The role of nucleus reuniens thalami for cortical-hippocampal interaction underlying spatial memory in the rat

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Contents

1. SUMMARY

The hippocampus (HPC) and prefrontal cortex (PFC), two critical brain regions which support several basic brain functions, are essential for our daily life. For instance, as one of the most important brain functions, the memory process has been demonstrated to be correlated to both HPC and PFC. By remembering (memory consolidation) and recalling (memory retrieval) the episode we experienced before, the memory process guides our current life. Considering their contribution to memory and the anatomical connections between them, the interaction between PFC and HPC has drawn a lot of attention and there is an increasing number of studies talking about the PFC-HPC interplay. However, the mechanism of the PFC-HPC interaction still remains relatively unclear by now.

The cross-regional coordination between spatially-distributed brain structures must rely on the direct/indirect anatomical connections. In rodents, while the direct connection between medial prefrontal cortex (mPFC) and HPC have already been elaborately studied, the indirect mPFC-HPC communication through the nucleus reuniens (RE) has not yet been well understood. In thesis, we aimed to investigate the role of RE in the mPFC-RE-HPC circuit. We first studied the RE contribution to the spatial memory process in a crossword maze task. By temporarily inactivating RE with muscimol injection, we revealed the important role of RE in spatial memory retrieval and/or “online” processing of spatial memory. Next, we performed multi-site recording in mPFC, RE and HPC in freely-moving rats. We observed the synchronization between RE and mPFC, between RE and HPC in high gamma frequency range. Moreover, we found a strong RE-HPC cross-frequency coupling around the high gamma synchrony event, which could be correlated to the memory demand. In the end, it was revealed that all nodes of mPFC-RE-HPC circuit could synchronize in high gamma range, during which the cross-frequency coupling between RE and HPC was significantly stronger. To summarize, the current work sheds light on the RE contribution to mPFC-HPC interaction and reveals the potential mechanism for mPFC

and HPC to communicate/coordinate indirectly.

2. SYNOPSIS

2.1 Introduction

2.1.1 Functional Interaction between mPFC and HPC

Since the finding of patient H.M., the importance of hippocampus (HPC) in memory processing has been demonstrated and emphasized by numerous studies[1-6]. Besides, the finding of place cells in hippocampus revealed the key role of hippocampus in spatial navigation[7]. The importance of these hippocampus-dependent brain functions places the hippocampus in a critical position in human daily life and makes it one of the most important research objects in modern neuroscience. Another brain region that is absolutely critical for adaptive behavior and many cognitive functions is the prefrontal cortex[8]. The medial prefrontal cortex (mPFC) in rodents is one of the most studied brain regions in the context of the mechanism of executive control. It has been shown that mPFC plays a critical role in a number of cognitive functions including decision making and working memory[9-14]. Considering the common functions which rely on both HPC and mPFC, it is natural to hypothesize that these two areas can work interactively to enable specific brain functions. Indeed, the interaction between mPFC and HPC has been found to contribute to memory and spatial navigation. The mPFC and HPC mainly interact in a form of synchronization of neuronal activity. For instance, Jones and Wilson revealed an enhanced phase-locking of mPFC spikes to HPC theta and also strong theta coherence between mPFC and HPC when the working memory was supposed to be recruited[15]. The mPFC-HPC task-specific interactions were observed by Benchenane and colleagues[16], there was enhanced theta coherence between mPFC and HPC at the choice point when rats were trained to learn new rules on maze. The mPFC neuron firing phase-locked to the HPC theta was also observed by[17]. Besides the theta-mediated interactions, in a recent work by Tamura et al.[18], the theta-gamma cross-frequency coupling between HPC and mPFC during spatial working memory task was also studied and this coupling was found to become stronger when the task difficulty increased, suggesting the role of

HPC-mPFC coupling in supporting the animal's performance in the task. Although the importance of mPFC-HPC interaction has been relatively well established, it is still not clear enough how these two distant brain areas coordinate with each other. Evidently, the functional interaction between mPFC and HPC must rely on anatomical connections.

2.1.2 Anatomical Basis for mPFC-HPC Interaction

The mPFC and HPC can be connected via direct and indirect pathways. There is a dense projection arising from the ventral HPC and terminating in the ventral mPFC[19-21]. This direct hippocampal projection to mPFC has been demonstrated to contribute to the spatial memory encoding[22]. The inactivation of this projection impaired the rats' performance in a spatial working memory task while spared memory consolidation and memory retrieval functions. The direct projection from the mPFC to HPC was unknown until very recently. A sparse projection from the mPFC (anterior cingulate cortex) directly to HPC (CA1 and CA3) was found and this sparse projection could affect spatial memory retrieval[23]. Considering the importance and complexity of cognitive processes that rely on the mPFC-HPC interaction, it is natural that the circuit underlying mPFC-HPC interaction likely involves other brain areas. A candidate structure mediating mPFC-HPC interactions via indirect pathways is the midline thalamic nucleus reuniens (RE). RE is reciprocally connected with both mPFC and HPC and therefore was suggested to be a hub for linking the mPFC and HPC indirectly[19,24-28]. The RE projects densely to the ventral and dorsal HPC and forms terminals in the stratum lacunosum moleculare of CA1, meanwhile the ventral subiculum has dense projection to caudal RE. In addition, the RE has strong connections to all sub-regions of mPFC. Strikingly, some RE neurons were found to project to mPFC and HPC, simultaneously[26,27]. Besides the RE, the entorhinal cortex (EC) was also found to connect to both mPFC and HPC. Interestingly, EC[29,30] is also the main target of RE and the RE projections to EC and HPC were found to arise from different neuron populations [31], indicating that RE may affect the interplay between mPFC and HPC through multiple indirect pathways. The RE electric stimulation was

found to induce subthreshold excitation in pyramidal neurons and supra-threshold excitation in putative interneurons in CA1[32], while the excitation in mPFC could also be elicited by RE stimulation[33]. The above findings further support the hypothesis that the RE may be a functional link between the mPFC and the HPC.

2.1.3 RE Contribution to Memory Process

As mentioned above, the RE is thought to be a putative node for coordinating activity in both mPFC and HPC and may play an important role in different cognitive functions which depend on the mPFC-HPC interaction. As a midline thalamus nucleus, RE receives diverse projections from many brain areas, mainly from limbic/limbic associated structures[24]. Meanwhile, the main targets of RE projections have been demonstrated to be the hippocampus and limbic cortical areas[30]. The research on the role of RE for cognition is largely limited lesion or RE inactivation studies, while physiological recordings in RE remain extremely rare. For example, it has been shown that the RE contributes to impulsive activity inhibition[34] or to task performance strategy shifting[35]. It was found that the lesion of RE affected performance in a spatial memory task, but not in tasks that rely on sensory guided responding or sequence learning[36]. Consistently, temporary RE inactivation affected spatial working memory[37,38]. A recent work by Ito et al. revealed that RE spikes were phase-locked to HPC theta during a working memory task[39]. Hallock and colleagues found that the temporary RE inactivation affected the phase-locking and theta coherence between mPFC and HPC[40]. Recently, the RE was found to contribute to dopamine release in the ventral tegmental area; the latter could be an indirect way for RE to affect performance in various reward motivated tasks[41].

We now review a few most critical studies. Loureiro et al.[42] carefully investigated the RE contribution to recent and remote spatial memory was. I First, they trained rats a in the Morris water maze and performed the immediate early gene imaging (*c-Fos*) at a short (5 d) and long (25 d) delay after learning. They found that the *c-Fos* expression was dramatically increased in RE at long (but not short) delay, suggesting a potential contribution of RE to the long-term memory consolidation. The RE was

then permanently lesioned and rats were trained in the same water maze. After RE lesion, rats were able to learn the spatial task and no deficit was observed when rats received a probe test 5 days later after the task acquisition. However, when tested 25 days later, the rats' performance was significantly affected and there was no sign of previously acquired spatial memory trace. Intriguingly, reversible RE inactivation during the probe test at 5 d or 25 d delay caused no behavioral deficit. These findings clearly revealed the RE contribution to long-term spatial memory consolidation while no critical role of RE for memory retrieval was evident.

Recent study by Xu and colleagues demonstrated that RE is critical for fear memory generalization[43]. Specifically, in this study animals were fear conditioned in one chamber and then tested in a similar but altered chamber. Freezing behavior (expression of fear memory) in two chambers was then compared. By specifically inactivating the mPFC projection to RE or directly inactivating the RE projections using optogenetic method, an over generalization of fear memory was observed, that is the animals could not efficiently discriminate the two different chambers and showed strong freezing behavior in both the training and the altered chamber. Authors of this study proposed that mPFC-RE-HPC circuit contributes to memory generalization. They suggested specific directionality of signal flow within this circuit. Motivational and emotional aspects of the memory from the mPFC is transferred to the RE then conveyed to the HPC. Eventually the signal in HPC is transmitted back to mPFC for memory generalization. It was hypothesized that fear memory generalization is supported by the mPFC-RE-HPC circuit by RE regulating excitation of HPC remapping, which in turn, makes 'remapping' process more efficient when similar memory is encoded in HPC. .

A similar idea about the direction of information flow within the mPFC-RE-HPC circuit was also suggested by Ito[44]. Ito and colleagues recorded spiking activity in the mPFC, RE and HPC and found trajectory-specific firing in all three brain regions. When the animals were navigating on the central arm of a modified T-maze, neurons showed different firing rates on the left- and right-turn trajectories. This trajectory preference of neuronal activity was found in all three brain areas. The lesion and

inactivation of RE dramatically reduced the trajectory-specific firing in CA1. It was suggested that the indirect mPFC projection to HPC via RE may play a critical role in representing the future path in the goal directed behavior. Intriguingly, although the trajectory-specific firing in CA1 was impaired, the rat's performance was not affected by the RE inactivation/lesion. It was suggested that, in a simple alternation task, the trajectory information that is presumably stored in the mPFC may reach other critical brain regions without passing through the HPC. This explained why the rat's performance was intact event under RE inactivation. It was also shown that the RE processing and the directed information transfer might become critical under conditions when the trajectory information in the mPFC had to be combined with information about acute location in the HPC. Although Xu et al., and Ito et al. studies focused on different cognitive aspects of brain functions, they both emphasized the importance of indirect mPFC projection to HPC via RE and that the RE may play a key role in relaying the information. Moreover, Ito and colleagues also proposed that the importance of this circuit may vary with different cognitive load. The above reviewed studies inspired us to use a relatively complex task to address the role of RE in consolidation and retrieval of spatial memory.

2.2 Thesis Overview

In this thesis, we aim to:

1) define the time window when the RE contributes to information processing and memory storage; interaction;

2) identify the type of cross-regional coordination of neural activity underlying complex navigation behavior and spatial decision making.

We first investigated the involvement of RE in spatial memory consolidation and retrieval using a complex maze task by transiently inactivating the RE at different phases of leaning. (***A.1***). Based on our results, we designed the second study for this thesis (***A.2***). We recorded electrophysiological activity in three brain regions and focused on the cross-regional interactions during spatial navigation.

2.2.1 Part one (A.1): RE contribution to spatial memory process

We tested the hypothesis that RE may be critical for consolidation of spatial memory. To this end we conducted a behavioral experiment and used conventional pharmacology method to transiently inactivate the RE by local injection of muscimol. We designed a reward-motivated task on a complex crossword maze. Rats were released from one of the two start locations and had to find the reward location following a complex maze trajectory consisting of 6/7 right or left turns. Two extramaze cues were fixed on a black curtain surrounding the maze. Inside the maze, several barriers blocked access to specific maze sections leaving 4 or 6 decision areas where animals had to select a particular path. For the first five training sessions (day1-day5), the microinjection of muscimol was performed immediately after the training. On day 6 and day 7, muscimol was injected before the training session. From day 8, rats were given 20 days long 'forgetting' period during which no training took place and rats were kept in their home cages. On day 30, rats were tested again on the maze task without any microinjection.

We found that the post-learning inactivation of RE did not affect learning of the spatial task, which is consistent with previous studies. In contrast, muscimol injection into RE before the probe test dramatically impaired the rats' performance during the entire training session without affectingrats' locomotion. The significantly increased error numbers indicated a deficit in retrieving the obtained spatial memory and suggested a critical role of RE for 'on-line' memory process, which is the memory retrieval and memory reconsolidation. This finding seems to be different with the study by Loureiro et al[42] in which the pre-test inactivation of RE in the Morris water maze task did not affect the animal performance. The probe test on day30 did not reveal any difference between groups, suggesting that post-learning RE inactivation did not affect the system level memory consolidation.

2.2.2 Part two (A.2): Synchronization/coupling between RE and mPFC/HPC

After showing the critical contribution of RE activity to spatial memory retrieval tested in a complex maze, we further investigated the neurophysiological mechanism

underlying this phenomenon. We hypothesized that the RE may support coordination between the mPFC and HPC. We performed LFP recordings in the mPFC, RE and HPC while rats performed the same spatial task as in the study A.1. Based on the results of According to the finding in our first study (A.1), we mainly focused on the 'on-line' information processing, that is, a period when rats were navigating on the maze.

