrode that was placed on the cholinergic projection pathway to the hippocampus (Fig. 2a); the latter also activated other neurotransmitter pathways (e.g., glutamatergic and GABAergic), but usually achieved similar theta-inducing effects as optogenetic activation. The hippocampal SC pathway was stimulated with another stimulating electrode (Fig. 2a), and the field-potential responses were recorded through a recording glass pipet that was usually placed in CA1 slm. As shown in Fig. 2b, a single stimulation of the SC pathway induced an immediate and fast field response, and ten stimulations of the cholinergic pathway at 10 Hz did not induce any detectable field-potential responses in CA1. However, when the SC pathway was activated immediately after cholinergic activation (within 100 ms after the last pulse of cholinergic activation), a bursting activity with theta frequency (referred to as theta oscillations in this study) could readily be observed in CA1 field-potential recordings that usually lasted for 1–2 s (Fig. 2b–e). Moreover, after several pairings, theta oscillations could be induced by SC stimulation alone, even long after the pairing. Theta oscillations could usually be induced for 30 min after the pairing, with similar theta power and frequency as at 5 min after the pairing (Fig. 2d, e).

The origins of theta rhythms

Once stable theta was established, we then examined the mechanisms underlying the theta current and theta rhythm generation. Simultaneous field-potential recordings from up to three different locations were used to map the theta current and rhythm generators. First, we looked at the field-potential responses from different layers in the hippocampal CA1 region (Fig. 3a, b), and we found that the theta responses were most prominent in CA1 slm (Fig. 3e), with theta power gradually decreasing when moving to the superficial layers. Next, we examined the theta properties in the slm from different hippocampal sub-regions and found similar synchronized theta responses from the slm of the subiculum, CA1, and CA3 (Fig. 3c, e). These results suggest that the theta currents of these three sub-regions came from a common input instead of being generated in one region and spread to the other regions. We further compared the timing of activities from the CA1 and CA3 pyramidal layer peaks with the CA1 slm theta current trough to determine whether the CA1 theta current was generated by inputs from local CA3 or CA1 cell assemblies (Fig. 3d). We found that activities from the CA1 and CA3 pyramidal layers appeared at similar times, and both appeared immediately after (instead of before) the CA1 slm theta current trough, spreading through 0–40 theta degrees after the CA1 slm theta trough (Fig. 3f, lower panel). The mean theta frequency was 8.5 ± 0.6 Hz and the mean theta cycle time (360°) was 122 ± 8 ms ($n = 10$ slices). Therefore, 40 theta degrees corresponds to about 13.5 ms. These results again strongly suggest that the theta current was not initiated by activities in the hippocampal sub-regions, and that the CA1 and CA3 pyramidal cells simply responded to rhythmic extrinsic inputs that come through the slm. The most likely candidate for these inputs is the PP/TA pathway from the EC.

We then examined the temporal relationship between EC neuronal activities and hippocampal CA1 theta current. We found that theta oscillations in EC layer I were mostly synchronized with, but slightly ahead of, CA1 theta (peaked 10 theta degrees earlier than the CA1 slm trough, Fig. 4). The mean theta frequency was 8.3 ± 0.4 Hz and mean theta cycle time (360°) was 122 ± 6 ms ($n = 12$ slices). Therefore, 10 theta degrees correspond to about 3.4 ms. EC layer II/III activities were also synchronized with EC I theta, but with opposite polarity. These results are consistent with in vivo theta observations (Mitchell and Ranck 1980; Mizuseki et al. 2009), again suggesting that the CA1 theta current is the direct synaptic response from the EC through the PP/TA inputs. We then examined the activities in the deep layers (V/VI) of the EC and found that they were also synchronized with EC I theta, but with opposite polarity and with peaks occurring slightly earlier (Fig. 4). These results strongly suggest that the electrical activities originated in EC deep layers (e.g., V/VI), then spread within the local circuit and passed to the EC superficial layers.