To characterize cross-regional interactions we first applied independent component analysis (ICA) to study if there is a cross-frequency coupling between the RE LFP oscillations and theta oscillations in the hippocampus. The ICA analysis revealed that power of high gamma (60-120 Hz) oscillation in the RE LFP was strongly correlated with HPC theta oscillation. We next calculated the pair-wise gamma synchrony between the RE and mPFC LFP and RE and HPC LFP and detected high gamma synchrony event (HGS event). We then examined spatial distribution of HGS events in the maze and did not observe any specific pattern of HGS event occurrence. We next compared the amplitude and duration of HGS eventbefore the correct and incorrect choices and found no difference For more detailed analysis, we combined the gamma synchronization analysis and cross-frequency coupling. Specifically, we used the times of HGS event as triggers and studied the phase-amplitude coupling between the RE and HPC around the HGS event. We found that, for the HGS event from both mPFC-RE and HPC-RE pair, incorrect choices were preceded by stronger coupling between the RE and HPC compared to the coupling strength before correct choices. The difference in cross-regional coupling strength around HGS event may reflect the level of animal uncertainty at the maze crossing and therefore is indicative for the efficiency of memory retrieval. We then compared the strength of RE-HPC coupling on the maze sections along the correct trajectory and on the maze segments outside the correct trajectory. Stronger HGS (both pair) event-triggered RE-HPC coupling accompanied rat traversing incorrect maze segments. This observation suggests that the RE-HPC coupling increase with the increase of memory/cognitive demand. When animals deviated from the correct trajectory, to return to the correct path they may need to

integrate previously acquired information (memory retrieval) and current spatial information (spatial orientation) , which is evidently cognitively more demanding. Thus, stronger functional interactions between the RE and HPC may reflect this cognitive effort.

The gamma synchrony events detected from one LFP signal pairs (e.g. mPFC-RE) could co-occur with the gamma synchrony event detected from another signal pair (e.g. HPC-RE). Co-occurring HGS events will be further referred as Co even and the HGS events occurring within each signal pair non-synchronously will be referred as Nonco event. For each LFP signal pair and according to the HGS event type used as trigger, we further split the HGS event-triggered RE-HPC coupling into two groups (Co and Nonco group). On the incorrect maze segments, the Co event-triggered cross-reginal coupling was stronger for both LFP signal pairs (mPFC-RE and HPC-RE), comparing to the Nonco group. This finding suggests a possible mechanism for cross-talking between all three nodes in the mPFC-RE-HPC circuit. The difference in coupling strength between the Nonco events in mPFC-RE and HPC-RE pair further supported the hypothesis that when it is necessary, the memory-related information may be transferred from mPFC via RE to HPC.

2.3 General Discussion

The midline thalamic nuclei including the RE belong to so called ‘nonspecific’ thalamus [45,46]. Since recently, the RE started drawing attention due to the reports about its involvement in higher-order brain functions like fear memory generalization and working memory. The RE was suggested to contribute to memory processing by its functional integration into memory supporting mPFC-HPC network[36-38,43]. The results of the first study of this thesis are consistent with previous studies. We have demonstrated that the RE does not appear to play a critical role during ‘off-line’ phase of memory consolidation as its inactivation after learning did not affect the learning efficiency of a spatial task In contrast, RE inactivation before the task caused a significant deficit during ‘on-line’ phase of learning (processing of current spatial information and/or spatial memory retrieval). At first glance, our findings appear to

contradict to previous studies that emphasized the importance of RE for memory consolidation. Earlier studies also did not indicate the RE contribution to memory retrieval[42]. However, this discrepancy in the results could be due to the use of different learning tasks. It has been demonstrated that the Morris water maze (MWM) task, which was used in Loureiro's study, is hippocampus-dependent, but does not critically engage the mPFC Moreover, memory retrieval in MWM task was found to require the PFC for the retrieval of remote (25-30d after learning), but not for recent memory retrieval (1-5d). This task-dependent effect was also observed when the RE contribution was compared in the spatial memory task that only depends on the HPC or in the task that depends on both mPFC and HPC[38]. In another study by Cholvin et al., RE was inactivated in different spatial tasks and its involvement was found to be cortical only in the task which depends on both mPFC and HPC[35]. The mechanism behind the differential RE involvement in different cognitive situations remains to be understood. The most consistent effects that are observed in situation when both the mPFC and HPC are simultaneously recruited during the task performance further support the idea that the RE contributes to the mPFC-HPC interaction.

The population synchrony in gamma range between distant brain areas has been suggested to be a plausible mechanism underling cross-talking between brain areas a constituting functional network. The gamma oscillations are thought to represent local neural activity and reflect activation of both local excitatory and inhibitory networks[47-49]. The gamma synchrony between two brain regions could reflect coordinated excitation-inhibition state within each local area opening transient communication windows for interplay and information transfer between two or more brain regions forming a functional network. In addition, cross-regional gamma synchronization can make inter-regional communication more efficient[50]. Thus two brain structures synchronized in the gamma range could be in a fine window which makes the cross-talking possible and they can affect each other more efficiently. Besides gamma synchronization, cross-frequency coupling between within and also between brain regions has been suggested as another mechanism for information processing. For example theta-gamma coupling has also been described for other brain areas[18,51,52].

Transient increases in gamma power may represent specific units of information. Phase coupling of gamma transients (at different phases of a single theta cycle) may organize multiple information units in a temporal pattern.

In line with previous studies, we observed gamma synchronization within the circuit formed by the mPFC, RE and HPC. Using the HGS event as trigger, we have demonstrated that stronger gamma-theta coupling between the RE and HPC occurs before incorrect choice in the maze and also during navigation outside the correct path. In addition to detecting a pairwise (between two LFP signals) gamma synchronization that reflects crosstalk between the two brain regions (e.g. mPFC-RE or HPC-RE), we also detected joint gamma synchronization within the entire mPFC-HPC-RE circuit (or Co-event). Concurrent gamma synchrony within mPFC-RE and HPC-RE may further indicate a network state when all nodes of the circuit are activated and the information transfer is possible. Stronger gamma-theta coupling between the RE (gamma) and HPC (theta) occurring around Co event further supports the idea that the synchronization within the entire circuit could facilitate communication within functional network [51-53].

A relatively dense direct projection from the ventral HPC to ventral mPFC has been known since long time ago and only recently a sparse projection from the mPFC to HPC has been described[23]. It is natural to hypothesize that the RE could mediate mPFC-HPC communication via indirect pathways. In the study by Xu et al.[43], it was shown that the inactivation of mPFC projection to RE affected fear memory generalization and the authors suggested that information flow from the mPFC to HPC via RE could be critical for fear memory consolidation. Ito et al.[44] proposed that a copy of spatial memory trace may be transferred from the mPFC via RE to HPC. The transferred information from the mPFC would be then integrated with the current spatial information encoded in the HPC. The results of our second study (manuscript in preparation) also support the idea of directed information flow. The instances of RE-HPC coupling around co-occurring and nonco-occurring mPFC-RE HGS event had some similarities. Intriguingly, around nonco-occurring HPC-RE HGS event, high frequency oscillations in the RE LFPs which strongly coupled with the HPC theta

cycles were missing. The phase-amplitude coupling around Nonco event for mPFC-RE and HPC-RE LFP signal pairs further supports the potential information flow from the mPFC to HPC, through RE. The successful information transfer from the mPFC to RE could be the first step of the memory retrieval and it is successive transfer to the HPC would allow integration of the retrieved and acute information about spatial environment that would facilitated goal-oriented navigation. This plausible scenario could underlie the tight temporal relationships of the RE gamma oscillations with HPC theta that are task-dependent; it also explains why such cross regional coupling is attenuated during time windows when only two hubs of the network (RE and HPC) are synchronized in the gamma range.

2.4 Conclusion and Outlook

In this thesis, we first confirmed the critical role of the RE in the spatial memory processing. Although the anatomical connectivity suggested the RE as a possible modulator of the interactions between the mPFC and HPC via indirect pathways, very limited number of studies examined the role of RE in spatial learning and memory. We have demonstrated that temporal RE inactivation significantly impaired memory retrieval in rats which had previously learned a complex spatial task. The subsequent analysis of population activity in the mPFC, RE and HPC revealed that a network state when all critical nodes are synchronized in the high gamma range maybe beneficial for successful information flow within this circuit and therefore supports memory retrieval and spatial information processing. We propose that within the mPFC-RE-HPC circuit, the memory retrieval related information may be transferred in a specific direction from the mPFC to HPC through RE, yet this prediction needs to be tested.

Our results expand the current knowledge about the contribution of the RE to memory supporting network, but many open questions remain. For instance, it is unknown what drives and coordinates the synchronization of all three brain regions. One possibility is that, collateral projections of some RE neurons to both mPFC and HPC[26,27] may simultaneously broadcast to both brain regions and thus contribute to

cross-regional synchronization. Simultaneous activation of local networks as reflected by high gamma oscillations facilitates cross-talk within the functional network. Thus, the RE neurons projecting to both mPFC and HPC may serve as a pace maker and network coordinator. To test this idea, the specific activation/inactivation of these neurons should be done in the future studies. Another possibility is that, these three brain areas may receive a common input from another brain region.

In the second study, we correlated cross-regional coupling with behavioral variables during rat's performance of a spatial task. Our result suggested that the strength of the RE-HPC cross frequency phase-amplitude coupling can indicate some specific aspects of memory processing such as memory retrieval. However, the precise mechanism underlying the high frequency oscillation in RE to phase lock to the hippocampal theta is still unknown. Dolleman-Van der Weel and colleagues suggested that the RE and HPC could also form a sub-loop[32], if this RE-HPC loop can contribute to the RE-HPC coupling could be an interesting question and should be answered in the future. The projections from rostral RE terminate in the stratum lacunosum moleculare of CA1 and could induce subthreshold excitation of the pyramidal neurons and supra-threshold excitation of putative interneurons. This RE contribution to the excitation level in the CA1 may be the mechanism for the RE to actively adjust the rhythms in CA1, which in turn, can phase-lock the RE gamma to hippocampal theta. Since it has been suggested that the different gamma oscillations which lock to different HPC theta phase may represent the sequence of the events, this mechanism could also provide a way for RE to transfer the mPFC information to HPC. Moreover, the dense projection from the subiculum to caudal RE could be a feedback mechanism for the HPC to adjust RE activity and eventually further adjust the rhythms in both RE and HPC. In the future, the multi-cite recording in RE, CA1 and ventral subiculum should be performed. To test the idea that the RE could affect the oscillation in HPC, the transient inactivation in RE during the RE-HPC coupling could be applied.

In the end, the potential directed information flow within the mPFC-RE-HPC circuit should also be carefully studied in the future. In the thesis, we hypothesized

that the RE mainly contribute the information transferring from the mPFC to HPC. To test this idea, the activation/inactivation of the mPFC projection to RE should be done. If the inactivation of aforementioned projection could abolish/dramatically impair the RE contribution, the mPFC could then be considered as the origin of the information flow in the mPFC-RE-HPC circuit.

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2.6 List of Papers/Manuscripts

This thesis comprises two manuscripts that are either published or in preparation.

Details of the manuscripts are as following:

2.6.1 Summarized Papers/Manuscripts

A.1

H Mei, NK Logothetis, O Eschenko

The activity of thalamic nucleus reuniens is critical for memory retrieval, but not essential for the early phase of 'off-line' consolidation.

Learning & Memory, 2018, 25, 129-137, doi: 10.1101/lm.047134.117

A.2

H Mei, NK Logothetis, O Eschenko

Investigation of cross-regional interactions within the prefrontal-thalamo-hippocampal circuit associated with spatial cognition in the rat

In preparation for submission

2.7 Statement of Contributions

A.1

The activity of thalamic nucleus reuniens is critical for memory retrieval, but not essential for the early phase of 'off-line' consolidation.

H Mei, NK Logothetis, O Eschenko

Learning & Memory, 2018, 25, 129-137, doi: 10.1101/lm.047134.117

H.M, N. L. and O.E. designed the research, **H.M** performed animal experiments, **H.M** acquired data, **H.M**, and O.E. analyzed data, and **H.M**, N. L. and O.E. wrote the manuscript.

A.2

Investigation of cross-regional interactions within the prefrontal-thalamo-hippocampal circuit associated with spatial cognition in the rat

H Mei, NK Logothetis, O Eschenko

In preparation for submission

H.M, N. L. and O.E. designed the research, **H.M** performed animal experiments, **H.M** acquired data, **H.M**, and O.E. analyzed data, and **H.M**, N. L. and O.E. wrote the manuscript.

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4. APPENDIX

4.1 A.1: The activity of thalamic nucleus reuniens is critical for memory retrieval, but not essential for the early phase of ‘off-line’ consolidation

The activity of thalamic nucleus reuniens is critical for memory retrieval, but not essential for the early phase of ‘off-line’ consolidation

H Mei, NK Logothetis, O Eschenko

Learning & Memory, 2018, 25, 129-137

doi: 10.1101/lm.047134.117

Research

The activity of thalamic nucleus reuniens is critical for memory retrieval, but not essential for the early phase of "off-line" consolidation

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Spatial navigation depends on the hippocampal function, but also requires bidirectional interactions between the hippocampus (HPC) and the prefrontal cortex (PFC). The cross-regional communication is typically regulated by critical nodes of a distributed brain network. The thalamic nucleus reuniens (RE) is reciprocally connected to both HPC and PFC and may coordinate the information flow within the HPC–PFC pathway. Here we examined if RE activity contributes to the spatial memory consolidation. Rats were trained to find reward following a complex trajectory on a crossword-like maze. Immediately after each of the five daily learning sessions the RE was reversibly inactivated by local injection of muscimol. The post-training RE inactivation affected neither the spatial task acquisition nor the memory retention, which was tested after a 20-d "forgetting" period. In contrast, the RE inactivation in well-trained rats prior to the maze exposure impaired the task performance without affecting locomotion or appetitive motivation. Our results support the role of the RE in memory retrieval and/or "online" processing of spatial information, but do not provide evidence for its engagement in "off-line" processing, at least within a time window immediately following learning experience.

[Supplemental material is available for this article.]

The interactions between the hippocampus (HPC) and the prefrontal cortex (PFC) are essential for many aspects of spatial cognition, particularly those engaging working or declarative-like memory (Buzsáki 1996; Benchenane et al. 2011; Gordon 2011; Eichenbaum 2017). It has been shown, for instance, that the oscillatory rhythms generated in the HPC may synchronize firing of the PFC neurons (Hyman et al. 2005; Siapas et al. 2005). The HPC–PFC oscillatory coupling accompanies rule-guided behaviors (Jones and Wilson 2005; Benchenane et al. 2010), but is also observed during "off-line" states (e.g., sleep), when fluctuations of the hippocampal and cortical population excitability (indicated by ripples and sleep spindles) are coordinated by cortical slow rhythms (Siapas and Wilson 1998; Moelle et al. 2009; Wierzynski et al. 2009). The HPC–PFC interlay occurring "off-line" is thought to underlie the information transfer from the HPC, where new information is first encoded, to the PFC and other neocortical regions for the long-term storage (Buzsáki 1996; Frankland and Bontempi 2005; Peyrache et al. 2009; Colgin 2011). The HPC–PFC interactions are supported by their anatomical connectivity, including reciprocal direct projections. In the rodent brain, the CA1 subfield of ventral HPC and the ventral subiculum (main HPC output) project to the prelimbic and infralimbic areas of the PFC (Thierry et al. 2000; Hoover and Vertes 2007). The top-down prefrontal (predominantly anterior cingulate) projections target the CA1 and CA3 subfields of the dorsal HPC; this recently identified direct PFC–HPC pathway has been implicated in the contextual fear memory retrieval (Rajasethupathy et al. 2015). Given the variety and complexity of cognitive processes that appear to depend on HPC–PFC interactions and the widespread anatomical connectivity of each structure to other brain regions, it is evident that the neural circuits supporting the performance of cognitively demanding tasks include brain structures beyond these two regions (Mizumori et al. 2000). Identifying the critical "hubs" of large-scale functional networks would advance our understanding of the neurophysiological mechanisms related to cognitive capacities, such as memory encoding, consolidation, and its long-term storage.

The nucleus reuniens (RE) is one such potentially critical node. As part of the midline thalamus, RE has been hypothesized to play a role for the HPC–PFC interactions (Vertes 2006; Vertes et al. 2007; Varela et al. 2014). This hypothesis originated from the studies of anatomical connectivity between the RE, HPC, and PFC and by now has received some support from functional studies. The RE is reciprocally connected to all subregions of the PFC, it densely projects to the dorsal and ventral HPC and also receives afferents from the ventral HPC and subiculum (McKenna and Vertes 2004; Vertes et al. 2006; Hoover and Vertes 2007). Notably, some of the RE neurons send their collaterals to both brain regions (Hoover and Vertes 2012). Such an anatomical connectivity pattern evidently places the RE in a key position for regulating the information flow between HPC and PFC (Vertes et al. 2007; Griffin 2015; Hallock et al. 2016; Roy et al. 2017). Indeed, RE lesion and/or inactivation impairs performance of previously learnt spatial and nonspatial tasks, particularly those requiring working memory (Davoodi et al. 2009; Hembrook and Mair 2011; Hembrook et al.

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2012; Hallock et al. 2013, 2016; Layfield et al. 2015). Nonetheless, the role of RE for spatial learning remains controversial as several studies were unable to demonstrate any effects of RE lesion or inactivation on the rat ability to acquire a spatial task (Dolleman-van der Weel et al. 2009; Loureiro et al. 2012; Ito et al. 2015), but see also (Davoodi et al. 2009). Furthermore, despite the lack of evidence that RE neurons encode spatial representations, the RE output appears to affect the hippocampal spatial coding (Ito et al. 2015). The "head direction" coding by a population of RE neurons may also contribute to spatial navigation (Jankowski et al. 2014). At present, little is known about the role of RE for the HPC–PFC interaction occurring "off-line." Experimental evidence exists that the RE may be involved in the spatial memory consolidation (Davoodi et al. 2009) and/or its long-term maintenance (Loureiro et al. 2012; Ali et al. 2017). The results from studies on aversive learning also suggest that the RE may be an intermediate processing step within the PFC–HPC pathway that enables fear memory consolidation and retrieval (Davoodi et al. 2011; Xu and Sudhof 2013). Yet, in contrast to the well-established role of the HPC–PFC interactions for both "online" and "off-line" memory-related processing, the exact role and the functional significance of RE activity within this highly interconnected neural circuit in different stages of memory formation remains insufficiently understood.

The present study was thus designed to examine the extent to which the RE is involved in the consolidation of spatial memory. To this end, we trained rats to perform a spatial task and evaluated the behavioral effects of post-learning reversible inactivation of the RE. From a methodological perspective, inactivation of a brain region after encoding phase (or "online" processing) tests its involvement in the consolidation phase, taking place "off-line." We also tested rat spatial memory after 20-d "forgetting" period; any deviation of the rat performance on the remote memory test would indicate the involvement of post-learning RE activity in memory retention.

Rats were trained on an elevated crossword-like maze (Fig. 1A), the configuration of which resembled a multiple-unit T-alley maze, originally designed to study rodent spatial cognition (Tolman 1948). In the beginning of each trial a rat was randomly released from one of the two start locations and allowed to reach reward by navigating along maze alleys (Fig. 1A). Two salient distal visual cues were fixed on the black curtains surrounding the maze. The experimental design, namely, availability of distal cues, variable start positions, and goal-oriented navigation along the two rather complex trajectories, assumed that the rat's performance will be relying on allocentric cues and a path integration, both known to depend on HPC (McNaughton et al. 2006; Buzsáki and Moser 2013). Performing this maze task relying on procedural memory (e.g., acquired motor habit) is unlikely at the early stages of learning, but it may eventually prevail following extensive training (Packard and McGaugh 1996), which however did not take place in the present study. Based on the literature reviewed above, efficient learning and successful performance of such a spatial task most likely require a coordinated interplay between HPC and PFC, whereas the "off-line" HPC–PFC interactions may be essential for the stabilization of encoded information.

Our present findings demonstrate that RE activity is essential for the performance of a spatial target-oriented spatial task, however not required for the early phase of "off-line" processing enabling memory stabilization and long-term storage. In other words, the contribution of the RE is likely limited to the network that is activated "online" or during active phases of information encoding.

Results

Rats were trained on the crossword-like maze (Fig. 1A). The rat performance on the maze was evaluated using the trial latency (time required to reach reward), the trajectory length (total number of maze alleys visited by a rat before reaching reward), and the number of errors. Each deviation from the "correct" (shortest) path was considered an error, regardless of how many maze alleys the rat would cross before returning to the "correct" path (see example on Supplemental Fig. S1A). Traversing along the "correct" path, but in the opposite direction was also classified as an error (Supplemental Fig. S1B). Entering a "wrong" maze alley with all four paws was considered an error.

Immediately after each of the five learning sessions, muscimol (MUS) or saline (SAL) was injected via chronically implanted cannulas targeting the RE (Fig. 1B). On days 6 and 7, MUS or SAL was injected prior to the maze exposure to test the effect of RE inactivation on the performance of a recently acquired spatial task (Fig. 1B). On day 8, we tested the drug-free rats on the maze again, but included two probe trials to verify that rats used distal visual cues for navigation on the maze. Specifically, the first three trials were run under the standard conditions, then all lights were turned off and rats had to perform the trial 4 in the darkness. Next trials (5–7) were standard and on the trial 8 rats were tested in the

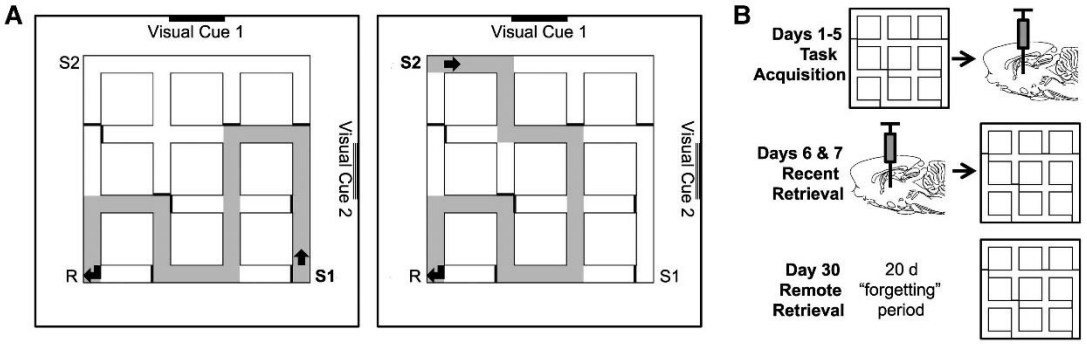

Figure 1. The experimental setup. (*A*) Top-down view of the experimental environment. The crossword-like maze consisted of perpendicular alleys; vertical barriers (thick black lines) blocked the access to some maze sections. There were two start locations (S1, S2) and one reward port (R). The "correct" (shortest) trajectory from Start 1 (*left* panel) and from Start 2 (*right* panel) are shown in gray; arrows indicate the direction of navigation. Two distal large visual cues were fixed on the black curtains (outer frame) surrounding the maze. (*B*) The experiment timeline. (*Top*) During the task acquisition sessions (days 1–5) rats received intrabrain injection of MUS or SAL immediately after the maze exposure. Middle, On days 6 and 7 the drug (MUS or SAL) was injected 30 min prior to the maze exposure. Bottom, After two additional injection-free training sessions on days 8 and 9 (not shown), rats were allowed 20 d of "forgetting" period and tested on the maze again on day 30.

darkness and without any vertical barriers that prevented them entering some maze alleys during the initial learning (Fig. 1A); the trials 9 and 10 were again standard. On trial 4 (darkness test), rats made significantly more errors (Supplemental Fig. S2), which was suggestive that rats used allocentric, but not procedural (e.g., sequence of turns) strategy to perform the task. On trial 8 (no barriers test), all rats easily reached reward, but followed completely different trajectories from the "correct" ones (Supplemental Fig. S3); thus rats did not appear to rely on the acquired motor habit (e.g., sequence of turns), but likely used path integration. On day 9, rats were trained in the standard conditions to stabilize the task performance and were tested on the maze again after 20-d "forgetting" period (Fig. 1B).

The histological examination confirmed the location of the cannula tip in the direct proximity to the RE in 17 rats (Fig. 2). The cases with the cannula placement outside the RE served as additional control. The histology result was in agreement with a post hoc analysis of the presence or absence of a motor deficit following intrabrain MUS injection. Typically, the RE inactivation did not affect motor activity. In contrast, MUS injection outside the RE or into the third ventricle often produced a motor deficit of different degrees. To quantify motor activity, for each of 10 trials after the MUS injections we extracted the rat maximal speed using video recording and a custom software (MathWorks). The K-means clustering analysis revealed three main patterns with motor activity stable across trials (Cluster1, Supplemental Fig. S4), gradually decreasing over time (Cluster2), and severely suppressed (Cluster3). In two rats with the cannula tip placed in the RE, the onset of motor deficit was delayed, probably due to MUS diffusion outside the RE. Conservatively, the data from these two rats were excluded from the analysis of the effects of the RE inactivation on memory consolidation. Hence, the rats were distributed between three experimental conditions with MUS injections restricted to the RE (RE-MUS, $n = 7$), with MUS injections outside of RE (notRE-MUS, $n = 7$) and SAL injections (SAL, $n = 16$). During the first training session (before any intrabrain injections), the rats' behavior on the maze was similar across three experimental groups (one-way analysis of variance [ANOVA]; errors: $(F_{(2,27)} = 0.39$, ns; trial latency: $F_{(2,27)} = 1.32$, ns; trajectory length: $F_{(2,27)} = 0.24$, ns).

The RE is not essential for "off-line" memory consolidation or long-term storage

To assess the effect of post-learning inactivation of the RE on the learning rate, we submitted behavioral variables across five training days to the repeated-measures ANOVA and compared across three experimental conditions. There was a significant day effect for the trial latency ($F_{(2.7,71.6)} = 154.5$, $P < 0.001$; Greenhouse–Geisser correction was applied whenever the assumption of sphericity was violated), the trajectory length ($F_{(2.4,65.3)} = 84.3$, $P < 0.001$) and the number of errors ($F_{(4,108)} = 184.5$, $P < 0.001$), yet no significant interaction or group effect. The task performance gradually improved over time as reflected by decreasing number of errors, but the learning rate was equal for all three groups (Fig. 3A). Post hoc multiple comparisons (Bonferroni corrected) between training days showed that rats learned the task during four sessions; the number of errors reached an asymptote level on day 4 and there was no further improvement on day 5 (Fig. 3B). There was also no between-group difference on the last day of training (one-way ANOVA, errors: $F_{(2,27)} = 1.25$, ns; trial latency: $F_{(2,27)} = 1.44$, ns; trajectory: $F_{(2,27)} = 1.40$, ns). Since the rats had to learn two partially overlapping trajectories (Fig. 1A), we also compared the learning rate between Start1- and Start2-trials. No trajectory preference was revealed in any experimental group by any of the behavioral variables analyzed (not shown). Finally, to evaluate the efficiency of spatial memory consolidation, we compared the rat performance during the first trial on days 2–5, which is equivalent to the conventional memory retrieval test. We found a significant day effect (latency: $F_{(3,81)} = 34.14$, $P < 0.001$; errors: $F_{(3,81)} = 19.79$, $P < 0.001$; trajectory: $F_{(3,81)} = 11.04$, $P < 0.001$), but no interaction or group effect. Overall, our results did not provide any evidence that post-learning inactivation of the RE affected the learning rate. Notably, post-learning MUS injections outside the RE, while caused a transient motor deficit, did not affect the next day task performance; the latter result suggests that brain regions surrounding the RE are not likely to contribute to the "off-line" consolidation.