To further verify the temporal relationship between EC and hippocampal local neuronal activities in generating the CA1 theta current, we examined the timing of action potential firing of individual EC and CA1 pyramidal neurons relative to the CA1 slm theta trough. As shown in Fig. 5, EC II/III neurons fire approximately 40 theta degrees (13.9 ms) before the CA1 slm theta trough. The mean theta frequency was 8.4 ± 0.3 Hz and mean theta cycle time (360°) was 125 ± 5 ms ($n = 36$ cells). Therefore, 40 theta degrees correspond to about 13.9 ms. EC V/VI neurons fire approximately 20° (6.9 ms) before EC II/III neurons, and with a more distributed pattern. In contrast, CA1 neurons fire approximately 20° – 40° (6.9–13.9 ms) after the theta trough. These results are consistent with those from the field-potential recordings and suggest that the theta rhythm most likely originated in EC V/VI. The temporal results strongly suggest that hippocampal theta is directly entrained by EC inputs, but the EC rhythm is generated locally instead of being directly entrained by hippocampal inputs.

We further recorded excitatory post-synaptic currents (EPSCs) and inhibitory post-synaptic currents (IPSCs) from EC and CA1 neurons to determine whether the rhythm was generated by the intrinsic oscillatory activities of EC neurons, or driven by network activities. As shown in Fig. 6, we found that the EPSCs of EC neurons occur before CA1 theta, with long accumulative rising phases of the currents, suggesting multi-synaptic responses. These results are consistent with the field-potential recordings that showed the continuous accumulative neuronal activities that led to the peak field-potential current in EC layers before CA1 theta. The IPSCs of EC neurons also occurred before CA1 theta, but slightly after the EPSCs and with a shorter rise time; this suggests that large IPSCs were only generated after stronger (synchronized) neuronal activities in the local circuit, resulting in the termination of excitatory activities. On the other hand, the EPSCs and IPSCs of CA1 neurons occur right after CA1 slm theta trough, suggesting that the activities in the CA1 arise from the PP/TA inputs.

To further verify the roles of the EC in the hippocampal theta generation, we examined the theta generation in co-cultured slices without the EC. As shown in Fig. 7, the theta in the CA1 slm was much weaker in these slices, and the subiculum now appears to play a critical role in theta generation. While no significant rhythmic activities were detected in the

subiculum cell layer during theta in EC-containing slices, rhythmic activities in the subiculum were observed in slices without the EC; these rhythmic activities in the subiculum were very similar to those observed in the EC layers in EC-containing slices during theta. For example, the peaks of rhythmic subiculum activities preceded the CA1 theta troughs, and there were accumulative neuronal activities before the peaks. These results support the idea that the EC may be the natural theta rhythm generator, but that there may be other regions that can function as potential rhythm generators, or instead that there may be various forms of theta that can be generated by different mechanisms under various conditions. One recent study, indeed, showed that theta rhythms generated in the rat subiculum flowed backward to actively modulate CA1 and CA3 activities in the whole-hippocampus preparation (Jackson et al. 2014).

EC–hippocampus interactions in generating continuous theta rhythms

We next examined whether the EC can generate theta by itself, or whether external inputs are required. To evaluate the contribution of local EC and hippocampal circuits to theta expression, we locally perfused glutamate receptor antagonists to the EC or hippocampus immediately after the SC stimulation for 1 s with a Picospritzer. As expected, local perfusion of the AMPA receptor antagonist CNQX to the EC blocked theta expression in both EC and hippocampus. However, local perfusion of CNQX to hippocampus not only blocked hippocampal theta as expected, but also blocked EC theta (Fig. 8a, c). CNQX was perfused to hippocampus immediately after the SC stimulation and thus did not block the SC-induced immediate responses in the hippocampus. To further confirm that hippocampus is required in EC theta expression, we directed the CNQX puff pipette to the CA1 slm PP/TA pathway instead of the pyramidal layer. Still, CNQX blocked both hippocampal theta and EC theta, with the SC-induced immediate hippocampal and EC responses intact (Fig. 8b, c). These results suggest that the EC cannot function as an independent rhythm generator or pacemaker; it requires hippocampal feedback to generate continuous theta oscillations. Even though EC local activities may prove to be the major driving force behind theta oscillations, the hippocampus is still required to complete the loop to maintain theta.

We then further tested the involvement of glutamate NMDA receptors that have been implicated in the atropine-resistant *in vivo* theta oscillations (Buzsaki 2002). As shown in Fig. 8d and e, local perfusion of the NMDA receptor antagonist APV to the EC, but not to the hippocampus, blocked theta expression in both EC and hippocampus, further confirming that the local EC circuitry dynamics play an active role in theta rhythm generation.