We also tested if post-learning inactivation of the RE affected the long-term stability of acquired memory. After the initial learning phase was completed (days 1–5), rats were additionally tested

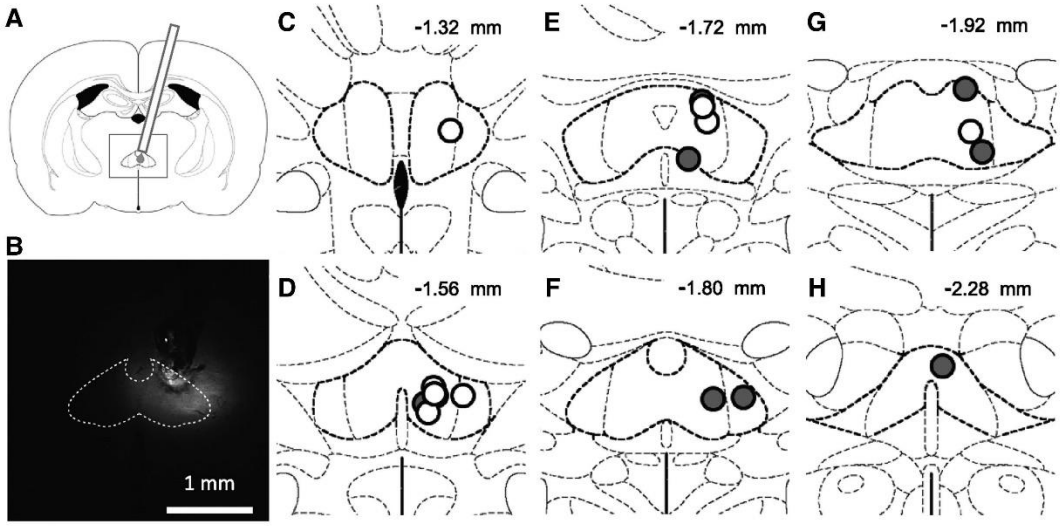

Figure 2. Histological reconstruction of the injection sites in the RE. (*A*) Schematic of the rat brain coronal section with injection cannula targeting the RE. (*B*) Enlarged brain section (indicated in *A*) showing the spread of fluorophore-conjugated MUS. Dashed line shows the RE boarders. (*C–H*) Reconstruction of the injection centers in the RE on different anterior-posterior planes. Placements of the post-learning injections of SAL (open circles) and MUS (filled circles) within the RE are shown; cases with injection centers outside the RE are not shown.

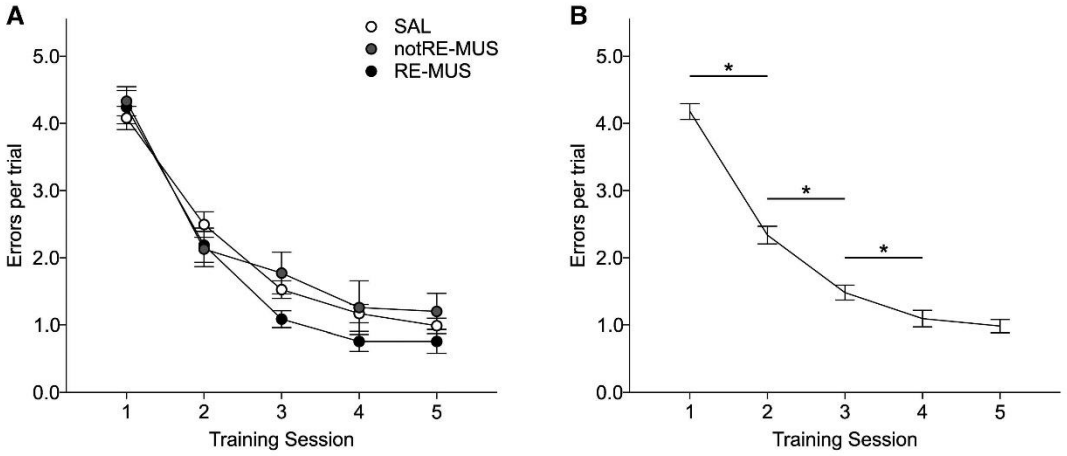

Figure 3. Reversible inactivation of the RE after each of the five task acquisition sessions did not affect the efficiency of spatial learning. (*A*) The average number of errors in each trial is shown across five training sessions for rats that received intrabrain injections of SAL (open circles, $n = 16$), MUS outside the RE (gray circles, $n = 7$), or MUS in the RE (black circles, $n = 7$). The accuracy of rat performance improved with equal rate in each experimental group. (*B*) The average number of errors in each trial is shown across five training sessions for all rats tested ($n = 30$). Rats reached asymptote performance on day 4. Error bars represent ±SEM; (*) $P < 0.01$ (Bonferroni corrected).

on the maze on days 6–9 and then were allowed 20 d of "forgetting" period, during which rats were kept in their home cages with unlimited access to food and water (Fig. 1B). At the end of the forgetting period rats were food deprived and tested again on the maze. The remote memory retrieval was evaluated by rat performance on the first trial. No intrabrain injections were made during "forgetting" period or prior to the maze exposure on day 30. Substantial forgetting was clearly evident as rats made significantly more errors on day 30 than on day 5, when the asymptote performance was reached (repeated-measures ANOVA, day effect: $F_{(1,26)} = 40.607$, $P < 0.001$) (Fig. 4). However, memory decay was equal in all experimental groups ($F_{(2,26)} = 0.960$, ns), there was also no significant interaction ($F_{(2,26)} = 1.886$, ns) (Fig. 4). Although, on day 30 rats made even more errors as on day 1 (6.4 ± 2.5 vs. 4.9 ± 2.9 $P < 0.05$), more detailed analysis of rat behavior revealed some important differences. First, on day 30 rats actively explored the maze and most of them reached reward within 3-min cut-off time (SAL: 73.3%, RE-MUS: 85.7%, notRE-MUS: 42.9%). Importantly, the proportion of completed trials on day 30 was much higher than on the very first learning trial on day 1 (6.9%, all rats combined); the latter indicated that rat behavior on day 30 was clearly target-oriented. Second, the average length of correct path (traversing along the correct trajectory without deviation) was much longer on day 30 (3.8 ± 1.5 maze sections) than on day 1 (1.3 ± 0.3 maze sections) and, in fact, it was comparable to the performance on day 3 (4.4 ± 1.9 maze sections). Thus, despite substantial memory decay on day 30, the rat behavior on the maze was not random and indicated that memory about the task was, at least, partially preserved. Finally, the trial latency on day 30 greatly varied across rats. The latencies distribution was bimodal with peaks around 63 and 162 sec. Using the K-means clustering, we assigned the rats to "slow" and "fast" performers. The "fast" performers showed a somewhat better preserved memory as they made fewer errors (2.55 ± 1.57 vs. 6.39 ± 2.55 for "fast" and "slow" performers, respectively; $t_{(26.978)} = 5.024$, $P < 0.001$) and had a shorter trajectory (18.0 ± 5.9 vs. 45.3 ± 11.2 maze sections, $t_{(27)} = 7.425$, $P < 0.001$). However, the proportion of "fast" performers was similar between three groups (SAL: 40.00%, notRE-MUS: 28.57%, and RE-MUS: 42.86%, Kruskal–Wallis test for independent samples, ns).

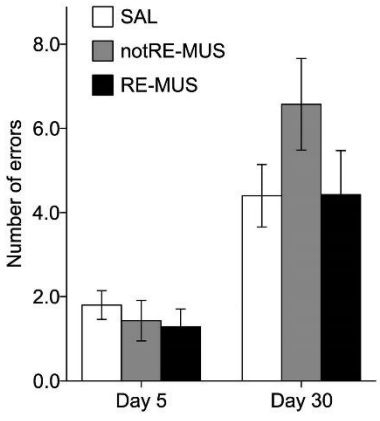

Figure 4. Reversible inactivation of the RE after each of the four task acquisition sessions had no effect on short- or long-term memory retention. The accuracy of task performance during the first trial on day 5 (memory retrieval test 1 d after the task acquisition) and on day 30 (remote memory retrieval) is shown for three experimental conditions. No drug injection was made during 20-d "forgetting" period or prior memory retrieval tests. Memory expression on days 5 and 30 was equal across three experimental groups. Error bars represent ±SEM.

The RE is critical for spatial memory retrieval

To assess the effects of RE inactivation on the retrieval of recently acquired spatial memory, we selected rats with injection centers in the RE and with no MUS-induced motor deficits during the retrieval trial ($n = 16$) (Fig. 2). Given that post-learning inactivation of the RE did not affect task acquisition (Fig. 3), we combined rats which received either SAL or MUS injections into the RE during the initial learning phase (days 1–5). After five learning sessions, on days 6 and 7 rats received either MUS or SAL injection 30 min before the maze exposure (Fig. 1B). The RE inactivation dramatically affected the rat behavior on the maze. On the first trial, 9 out of 16 rats (56.3%) did not find the reward location within a 3-min cut-off time. The first trial latency was significantly longer compared to SAL injection (149.9 ± 45.4 sec vs. 41.9 ± 38.5 sec after MUS and SAL injection, respectively; $t_{(15)} = -8.4$, $P < 0.001$). Notably, the MUS-injected rats were actively exploring the maze (Fig. 5A). The

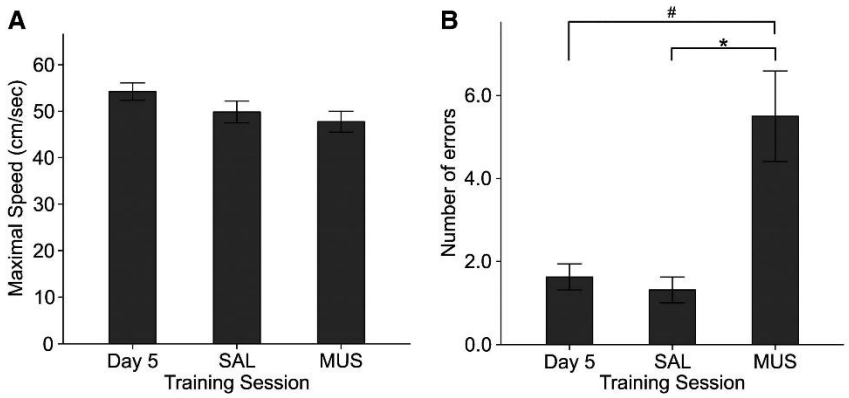

Figure 5. The RE inactivation impairs performance of a spatial task. (*A*) The maximal movement speed on the maze during the drug-free session (day 5, $n = 16$), after intrabrain injection of SAL ($n = 16$), and after MUS injection in the RE ($n = 16$). (*B*) The number of errors was significantly higher in the MUS-group. (*) $P < 0.01$ and (#) $P < 0.05$ (Bonferroni corrected). Error bars represent ±SEM.

trajectory length was twice as long after the RE inactivation (33.0 ± 17.2 vs. 16.0 ± 11.1 maze sections for MUS and SAL, respectively; $t_{(15)} = -4.6$, $P < 0.001$). Remarkably, the MUS-induced performance deficit was preserved during all 10 trials of the maze session (Supplemental Fig. S5). To quantify this observation, we restricted the analysis to rats with unaffected motor activity (Supplemental Fig. S3, Cluster 1). The number of errors was stable across trials and significantly lower on day 5 or after SAL injection than after MUS injection (repeated ANOVA, trials: $F_{(1.419,21.28)} = 0.864$, ns). Thus, the RE inactivation caused a persistent spatial memory deficit as the task performance did not improve with repeated trials.

Discussion

The findings of the present study suggest that nucleus RE has a selective regulatory effect on the network that support the active phase of learning (information retrieval and encoding), but not involved in the mechanisms of systems consolidation occurring after learning or "off-line." Specifically, we found that the RE inactivation after each of the five learning sessions did not affect the rat's ability to acquire a spatial task, nor did it result in a faster memory decay over a 20-d "forgetting" period. In contrast, the RE inactivation in well-trained rats prior the maze exposure strongly impaired their task performance. Notably, the unaffected motor activity and appetitive motivation were both suggestive of a memory retrieval deficit. Strikingly, there was no improvement in the task performance across subsequent trials of the same session; thus, neither "delayed" memory retrieval (e.g., after first few trials) nor relearning occurred in the RE-inactivated rats. It is possible, that the RE inactivation affected the ability to navigate the maze or to make goal-directed decisions. Indeed, at the start position a rat was required first to localize itself in the environment, likely by using intra- and extramaze cues, then "retrieve" the reward location and navigate along the shortest trajectory based on previously acquired (and stored) information. At each maze crossing, a decision-making process (e.g., turn right or left) could, in principle, take place based on the rat's current position and information stored in working memory and/or retrieved from remote memory. However, deficit in navigation or decision-making would also affect spatial learning, which remains largely preserved after the RE lesion or inactivation (Dolleman-van der Weel et al. 2009; Loureiro et al. 2012; Ito et al. 2015). The intriguing possibility of the RE involvement in decision-making or in cognitive flexibility shall be certainly addressed in future studies by testing animals in both spatial and nonspatial tasks. It is also unlikely that the RE inactivation impaired the rat ability for stimulus–response association as an earlier study showed no effect when the RE-lesioned rats were tested in a visually guided task (Hembrook and Mair 2011).

Our findings may appear to contradict the result of other studies that revealed no effects of the RE inactivation on performance of the Morris water maze (MWM) task (Loureiro et al. 2012; Cholvin et al. 2013). Yet, the discrepancy in the results may actually be explained by the task- and time-specific activation of the memory supporting large-scale network. It is well established that learning of the MWM task depends on the HPC, but does not require the PFC (de Bruin et al. 1994; Sloan et al. 2006). Notably, the recruitment of the PFC is required for the retrieval of remote (25–30 d after learning), but not recent (1–5 d) memory in the MWM (Teixeira et al. 2006; Lopez et al. 2012); the latter is consistent with time-dependent reorganization of the circuit supporting memory storage (Frankland and Bontempi 2005). Interestingly, in a modified version of the MWM task, namely, under partial-cue conditions, the PFC is required also for recent memory retrieval (Jo et al. 2007). Furthermore, when rats are tested on a double-H water maze or on a T-maze, both the HPC and the PFC are engaged in the spatial task performance (Cholvin et al. 2013; Layfield et al. 2015). Therefore, the RE inactivation appears ineffective when the HPC–PFC interaction is not required for behavioral execution. Besides, as noted by Hembrook et al. (2012), cognitive demands of the MWM maze task may be insufficient to reveal less pronounced memory deficits like, for example, in cases of lesion of the ventral midline thalamic nuclei. Collectively, our results suggest that the RE may critically contribute to the retrieval of spatial memory and, possibly, to spatial navigation; yet it does not appear to be involved at least in the early phase of "off-line" processing leading to memory stabilization. Our results also support the view that the RE is important for cognitive functions that depend on the HPC–PFC interaction (Hembrook et al. 2012; Cholvin et al. 2013, 2016; Layfield et al. 2015); memory retrieval, working memory, and spatial navigation belonging to such brain functions (Churchwell et al. 2010; Benchenane et al. 2011; Gordon 2011; Eichenbaum 2017).

The behavioral effects of the RE inactivation reported here support the idea that the RE may coordinate the PFC–HPC interactions. Specifically, gamma-range synchronization between the HPC and PFC is thought to mediate encoding and updating task-related spatial information (Spellman et al. 2015). The PFC–HPC synchrony at slower (4–12 Hz) frequency range presumably facilitates integration and maintenance of information in working memory (Jones and Wilson 2005). The spike-phase coherence and cross-frequency coupling between the HPC and PFC have been attributed to memory encoding (Siapas et al. 2005; Benchenane et al. 2010). Finally, both HPC and PFC function is critical for memory retrieval (Churchwell et al. 2010; Lopez et al. 2012; Cholvin et al. 2016).

The following mechanisms described by now in the literature can account for our results: (1) the excitatory input from the RE may facilitate the HPC–PFC coupling (Dolleman-van der Weel et al. 1997; Di Prisco and Vertes 2006; Hallock et al. 2016; Roy et al. 2017); (2) the neural activity in the CA1 may be adjusted via the HPC output to the RE (Dolleman-van der Weel et al. 1997); (3)

the RE output may affect spatial coding in the HPC and/or spatial information from the HPC to the PFC may be transferred via the RE (Jankowski et al. 2014; Ito et al. 2015). Besides, the RE, as a part of the circuitry mediating top-down control of dopamine neurons in the ventral tegmental area (Zimmerman and Grace 2016), may influence reward-motivated behaviors. Therefore, if the RE, indeed, gates the bidirectional information flow within the HPC–PFC pathway, it is not surprising that the inactivation of RE impaired the spatial task performance, which is dependent on this functional circuit.

It is also possible that the RE contributes to the systems consolidation, but the RE inactivation in our experiments was not sufficient to cause interference of "off-line" processing. Most studies are consistent in reporting that the effects of MUS reach maximum within 30 min after injection and last for at least 2 h (Martin 1991; Edeline et al. 2002; Allen et al. 2008). Depending on the injection volume and concentration, it has been shown that the effects of MUS injection can last up to 6 h (Brandon et al. 2011). In our study, we made an effort to make a rather small injection by using MUS concentration of 0.27 μg/μL and volume 0.19 μL. The acute effects of MUS injection (30–60 min) were rather robust as reflected by impaired task performance on the memory retrieval test or by motor deficit in case of injection outside the RE. The diffusion of fluorophore-conjugated MUS was ~1 mm radius, which was likely smaller than the diffusion of unlabeled MUS. Taking into account MUS diffusion over time (Edeline et al. 2002), it is unlikely that inactivation of the RE was insufficient; moreover, the adjacent brain regions including the rhomboid nucleus were likely affected by MUS. A higher concentration of MUS or a larger volume would compromise even more the spatial selectivity of the affected brain area. In our experiments, the RE activity was substantially suppressed within at least the first hour after learning when the experience-induced neuronal ensembles replay (Wilson and McNaughton 1994; Peyrache et al. 2009) and other learning-induced changes of HPC and PFC population activity occur (Eschenko et al. 2006, 2008). Consistently, several studies reported effects on memory consolidation due to manipulation of neural activity during the first hour after learning experience (Girardeau et al. 2009; Ego-Stengel and Wilson 2010; Maingret et al. 2016; Novitskaya et al. 2016). It is possible that the duration of the RE inactivation was insufficient to interfere with the mechanisms of systems consolidation or the RE activity may be critical within a delayed post-learning time window.

Finally, any behavioral study of memory unavoidably faces methodological drawbacks, which may complicate the interpretation of results. We used the crossword-like maze, which belongs to a family of multi-unit T-mazes (Tolman 1948) or can be considered as one of configurations of the Hebb–Williams maze (Hebb and Williams 1946); both mazes have been designed and intensively used for studying spatial cognition in rodents. Our experimental design fulfilled the requirements for allocentric navigation (Vorhees and Williams 2014). Successful task performance required spatial orientation at the start position, retrieval of acquired cognitive/spatial map, and use of path integration for updating the animal current position on the maze. The essential role of the HPC in spatial learning and memory is well-established; there is also substantial evidence that the PFC is involved in goal-directed spatial navigation (Porter and Mair 1997; Ragozzino et al. 1998; Dias and Aggleton 2000; Hok et al. 2005; Fujisawa et al. 2008; Churchwell et al. 2010). Moreover, extensive literature exists that learning and performance of various maze tasks depends on the HPC–PFC interaction (Floresco et al. 1997; Jones and Wilson 2005; Benchenane et al. 2010; Hyman et al. 2010; Gordon 2011). The task performance deficit after the RE inactivation observed in our study is consistent with a notion that the RE contribution is critical for the cognitive functions requiring coordinated activation of both the HPC and the PFC (Hembrook et al. 2012; Cholvin et al. 2013; Layfield et al. 2015). However, the involvement of other memory types (e.g., working or procedural) was also—in principle—possible. The working memory, for instance, may have played a role for storing information about visited maze alleys on a given trial. The engagement of procedural memory (e.g., remembering the sequence of turns) cannot be excluded, however it would only be expected at later stages of learning (Packard and McGaugh 1996). The task performance based on procedural memory may be, indeed, advantageous in a stable and highly predictable environment; yet, for example, the action sequence learning does not depend on the RE activity (Hembrook and Mair 2011). Finally, parallel use of different memory systems and navigation strategies could also take place (Iglói et al. 2009). The retrieval of cognitive map may speed up animal orientation at the start position, memory about the maze configuration may facilitate path integration, maintenance in working memory information about visited locations may help for updating the animal current position, and contextual decision-making would help targeted navigation. This scenario is generally consistent with the role of the RE for performance of working memory-dependent spatial tasks (Hembrook and Mair 2011; Hembrook et al. 2012; Hallock et al. 2013, 2016; Layfield et al. 2015). The aforementioned methodological concerns open a possibility that a compromised spatial behavior after the RE inactivation was due to an integrated deficit in the retrieval of spatial memory, in the working memory, in the ability to navigate or make decisions. To further clarify the nature of the behavioral deficit produced by the RE inactivation, testing animals in various situations differing by predominant cognitive demands is crucial.

All in all, our findings further support the hypothesis that the RE is critical for spatial navigation and memory retrieval, yet do not provide evidence for the RE involvement in the mechanisms of systems consolidation, at least during the time window immediately following learning experience.

Materials and Methods

Male Sprague Dawley rats (Envigo, Huntingdon, UK) weighing 300–350 g at the beginning of experiment were kept in pairs with food and water ad libitum on 12 h/12 h light–dark cycle. All the experiments were performed during the dark cycle. When rat appetitive behavior was tested, rats were kept on a food-restricted diet to ensure their appetitive motivation at times of behavioral testing. On these days, in addition to the chocolate milk (0.6 mL) obtained as reward during maze exposure, each rat received 15–20 g of food pellets and unlimited access to water in their home cage. Rat weight was monitored on a daily basis and kept at ~90% of ad libitum body weight. All experimental procedures were approved by the local authorities (Regierungspräsidium Tübingen, Germany, Referat 35, Veterinärwesen) in accordance with the regional animal welfare committee pursuant to §15 of the German Animal Welfare Act (Kommission nach §15 des Tierschutzgesetzes), and were in full compliance with the Directive 2010/63/EU of the European Parliament and of the council on the protection of animals used for scientific purposes.

Surgical procedures

We performed surgeries following standard aseptic procedures. Briefly, before surgery rats were deeply anesthetized with isoflurane (4% induction, ~1.5% maintenance) and placed in a stereotaxic frame (David Kopf Instruments). Subsequently, the skull was exposed, a burr hole was drilled to target the RE using the following stereotaxic coordinates: AP/ML = −1.8/−1.5 mm (Paxinos and Watson 2005). Furthermore, a stainless-steel guide tube (22-gauge, Plastics One Inc.) was inserted 4.9 mm deep relative to the surface of the brain and at a medial-lateral angle of 10° to avoid damaging the sagittal sinus. The guide tube was fixed to the skull using dental

cement and stainless-steel anchor screws. After the end of the surgery we placed a dummy cannula inside the guide tube to prevent the brain tissue growth. Rats were allowed to have a 1-wk post-surgery recovery before behavioral testing began.

Behavioral apparatus and training procedures

The crossword maze (130 × 130 cm) was custom-built from black polyvinyl chloride. The perpendicular maze alleys (4 × 4) formed nine identical square sections (Fig. 1A). Maze alleys were 10-cm wide and had 2-cm high rims on both sides. There were two start locations and one reward port connected via tubing with a pump (Izmatec) releasing liquid reward (chocolate milk). To reduce the number of alternative routes on the maze we placed nine vertical barriers (30-cm high and 25–40-cm wide) at specific crossing points, thus restricting the access of the animal to some maze sections. The maze was elevated 80 cm above the floor and surrounded by black curtains; two posters served as extramaze visual cues. All behavioral procedures were performed under dim light.

For habituation, the maze was converted to a single linear alley. A rat was released from one end of the alley, had to reach the reward port at the other end and obtain reward by nose poking. This procedure was repeated until the rat behavior became consistently reward oriented. The start and reward locations were different from the ones used for the main task; extramaze visual cues were removed. Rats were also habituated to the intrabrain microinjection procedure by handling. After three habituation sessions, rats were trained on a spatial task for five consecutive days. Two start locations were used in pseudo-random order to minimize the procedural component of learning; the two shortest (correct) trajectories leading to reward partially overlapped (Fig. 1A). A rat was released from the start location and allowed 3 min to reach the reward port. After reward consumption or after the trial cut-off time elapsed, the rat was removed from the maze and left in a waiting box for 3–5 min. During each intertrial interval the maze alleys were wiped to minimize local olfactory cues. Each training session consisted of 10 trials. Immediately after the learning session rats received either intrabrain injection of phosphate-buffered saline (SAL-group) or muscimol (MUS-group) and returned to their home cages. On days 6 and 7, rats received either SAL or MUS injection 30 min before the maze exposure (Fig. 1B). On days 8 and 9, rats were trained on the same task (without any drug injections) to further stabilize the acquired spatial memory and then kept in their home cages for 20 d without any behavioral testing; during this "forgetting" period rats had food and water ad libitum, except 24 h before the remote memory retrieval test (Fig. 1B).

Intrabrain microinjection

The MUS powder (Sigma-Aldrich) was diluted in SAL at a final concentration of 0.27 µg/µL. The injections were performed using Hamilton syringe loaded into a microinfusion pump (UMP3, WPI). For the drug injection procedure, a rat was gently restrained by hand, the dummy cannula was removed and the injection cannula was inserted inside the guide tube. The tip of the injection cannula extended 2 mm beyond the tip of the guide tube. We used polyethylene tubing to connect the injection cannula to the Hamilton syringe. MUS or SAL (0.19 µL) was infused over 60 sec. The injection cannula was left in place for additional 2 min before it was slowly retracted. The dummy cannula was then inserted back to the guide tube.

Data analysis

The rat behavior on the maze was video recorded by an infrared camera. The trial time was measured manually using a stopwatch. The rat trajectories on the maze were reconstructed manually from video and the movement speed was extracted and further analyzed using custom made program in MATLAB (MathWorks). Any deviation from the "correct" (shortest) trajectory leading to reward was considered as an error. Specifically, if a rat entered a maze alley, which did not belong to the "correct" path, with all four limbs, it was scored as an error. Further animal passage along other maze alleys outside of the "correct" path after deviating was not scored as additional errors unless the rat returned to the correct path (see examples on Supplemental Fig. S1A). Moving along the correct trajectory, but in opposite direction (away from reward port) was also considered as an error (see examples on Supplemental Fig. S1B). The trajectory length, the time to reach reward, and the number of errors were extracted for each trial. To assess the "content" of spatial memory from the rat trajectory, maze alleys were arbitrary divided into 24 equal-length sections separated by maze crossings; each maze section was assigned a unique number and the movement sequences analyzed. Thus, the shortest (correct) trajectory from the Start 1 (S1) consisted of nine maze sections (Fig. 1A). For each trial, we extracted the mean length of the rat trajectory (number of maze sections) overlapping with the correct path before deviation from the correct path.