Discussion

Using an *in vitro* model that incorporates hippocampus, septum, and EC, we were able to induce hippocampal theta oscillations that were primarily driven by EC inputs. It is known that the EC provides the major rhythmic excitatory inputs to drive *in vivo* hippocampal theta oscillations (Bragin et al. 1995; Brankack et al. 1993; Mitchell and Ranck 1980; Stewart et al. 1992). However, previous studies on theta generation have primarily focused on the septal-hippocampal pathways. To our knowledge, this is the first *in vitro* experimental preparation that functionally incorporates these three important brain regions and suggests

an active role of the EC region in generating hippocampal theta oscillations. Due to the simplicity and ease of access to the whole circuit formed by these three regions, we were able to simultaneously monitor and/or manipulate these regions independently, and observe the dynamic activities flowing among these regions during theta generation and propagation.

EC inputs to the hippocampus provide the major excitatory driving force for the theta current sink in the hippocampus (Bragin et al. 1995; Buzsaki 2002; Kamondi et al. 1998); however, little is known about how rhythmic EC activities are generated. Theoretically, the EC could receive rhythmic inputs from the hippocampus or directly from the septum, assuming rhythmic septal activities are the pacemaker for theta oscillations. Even though studies have shown that septal inputs are critical for EC theta generation (Brandon et al. 2011; Jeffery et al. 1995; Mitchell et al. 1982), pacemakers have never been clearly identified. Our study here reveals a potential mechanism for theta to emerge as the result of dynamic interactions among septum, EC, and the hippocampus, in the absence of clear pacemakers. Results from the simultaneous recordings and local glutamate receptor antagonist application suggest that each theta cycle appears first in the EC deep layers (the potential theta origin), then spread to EC superficial layers, then to the hippocampus slm, and finally to the hippocampal pyramidal neurons. However, the EC deep layers are not the automatic rhythm generator; hippocampal inputs or feedback to EC are required to start the next theta cycle. During the theta cycle, the incoming theta signal to a local circuit is actively processed and integrated with other inputs. The processed signals could then be passed down to the target region with updated information. In this way, theta rhythm can be seen as the functional product of the dynamic interactions of hippocampus-associated regions and a platform for ongoing inter-regional communication. This idea is consistent with recent observations that oscillations coupling EC and hippocampus that emerged during learning and memory processes may serve as a means for inter-regional communication (Igarashi et al. 2014; Yamamoto et al. 2014).

It has long been suspected that the atropine-resistant component of theta is largely NMDAR-dependent and mainly driven by EC inputs to the hippocampus. However, the site of NMDAR activation, and how it contributes to theta generation, is largely unknown (Buzsaki 2002). Our results suggested that NMDARs in the EC could be critically involved in theta propagation in the entorhinal-hippocampal loop. This observation also supports our hypothesis that the EC is actively involved, and plays a critical role in theta generation and propagation rather than passively passing rhythmic activities from the hippocampus.

In seeking clues on theta generation, many recent studies have focused on the local hippocampal circuit with close attention on the diverse GABAergic neurons (Buzsaki 2002; Dannenberg et al. 2015; Vandecasteele et al. 2014), assuming rhythmic EC activities were entrained by rhythmic septal or hippocampal inputs during theta. Our study here suggests that the EC may play an active or even central role in generating theta rhythms in the EC-hippocampal loop under certain circumstances. Although the local hippocampal circuit, especially with the diverse interneurons, may be the major target of septal cholinergic modulation, rhythmic activities may not be directly generated there. Instead, the cholinergic-modulated hippocampal circuit may permit or promote theta rhythm generation and

propagation in the EC-hippocampal loop, while EC local network dynamics could play a critical role in theta generation.

Understandably, in vivo theta generation is much more complex and may involve many brain regions and neuro-modulator systems other than cholinergic transmission. Besides, the in vitro model lacks the continuous spontaneous neuronal activities running in the network. The duration of this in vitro theta is also considerably shorter than that of in vivo theta, presumably due to the lack of constant inputs to the hippocampus from many other brain regions in vivo. After all, here, the in vitro theta oscillations were induced only by single-pulse stimulation. Nevertheless, the current model does incorporate the EC for the first time, and the theta was induced by physiologically possible stimuli. Moreover, the current study also revealed a potential active or even central role for EC local circuit in hippocampal theta generation. Due to the easy access to the whole network, more detailed cellular mechanisms can be studied, including the targets of the cholinergic inputs, the roles of local hippocampal and EC dynamics, and the interactions between the hippocampus and the EC in theta generation.