Behavioral variables during five learning sessions were submitted to the repeated-measures ANOVA; post hoc comparisons were made when appropriate using Bonferroni correction. Data were tested for equality of error variance and Greenhouse–Geisser correction was applied whenever the assumption of sphericity was violated. The one-way ANOVA or Student *t*-test was used for between-group comparisons. The statistical significance α-value was set at $P < 0.05$ level. The IBM SPSS Statistics (v.22) software package was used for statistical analysis.

Perfusion and histology

At the end of behavioral experiments, we injected each rat with fluorophore-conjugated MUS (Thermo Fisher Scientific). This helped to localize the site of injection and evaluate the extent of drug diffusion. After ~30 min post-injection, the rat was euthanized with a lethal dose of sodium pentobarbital (100 mg/kg i.p.; Narcoren, Merial GmbH) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was removed and stored in the same fixative. Before sectioning, the whole brain was placed in 30% sucrose solution at 4°C until they sank. Serial 50-µm-thick coronal sections were cut on a horizontal freezing microtome (Thermo Fisher Scientific). Every second section was Nissl-stained; adjacent sections were stored for examination under the fluorescent microscope (AxioVision, Carl Zeiss). Position of the injection cannula tip was assessed visually and digitized.

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Supplemental Figures

LEARNMEM/2017/047134: The activity of thalamic nucleus reuniens is critical for memory retrieval, but not essential for the early phase of 'off-line' consolidation

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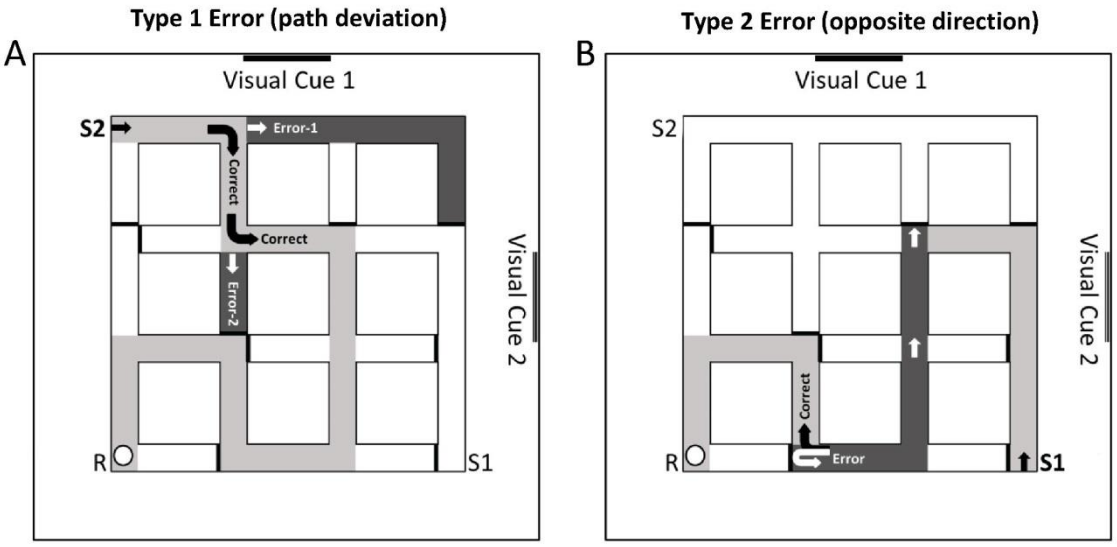
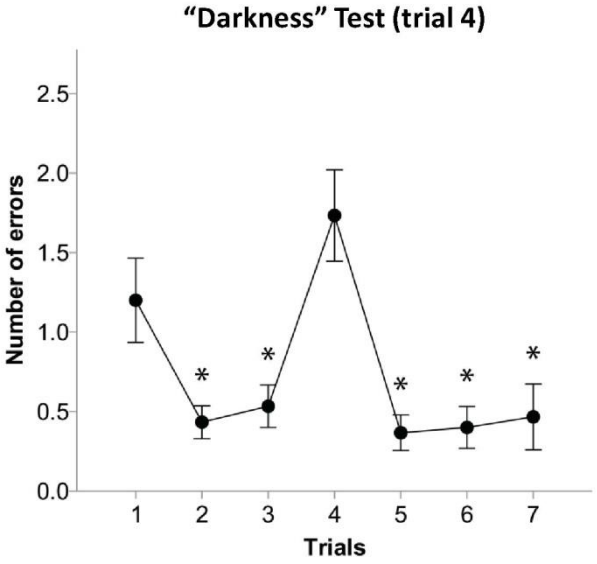
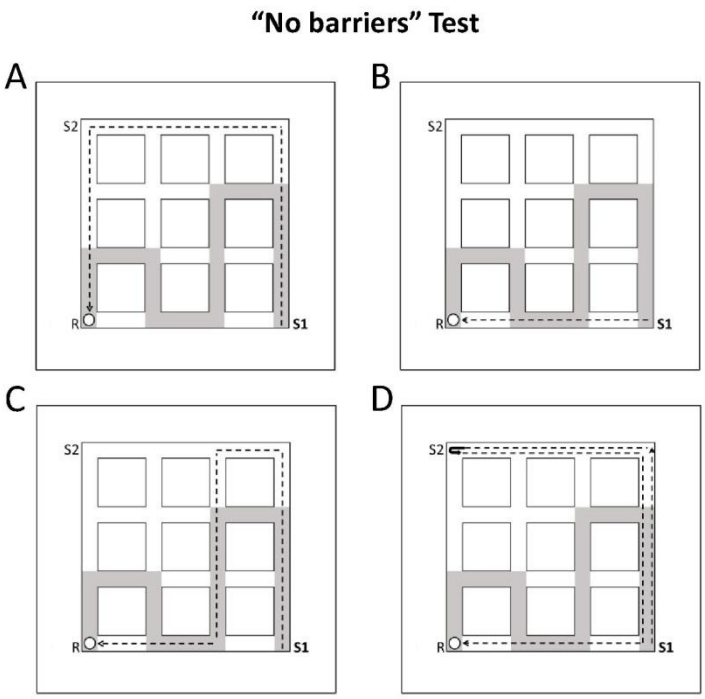


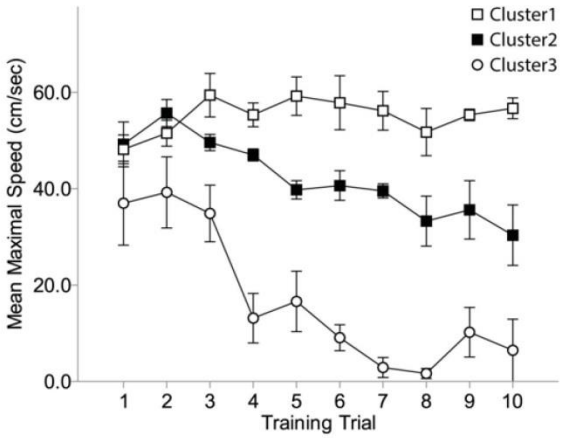
Supplemental Figure 1. Examples of navigation errors. Top-down view of the experimental environment is shown. The crossword-like maze consisted of perpendicular alleys; vertical barriers (thick black lines) blocked the access to some maze sections. Rat was placed on one of the two start locations (S1 or S2) and had to reach reward (R). The 'correct' (shortest) and 'incorrect' trajectories are shown in light and dark grey, respectively; arrows indicate the direction of navigation. A, Example of a "path deviation" error. A rat released from S2 continued straight forward (Error-1) instead of turning right at the first maze crossing. Note, 'incorrect' trajectory led to a dead-end and returning to the same maze crossing was required to complete the trial. A deviation from the 'correct' path was considered as a single error regardless of the total number of maze alleys visited. For example, after Error-1, the rat returned to the first maze crossing and followed the 'correct' path until the next maze crossing, where, instead of turning left, it continued straight forward (Error-2) along the blind alley. After returning to the 'correct' path, the rat reached reward without making further errors. Thus, in this example trial the total number of errors equaled 2. B, Example of a "navigation in opposite direction" error. A rat was released from S1 and navigated along the 'correct' path by crossing 6 maze sections until, instead of turning right, it made a U-turn. The rat continued moving in the wrong direction until it reached the barrier. The 'incorrect' path is shown in dark grey, white arrows indicate navigation in opposite direction. After correcting the navigation error, the rat reached reward without making further deviations. In this example trial, there was 1 error.



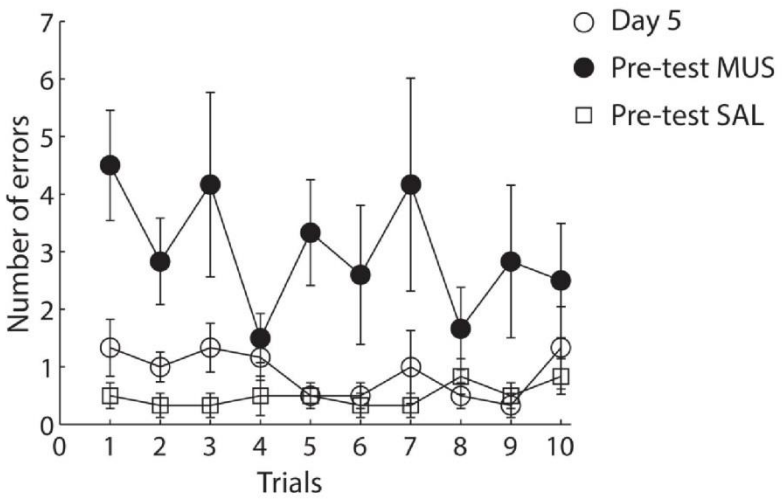
Supplemental Figure 2. The "darkness" test. The accuracy of task performance on day 6 is shown for each trial. Trials 1-3 and 5-7 were run under standard conditions. Before trial 4, room lights were turned off and rats had to navigate the maze in the darkness. The number of errors on trial 4 was significantly higher compared to all other trials (except trial 1). This result implied that during standard trials (lights on) rats used distal visual cues for navigation on the maze, but did not rely on a motor habit. * - $p < 0.01$ compared with trial 4 (Bonferroni corrected, N = 30). Error bars represent ± SEM.



Supplemental Figure 3. Representative rat trajectories during "no-barriers" probe trial. A-D, Top-down view of the crossword-like maze. On day 6, two probes were made on trials 4 and 8. Trial 4 was run in the darkness (see Fig. S2). During inter-trial interval before trail 8, all vertical barriers blocking access to some maze alleys (see Fig.1A) were removed and the room lights were turned off. All rats were released from the same start location (S1) and easily reached reward (R), however, by following the trajectories (dashed lines) that differed from the 'correct' one (grey). Most common trajectories on trial 8 are shown on A (N = 17) and B (N = 6); less common trajectories are shown on C (N = 2) and D (N =2). Note, none of the trajectories matched the 'correct' path indicating that rats did not rely on a motor habit.



Supplemental Figure 4. Effects of the intra-brain MUS injection on rats' motor activity. The maximal movement speed on the maze is shown for all trials of the post-injection session. Rats were classified according to the dynamics of their motor activity using the K-means clustering analysis. Cluster 1 - motor activity unaffected, Cluster 2 - gradual decay of motor activity, and Cluster 3 - motor activity suppression. Error bars show ± SEM.



Supplemental Figure 5. The RE inactivation caused a persistent performance deficit. The mean number of errors in each trial is plotted for the drug-free session (day 5) and after SAL or MUS injection in the RE. Note, MUS-induced performance deficit persisted throughout the session. Error bars show ± SEM.

4.2 A.2 : Investigation of cross-regional interactions within the prefrontal-thalamo-hippocampal circuit associated with spatial cognition in the rat

Investigation of cross-regional interactions within the prefrontal-thalamo-hippocampal circuit associated with spatial cognition in the rat

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In preparation for submission

Investigation of cross-regional interactions within the prefrontal-thalamo-hippocampal circuit associated with spatial cognition in the rat

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Abstract

The interplay between medial prefrontal cortex (mPFC) and hippocampus plays a critical role in spatial memory process. The nucleus reuniens (RE) which has anatomical connections with both prefrontal cortex and hippocampus may contribute to the indirect mPFC-HPC communication. We here report spatial cognition related RE activity within the mPFC-RE-HPC circuit. While the rats were trained to learn a crossword maze task, we found synchronized gamma activity between mPFC and RE as well as between HPC and RE. Before the incorrect decision, we observed strong coupling between RE high frequency oscillation and HPC theta around the gamma synchronization event. The enhanced RE-HPC coupling was also found when rats were on the incorrect maze segments. The mPFC-RE and HPC-RE gamma synchronization could happen concurrently and was associated with strong coupling between RE and HPC. The finding indicates that the gamma synchronization may be a mechanism gating the spatial information transfer within the mPFC-RE-HPC circuit.

Introduction

The interaction between prefrontal cortex (mPFC) and hippocampus (HPC) has been suggested to play a key role in navigation and memory processing[1-4]. By now, the mPFC and HPC were found to interplay in a way of synchronization. For instance, it was shown that

mPFC and HPC synchronized in theta range during a spatial task[5]. Siapas and colleagues also revealed that the mPFC spikes were phase locked to HPC theta[3], while the similar finding was also observed in the study by Benchenane et al.[2]. The anatomical connections between mPFC and HPC have been well established. There is a dense projection arising from ventral HPC and terminating in ventral mPFC[6-8], this direct projection has been demonstrated to contribute to spatial working memory encoding[1]. Recently a sparse projection from mPFC to HPC was found to contribute to spatial memory retrieval[9]. Besides these direct anatomical connections between mPFC and HPC, the indirect connection between mPFC and HPC through the nucleus reuniens (RE) starts drawing more attention and has been discussed recently. The nucleus reuniens (RE) has been considered to be a critical node which links the mPFC and HPC in an indirect way[6,10-13]. RE was found to have dense reciprocal connections with mPFC and HPC, moreover, some RE neurons show simultaneous projection to both mPFC and HPC[12,14]. The anatomical connections with mPFC and HPC place RE in a critical position in modulating the mPFC-HPC communication which underlies complicated brain functions. In addition, the studies on RE have found that the electric stimulation in RE could induce sub-threshold excitation of pyramidal neurons of CA1, supra-threshold excitation of putative CA1 interneurons[15], as well as excitation in mPFC[16]. These studies further suggested a potential role of RE in modulating the interaction between mPFC and HPC. The lesion/inactivation of RE has been found affect the animal's performance in various tasks which depend on both mPFC and HPC[17-19]. These findings further support the idea that the RE may contribute to mPFC-HPC interaction. Recently, Xu found that RE plays a critical role in fear memory generalization circuit which also involves both mPFC and HPC[20], while Ito et al. revealed the RE contribution to spatial navigation and proposed that RE could transfer the spatial information from mPFC to HPC[21]. To study the RE contribution to spatial memory which depends on the mPFC-HPC coordination, in our previous study, we temporarily inactivated RE during a spatial learning task and found a deficit in 'on-line' memory process(memory retrieval) after the RE inhibition[22], which is in line with the hypothesis that the RE is involved in spatial memory process. However, the electrophysiological mechanism behind the RE contribution to spatial memory is still unclear.

In current study, we applied LFP recording simultaneously in mPFC, RE and HPC when rats were trained to finish a spatial learning task. By applying ICA analysis, we first identified a high gamma oscillation (60-150 Hz) in RE which nested in HPC theta. As the gamma synchrony has been considered to be one of the mechanisms underlie the cross-talking between the spatially distributed brain regions[23-25], we then studied the gamma synchrony between mPFC/HPC and RE with the high gamma band we detected. It was found that the maximal amplitude and the duration of the mPFC-RE and HPC-RE gamma

synchrony events did not show significant difference before the decisions show different memory accuracy.

Besides the gamma synchrony, the cross-frequency coupling has also been suggested to be a mechanism for modulating the cross talking between different areas[4,26,27]. By using the high gamma synchrony event as trigger, we found that the event-triggered phase-amplitude coupling showed stronger RE gamma-HPC theta coupling before the incorrect decision making, indicating that the event-triggered cross-frequency coupling is correlated to the memory process in our task. The enhanced event-triggered phase-amplitude coupling was also observed when rats were in the maze segments which deviated from the correct trajectory. Thus, the stronger coupling between RE and HPC may reflect an enhanced memory demand. While the mPFC-RE and HPC-RE gamma synchrony event could happen individually (Nonco-event), we also observed these two types of events happened with a short interval (Co-event).It was found that when mPFC, RE and HPC all synchronized in gamma range with in a short time window (Co-event), there was significant stronger coupling between RE high gamma oscillation and HPC theta. Eventually, it was found that there was a difference between the event-triggered RE-HPC coupling when the mPFC-RE and HPC-RE gamma synchrony event happened individually. This finding further supports the hypothesis that the spatial information in mPFC could be transferred to HPC via RE.

Materials and Methods

6 male Sprague Dawley rats (Charles River) weighting 300-350g at the beginning of experiment were single housed with food and water *ad libitum* on 12h/12h light/dark cycle. All the experiments were performed during the dark cycle. When rat appetitive behavior was tested, rats were kept on a food-restricted diet to ensure their appetitive motivation at times of behavioral testing. On these days, in addition to the chocolate milk (0.6 ml) obtained as reward during maze exposure, each rat received 15-20g of food pellets and unlimited access to water in their home cage. Rat weight was monitored on a daily basis and kept at ~90% of *ad libitum* body weight. All experimental procedures were approved by the local authorities (Regierungspräsidium Tübingen, Germany, Referat 35, Veterinärwesen) in accordance with the regional animal welfare committee pursuant to §15 of the German Animal Welfare Act (Kommission nach §15 des Tierschutzgesetzes), and were in full compliance with the Directive 2010/63/EU of the European Parliament and of the council on the protection of animals used for scientific purposes.

Surgical procedures

The rats were first anesthetized with 4% isoflurane for induction, the anesthesia was then maintained with 1.5% isoflurane during the surgery. After the rat's head was fixed in stereotaxic frame (David Kopf Instruments), the skull was exposed and three craniotomies were performed on the same hemisphere. According to the brain atlas by Paxinos et al.[28], circular holes were drilled above mPFC (AP/ML = 3.1/0.8 mm), HPC (AP/ML = −3.8/2.4 mm) and RE (AP/ML = −1.8/−1.3 mm). The platinum-iridium electrode (FHC Inc.) was implanted to mPFC (DV = 3.4 mm), HPC (DV = 2 mm) and RE (DV = 6.8 mm, Medial-Lateral angle = 10°), respectively. Rats were allowed to have an at least 1-week post-surgery recovery before behavioral testing began.

Recording during the behavior task

As described previously in our previous study[22] , the custom-built black crossword maze (130 × 130 cm) was applied in our study. The perpendicular maze alleys (4 × 4, 10-cm wide with 2-cm high rims on both sides) formed nine identical square sections. Nine vertical barriers (30-cm high and 25–40-cm wide) were placed on the maze to restrict the navigation to specific directions at specific crossings (Figure 1A). Thus, to get the only reward port on the maze, the rats needed to navigate in specific trajectory. The maze was elevated 80 cm above the floor and surrounded by black curtains with two different posters on it. After the 3-day habituation period, during the training session, the rats were randomly released from one of the two start locations in each trial, rats received the reward from the nose-poking structure if they arrived the reward port in the time limit (3 min). For each training session, rats received 5 trials released from each of the two start locations. The sequence of the start locations was in a pseudo-random order and varied over the training sessions. After each trial the rats were kept in a waiting box for 3–5 min and the olfactory cues on the maze from last trial were wiped out during the inter-trial interval. The training was performed under dim light.

The simultaneous recording in mPFC, RE and HPC was performed with Cheetah recording software (Neuralynx) when rats were trained to finish the spatial task. A bandpass filter with 1-9000 Hz passband was applied while the electrophysiology signal was sampled in 32556 Hz. The LFP data were band-pass filtered with a pass band from 1 to 250 Hz and further down-sampled to 660 Hz. The LFP data were then used for all the following analysis.

Motion detection and behavior definition

A camera synchronized with the recording system was used to monitor the animal's behavior with 25 frame rate. Two LEDs on the headstage were detected with a custom-written MATLAB program and rat's position was represented as the middle point

between the two LED dots. The visit to a specific maze segment was defined when the rat's position was detected to cross the midline of that maze segment. When the LEDs that were detected on specific maze segment, it was considered to be the rat's movement on that segment. While the LEDs were detected to be out of the last segment but not yet on the next segment, it was also considered to be the movement of the last maze segment.

Data analysis

Theta-nested gamma oscillation in RE

The analysis for extracting the independent components which nest in theta oscillation was elaborately described in the study by Lopes-Dos-Santos[29]. Generally, we used the same method as used in the above study. The only difference was in current study we decomposed the signal by directly filtering the signal, rather than applied the Ensemble Empirical Mode Decomposition (EEMD) method. The theta signal in HPC was obtained by band pass filtering the signal with the band 5-12 Hz, the low-frequency signal was obtained by low pass filtering the HPC signal (with 5 Hz cut-off frequency). The supra-theta signal in RE was defined as the high pass filtered RE signal with frequency above 30 Hz. The theta cycle candidates were detected as the period between two consecutive troughs which surrounded one peak, the duration of qualified theta cycle candidate should be no less than 71 ms and no more than 200 ms. The valid theta cycle was defined as the theta cycle with absolute values of both peak and troughs higher than the corresponding values of envelope of the low frequency signal.

Once the qualified theta cycles were detected, for each theta cycle, the spectrum vector of supra-theta signal was calculated by averaging the wavelet spectrum along the time of that theta cycle. The matrix contained multiple spectrum vectors was then submitted to the principal component analysis (PCA), the first 5 components were then applied by the independent component analysis (ICA). Eventually, the statistically independent components of supra-theta signal in RE that nested in HPC theta were extracted.

Gamma synchrony calculation and gamma synchrony event detection

For gamma synchrony calculation, the data from two brain areas were first bandpass filtered with the frequency band 70-150 Hz. The Hilbert transform was then applied to both andpass filtered signals and the phase (P_1 and P_2) and amplitude (A_1 and A_2) of the two signals were obtained. As described in the study by Yamamoto[25], the gamma synchrony signal between paired brain structures was obtained by the equation: $A_1 \times A_2 \times cos(P_1 - P_2)$. The envelope was obtained by applying the Hilbert transformation to the gamma

synchrony signal. The envelope signal was then normalized and the 2-SD threshold was used to detect the gamma synchrony events candidates. The candidates with the duration above the threshold longer than 1/70 second (one cycle of lowest frequency) were considered to be qualified gamma synchronization events. The duration of the event was defined as the length of signal around the event peak with amplitude above 1-SD. The event amplitude was defined as the maximal value in the gamma synchrony signal corresponded to time of envelop signal with values above the threshold.

Phase-amplitude coupling matrix

The modulation index of phase-amplitude coupling was a measurement to quantify the coupling between the amplitude of high-frequency oscillation and phase of low-frequency oscillation. The modulation indexes were calculated with the 1000-ms long window centered at the gamma synchrony event. The modulation index was calculated between different high and low-frequency oscillation pairs, while the low frequency oscillation stepped in 1 Hz with 2 Hz band-width, the high frequency oscillation stepped in 2 Hz with 4 Hz band width. After the calculation of multiple/consecutive oscillation pairs, the phase-amplitude coupling matrix was obtained in the end. Each element in the matrix represents the modulation index of specific high and low frequency oscillation pair. The comodulogram was plotted with the phase-amplitude coupling matrix.

We calculated the modulation index with the method described in Tort's study[30]. The signals from RE and HPC were first band-pass filtered with corresponding pass bands. After the obtaining of high and low frequency oscillations, the amplitude of high frequency signal from RE and phase of low frequency signal from HPC were calculated with the Hilbert transform, respectively. The amplitude and phase signals were synchronized time series, the amplitude of each discrete phase point was defined as the value of the corresponding amplitude point. The phase signal was then binned with 20° interval and 18 bins were obtained. The amplitude of each bin *j* was calculated by averaging the amplitude over each phase bin and was eventually denoted as: $\langle A \rangle_P(j)$. As described in the study by Tort et al., the modulation index of the phase-amplitude was defined by:

$$MI = \frac{H_{max} - H}{H_{max}} .$$

While the *H* was defined as entropy and given by:

$$H = -\sum_{j=1}^{N} p_j \log p_j .$$

Where the N represents the bin numbers and the p_j is calculated as:

$$p_j = \frac{\langle A \rangle_P(j)}{\sum_{j=1}^{N} \langle A \rangle_P(j)} .$$

As described in Tort's study[30], to define the significance threshold of modulation index in the event-triggered phase-amplitude coupling, the base phase-amplitude coupling was

used. In current study, the base phase-amplitude coupling was calculated by using the 1000-ms window centered at the randomly chosen points during the base activity, which had no overlap with the evet-triggered window. After averaging the base phase-amplitude coupling matrices, the distribution of modulation index in the averaged matrix was then obtained. By assuming distribution as a normal distribution, the modulation index which indicates the *P*<0.01 was considered as significance threshold. In the main article, each event-triggered phase-amplitude matrix was subtracted with the threshold and any value in the comodulagram that was above 0 indicated statistical significance.

Statistics analysis

The statistical significance α-value was set at $p < 0.05$ level. When compared different event-triggered phase-amplitude coupling groups, for example, group A and group B, we applied an element to element statistics analysis. Each element (M_{ij}) in the modulation index matrix represented the strength of the coupling between specific high-frequency oscillation in RE and slow-frequency oscillation in HPC. In group A, the specific element in the same position of the modulation index matrix was subtracted as vector: $\langle M_{ij}\rangle_1, \langle M_{ij}\rangle_2 \ldots \langle M_{ij}\rangle_n$, while the *n* presents the size of group A (number of matrix). The corresponding vector of the same matrix element would be: $\langle M_{ij}\rangle_1, \langle M_{ij}\rangle_2 \ldots \langle M_{ij}\rangle_m$ in group B, while the m is the size of group B. The two vectors would be subjected to Wilcoxon rank sum test and the *P*-value for specific matrix element was calculated. After calculating the *P*-value for all the elements in the event-triggered modulation index matrix, a new *P*-value matrix was obtained. Any element with *P*-value>0.95 indicated a significant difference.

Results

Gamma synchronization event distribution

In order to study the learning associated cross-regional coupling within mPFC-RE-HPC circuit, we first validated the existence of cross-regional interaction. The theta oscillation in HPC was first subtracted from the HPC LFP and the qualified theta cycles were then detected. The corresponding RE power spectral density during each HPC theta cycle was calculated and the matrix contained multiple RE PSD vectors was subjected to ICA analysis. We then found the components in RE which nested in HPC theta (Figure 1B). According to the study by Yamamoto et al.[25], the cross-regional gamma synchronization (e.g. between the entorhinal cortex and HPC) can be related to the spatial task performance. To link the coordinated neural activity in the RE, HPC and mPFC with maze task performance, we calculated the gamma synchrony between the RE and HPC (HR pair) and between the RE and mPFC (PR pair) (see Materials and Methods) with the frequency range of the high

gamma component (60-120 Hz) we detected. The high gamma synchrony events (HGS) in each gamma synchrony signal were then detected.