Conclusions

Theta oscillations were induced by pairing septal cholinergic tone with hippocampal activities in an in vitro system that functionally incorporates septum, hippocampus, and EC. Our study strongly suggests an active or even central role of EC local circuit in generating hippocampal theta rhythm. The current study also reveals a potential mechanism for theta rhythm to emerge in the EC-hippocampal network as a result of network interaction in the absence of clear pacemakers, supporting the idea that theta rhythm may emerge as the result of and a means for inter-regional communication.

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Fig. 1.

Inter-regional connections among sub-regions in in vitro septo-entorhinal-hippocampal slice co-cultures. **a** The tri-culture slice preparation. *Left* horizontal mouse brain slice showing hippocampus with EC. *Middle* a coronal brain slice showing medial septum (MS), which hosts the hippocampus-projecting cholinergic neurons. *Right* a piece of MS tissue was placed next to the hippocampal-entorhinal slice to form a co-culture that includes the three major brain sub-regions for in vivo theta generation. **b** Major projections were formed in the co-cultured brain slice. The *upper panels* show the locations of neurons that express fluorescent proteins. The *lower panels* are the same as the *upper panels* but brightened to show the axonal projections in the target areas. *Left* cholinergic neurons in MS project to the hippocampus and EC. *Middle* EC neurons project to the hippocampus via TA/PP pathways. *Right* hippocampal CA1 neurons project to EC deep layers

Fig. 2.

Cholinergic tone converts discrete hippocampal activity to theta oscillations. **a** The basic experimental scheme. Field potentials were recorded in CA1 slm. The Schaffer collateral (SC) pathway was activated by a stimulating electrode placed in CA1 stratum radiatum. Cholinergic neurons were activated either via channelrhodopsin-2 or another stimulating electrode in MS. **b** Cholinergic tone converts discrete SC activation to theta oscillations that can be later reproduced by SC activation alone. From the top trace, one-pulse SC stimulation induced a short field-potential response recorded in the CA1 molecular layer. Ten pulses (at 10 Hz) of cholinergic activation did not induce detectable field-potential response. Ten-pulse cholinergic activation immediately followed (within 100 ms) by one-pulse SC stimulation induced oscillatory field-potential response with a frequency matching hippocampal theta oscillations. After 5–10 pairings, SC stimulation alone could induce oscillatory responses, which could be observed long after the pairing. **c** Spectrogram analysis of representative

traces before, during, and 5 min and 30 min after pairing showing oscillations with theta frequency. The red *arrows* show the time of SC stimulation. **d** Power spectral density analysis of representative traces before, during, and 5 and 30 min after the pairing showing the peak power around 8 Hz. **e** *Bar graph* showing similar peak power spectral density (six individual slices for each group, $p = 0.41$, t test) and the frequency at 5 and 30 min after the pairing ($n = 6$, $p = 0.76$, t test)

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Fig. 3.

Hippocampal theta oscillations were led by TA/PP pathway inputs. **a** Representative traces showing simultaneous recordings from CA1 stratum oriens (so), stratum pyramidale (pyr), and stratum lacunosum-moleculare (slm). Theta was most prominent in the deep layer. **b** Simultaneous recordings from CA1 stratum radiatum (sr) and slm showing that theta was more prominent in slm. **c** Simultaneous recordings from slm under subiculum (sub), CA1 and CA3, showing synchronized theta oscillations in all three sub-regions. **d** Simultaneous recordings from CA1 slm, CA1 and CA3 pyr, showing that the activities in CA1 and CA3 pyramidal layers were synchronized and both closely followed CA1 slm activities. **e** Bar graph of power spectral density analysis showing that theta power was most prominent in CA1 slm. **f** Upper panel: bar graph of the theta phases of CA3 and sub slm theta trough relative to CA1 slm theta trough, showing synchronized theta oscillations in all three sub-regions. Lower panel: bar graph of the theta phases of CA1 and CA3 pyramidal layer theta peak relative to CA1 slm theta trough, showing that the activities in CA1 and CA3 pyramidal layer were synchronized, and both closely followed slm activities. * $p < 0.001$ compared with control, $n = 5$ slices for each group, t test

Fig. 4.

Rhythmic EC activities preceded hippocampal theta oscillations. **a** Simultaneous recordings from EC II/III, EC I, and CA1 slm layers showing that synchronized activities in EC I and CA1 slm layers follow rhythmic activities in EC II/III. **b** Simultaneous recordings from EC V/VI, EC I, and CA1 slm layers showing earlier rhythmic activities in EC V/VI followed by activities in EC I and CA1 slm. **c** Bar graph of the theta phases of EC V/VI and EC II/III theta peak and EC I theta trough relative to CA1 slm theta trough, showing that EC V/VI activities occurred approximately 40° before and EC II/III activities 30° before CA1 slm theta. EC I oscillations occurred immediately before CA1 slm theta. Data were collected from six slices for each group

Fig. 5.

EC neurons fire before hippocampal theta. **a** Whole-cell recordings showing that EC II/III pyramidal neurons fired rhythmic action potentials that were closely followed by CA1 theta oscillations. **b** Whole-cell recordings showing that EC V/VI pyramidal neurons fired rhythmic action potentials that were loosely followed by CA1 theta oscillations. **c** Whole-cell recordings showing that CA1 pyramidal neurons fired rhythmic action potentials that closely followed CA1 theta oscillations. **d** The firing phase of neurons relative to CA1 slm theta trough showing that ECV/VI neurons fire approximately 60° before and EC II/III neurons fire approximately 40° before CA1 slm theta trough, while CA1 neurons fire approximately 20° after CA1 slm theta trough. Data were collected from 12 individual neurons for each group

Fig. 6.

Whole-cell recordings confirmed that EC activities preceded hippocampal theta, while CA1 activities followed. **a** Whole-cell recordings showing that EC II/III pyramidal neurons received rhythmic excitatory and inhibitory inputs that were closely followed by CA1 theta oscillations. **b** Whole-cell recordings showing that EC V/VI pyramidal neurons received excitatory and inhibitory inputs that were loosely followed by CA1 theta oscillations. **c** Whole-cell recordings showing that CA1 pyramidal neurons received rhythmic excitatory and inhibitory inputs that closely followed CA1 theta oscillations. **d** Bar graph showing the much longer EPSC current rise time of EC neurons than those of CA1 neurons, while the IPSC current rise time is similar. * $p < 0.001$ compared with CA1 neurons, $n = 6$ slices for each group, t test

Fig. 7.

Subiculum may serve as a potential rhythm generator in the absence of EC. **a** Simultaneous recordings from subiculum and CA1 pyramidal layer showing that no rhythmic activities observed in the subiculum pyramidal layer in the co-cultured slice with EC. **b** Simultaneous recordings from subiculum and CA1 pyramidal layer showing that rhythmic activities in the subiculum pyramidal layer preceded CA1 slm activities in the co-cultured slice without EC. **c** Power spectral density analysis showing much smaller theta power from slices without EC. **d** Bar graph showing that the theta power was much weaker in slices without EC, while the theta frequency was not significantly changed. $*p < 0.001$ compared with no EC group, $n = 5$ slices for each group, t test

Fig. 8.

Theta expression required continuous activities in both EC and hippocampus. **a** Local perfusion of an AMPAR antagonist to either EC or hippocampus blocked theta oscillations in both EC and hippocampus. **b** Local perfusion of an AMPAR antagonist to CA1 slm blocked theta oscillations in both EC and hippocampus. **c** *Bar graph* of peak power showing blockade of theta oscillations in both EC and CA1 by local perfusion of the AMPAR antagonist CNQX to either EC or CA1. **d** Local perfusion of an NMDAR antagonist to EC, but not hippocampus, blocked theta oscillations in both EC and hippocampus. **e** *Bar graph* of peak power showing blockade of theta oscillations in both EC and CA1 by local perfusion of the NMDAR antagonist APV to EC, but not to CA1. * $p < 0.001$ compared with control, $n = 5$ slices for each group, t test