We next analyzed in more detail the spatial pattern of occurrence of the HGS event while rats performed the maze task. We selected the data from the last training day (day5) and from error-free trials (rat passage without any deviation from the correct trajectory) and extracted the number of HGS event for each maze segment and for each signal pair. The correct maze segment sequences for S1 and S2 trial are illustrated on Figure 1A; there were total of 4 and 6 choice points (points where animal could deviate from the correct trajectory) for S1 and S2 trials, respectively (Figure 1B). The average occurrence of HGS event is shown in Figure 1D. Different from the finding in Yamamoto's study which used the T-maze[25], although the distribution of HGS event along the correct trajectory was uneven for both PR and HR pair, we did not find a spatial position where the high gamma synchrony events could specifically happen.

Cross-regional high-gamma synchrony precedes navigation decision making

For both PR and HR-HGS events, the segment10 of S1 trail and segment1 of S2 trial showed high event possibility. In both types of training trial, the corresponding segment was the maze section adjacent to a junction area where the first time animals need make decision between multiple choices. This raised a question: if the HGS events could be correlated to rat decision making process (e.g. selection of next maze segment to enter) or memory process (selection of the correct motor program) in our task. In the first case, the HGS event would be expected to occur before each maze junction, regardless if the rat followed the correct of incorrect trajectory. In case of memory-related processing, the HGS event before the correct and incorrect choice would be expected to be different, since these two decision types reflect different memory accuracy. We extracted HGS events detected between the entering of the maze segment preceding the choice point and the moving out of the choice point. The data were further split according to the rat choice accuracy (entering 'correct' or 'incorrect' maze section after the junction) and signal pair (PR and HR). If the HGS event is required for decision making, there should be no difference between the 'correct' and 'incorrect' choices. Alternatively, difference between groups would indicate that the cross-regional high gamma synchrony may be related to spatial memory. For PR and HR–HGS events, we compared the event duration and event peak amplitude of the first decision area (S1 and S2 trials pooled together). None of the variable predicted the rat choice at the first maze junction (Wilcoxon rank sum test).

To further examine if there were other LFP frequency bands that associated with PR/HR HGS event and could contribute to decision making or memory processing, we then applied cross-regional phase-amplitude coupling analysis (PAC) by centering a 1-second

window at each PR/HR HGS event. Briefly, the temporal relationships between the power of high frequency oscillations (hfo) (30-250 Hz) in the RE LFPs (amplitude component) and the phase of low frequency oscillations (6-20 Hz) in HPC LFPs (phase component) were calculated. The data of the last training day was first estimated.

To specifically test if the transient HPC-RE phase-amplitude coupling around HGS event contributes to some aspects of cognition during the maze task performance (e.g. decision making, memory retrieval), we selected the HGS events occurring prior the first choice point for both S1 and S2 trials (Figure 2) and compared the HGS event-triggered PAC before the correct and incorrect choices (as described above). On day5, we analyzed total of 39 correct and 26 incorrect choices (entering 'correct' or 'incorrect' maze segment after choice point, respectively) made prior the first choice point. Correspondingly, 84 and 39 PR HGS events were detected prior the correct and incorrect first choice, respectively. Similarly, 94 and 35 HR events were detected before the correct and incorrect first choice, separately. Significant coupling was present between the HPC-theta and RE-hfo around both PR and HR HGS event (Figure 2A, 2B). Notably, regardless of the HGS event type (PR or HR), incorrect choices were preceded by stronger phase-amplitude coupling (higher modulation index) between the HPC theta and RE hfo (60-100 Hz) compared to 'correct' choices (Wilcoxon rank sum test, $P < 0.05$).

Similar result was obtained when we extended the analysis to all choice points (Figure 3). On day 5, rats (n = 6) made total 274 correct and 32 incorrect choices. Correspondingly, 259/46 PR and 293/45 HR HGS events were detected prior correct/incorrect choices, respectively. The event-triggered coupling between the HPC-theta and RE-hfo was stronger prior incorrect choices (Wilcoxon rank sum test, $P < 0.05$).

We repeated the above analysis for an earlier (intermediate) stage of learning. On day3, rats made total 239 correct and 70 incorrect choices at all choice points. Correspondingly, 231/151 PR and 245/151 HR HGS events were detected prior the correct/incorrect choice, respectively (Figure 3C, 3D). With some notable differences compared to day 5, the strength of both PR and HR event-triggered RE-HPC coupling was relative week, regardless of the decision accuracy (correct and incorrect choice). Meanwhile, PR HGS event-triggered coupling did not show any difference between the correct and incorrect group. The HR HGS event-triggered coupling differed between correct and incorrect choices at two relatively narrow frequency ranges (~70-80 Hz and ~230-250 and; Wilcoxon rank sum test, $P < 0.05$).

Since S1 and S2 trials had the identical second-half of the trajectory (segments 13-18, Figure 1A), we next compared cross-regional coupling just before animal entering the common path for S1 and S2 trials (segment13, Figure 4). The data from correct choices only were included for this analysis. On day5, we detected total 31/19 PR HGS events and 35/27

HR HGS events for S1/S2 trials, respectively (Figure 4A, 4B). Despite relatively small number of HGS events, a strong phase-amplitude coupling was present between the HPC theta and RE-hfo around both PR and HR HGS events before rat entered the common maze trajectory (Figure 4). The RE LFPs showed strong HPC theta modulation was at ~110-130 Hz and ~160-200 Hz around PR HGS events, at ~160-230 Hz around HR events and stronger for S1 than for S2 trials.

Transient phase-amplitude coupling before entering a common path was also observed on day3 (Figure 4C, 4D). On day3, total of 19/14 PR and 17/22 HR HGS events were detected for S1/S2 trials, respectively. The strength of PR HGS event triggered cross-regional coupling on day 3 differed between S1 and S2 trials (Wilcoxon rank sum test, $P < 0.05$), but within higher frequency range (~220-240 Hz). The RE-HPC coupling around the HR HGS event did not show difference between S1 and S2 trials.

Collectively, our results demonstrate that transient functional coupling occurs between the phase of HPC-theta and the amplitude of RE-hfo prior choice point and this type of cross-regional interaction is gated by PFC-RE and/or HPC-RE high gamma synchrony events. The HGS event-triggered coupling could be more related to memory process than the decision making. However the strength of cross-regional coupling did not appear to predict the outcome of the rat spatial navigation. Yet, our present results do not rule out the contribution of this form of cross-regional interactions to some other aspects of cognition (e.g. memory demand, cognitive effort, choice uncertainty, etc.) that are varying across learning stages and accompany the maze task performance.

HPC-RE coupling is correlated to the spatial memory demand

If the RE-HPC coupling contributes to memory process, such as memory retrieval, we predicted that error corrections (when animal shortly deviates from the correct trajectory and returns back to the correct path) shall be accompanied by enhanced HPC-RE coupling as indication of increased memory demand. To test this prediction, we compared the strength of HPC-RE coupling on the ‘correct’ and ‘incorrect’ maze segments (Figure 5). Specifically, we computed the HPC-RE phase-amplitude coupling triggered by PR and HR HGS events as described above. On day5, total of 651 PR events and 656 HR HGS events were detected on correct maze segments. On the incorrect maze segments, total of 313 PR events and 263 HR HGS events were detected. In line with our prediction, the HGS event-triggered phase-amplitude coupling between RE and HPC was stronger on the incorrect segments. Around PR events, a clear RE hfo-HPC theta coupling at frequency range 160-204 Hz were only observed when rats were on incorrect maze segments. The PR HGS event-triggered RE-HPC coupling was also stronger on incorrect than on correct maze segments (Wilcoxon rank sum test, $P < 0.05$, Figure 5A). Around HR HGS events, the RE LFPs strongly HPC

theta-phase modulated at frequency range 160-200 Hz were also found only when rats deviated from the correct trajectory. The HR HGS event-triggered HPC-RE coupling was stronger on incorrect than on correct maze segments (Wilcoxon rank sum test, $P < 0.05$).

A stronger coupling between RE-hfo and HPC-theta on incorrect maze segments was already detected earlier in learning on day3. On day3, on the correct maze segments, total of 809PR HGS and 809 HR HGS events were detected. On the incorrect maze segments, total of 670 PR and 640 HR HGS events were detected. The PR and HR HGS event-triggered phase-amplitude coupling between RE and HPC was stronger on incorrect than on correct maze segments (Wilcoxon rank sum test, $P < 0.05$). On the incorrect maze segments, comparing to day5, the frequency range of RE-LFPs that was modulated by HPC-theta and triggered by both PR and HR HGS events was much broader and the modulation strength was relatively week.

Thus, differential cross-regional interaction day 3 and day 5 reflected a network dynamics over learning process. Our results indicate that transient epochs of high-gamma synchrony within PFC-RE-HPC circuit that are also organized by the HPC-theta phase may underlie some critical aspects of spatial cognition.

Co-occur gamma synchronization event

We also studied beyond pair-wise high-gamma synchronization within the PFC-RE-HPC circuit. Thus, we considered the epochs when PFC-RE and HPC-RE events occurred in synchrony or not and repeated the phase-amplitude analysis for above cases. We first plotted the cross-correlogram with PR and HR event trains. As shown in Supplementary Figure, there is a strong correlation between PR and HR events. Accompany with the occurrence of PR HGS event, there is strong tendency that a HR HGS event would happen within a short interval. By applying a 0.5 second long window centered at the occurrence of PR event, we found that 29.18% (274/939) of HR events detected in the window also happened in a range of 40 ms around the PR event. Thus we further defined the Co event, that is the two events from PR and HR event trains respectively with interval less than 40 ms would be considered as Co event of each pair-wise. The rest events of each event type which happened individually were then grouped as Nonco events. For both PR and HR events, the Co and Nonco event-triggered phase-amplitude coupling were compared (Figure 6). Since we have already revealed that there was stronger RE-HPC cross-frequency coupling when rats were on in correct maze segments, here we only focused on the Co and Nonco event detected on incorrect maze segments. On day5, 77 Co events were detected, while 236 and 186 Nonco events were found in PFC-RE and HPC-RE pair, respectively. Two frequency ranges (100-130 Hz and 150-200 Hz) of RE hfo showed strong coupling to HPC theta when Co event happened on incorrect segments. Comparing to the Co event, strong

coupling between the RE hfo at range of 150-200 Hz and HPC theta was still observed around Nonco PR HGS event, whereas, the coupling between lower frequency range (100-130 Hz) in RE and HPC theta was missing. Intriguingly, comparing to Co event, for cross-frequency coupling around the individually occurred HR HGS event, both of the two frequency ranges in RE which strongly coupled to HPC theta disappeared. On day3, Co (158/158) and Nonco (512/482) events were also detected in PFC-RE/HPC-RE pair. There was also stronger coupling between RE hfo and HPC theta was also found around Co event, however, frequency range of the theta modulated RE hfo was relatively broad.

The co-occurred PFC-RE and HPC-RE synchronization as well as the stronger RE-HPC cross frequency around Co event indicates that, the PFC-RE-HPC synchronization may be a mechanism underlying the cross-talking and information transfer in the PFC-RE-HPC circuit.

Discussion

There is a great body of studies discussing about the important role of prefrontal–hippocampal communication in memory processing and memory dependent navigation[1-3,5]. Considering the complexity of the brain functions depend the mPFC-HPC interaction, it is natural that other brain areas would also be involved in this mPFC-HPC circuit and work together with both mPFC and HPC. Because of its reciprocal connections with both mPFC and HPC, the RE has been suggested to play a key role in modulating the mPFC-HPC interaction. In line with this idea, recent studies observed RE contribution to the brain functions which depend on the hippocampal-prefrontal communication, for example, the fear memory generalization[20], strategy shifting[18], spatial navigation/memory [21,31]. In our previous study, we revealed the critical role of RE in spatial memory retrieval process[22]. The pre-training RE inactivation dramatically impaired the performance of well-trained rats in a spatial learning task. To further explore the neurophysiological mechanism underlies RE contribution to spatial memory retrieval, in current study, we performed simultaneous LFP recording in mPFC, RE and HPC when animals were trained to finish the same spatial task. We first observed epochs with synchronized high gamma oscillation in both mPFC-RE and HPC-RE signal pair. Next, we found a rats' performance dependent RE gamma-HPC theta coupling which associated with high gamma synchrony events. Although the high gamma synchrony in the mPFC-RE-HPC circuit could be observed since the first training day, we found a dynamic change of the HPC-RE cross-frequency coupling triggered by high gamma synchrony event over the training session. In the end, we found the co-occurred gamma synchrony between mPFC-RE and HPC-RE pair. The RE-HPC phase-amplitude coupling around the co-occurred gamma synchrony was dramatically stronger than the coupling triggered by the non-concurrent gamma synchrony.

The rats' performance dependent HPC-RE coupling

In current study, we found distinct patterns of HGS event-triggered HPC-RE cross-frequency coupling which depended on rats' performance. The synchronized gamma oscillation between brain areas has been extensively studied and is suggested to contribute to the information processing between spatially distributed brain areas. While the gamma oscillation in each brain structure reflects the local activity, the two brain regions showed synchronized gamma oscillations could be temporarily connected and make the information follow between them possible. For instance, the CA1-CA3 and CA1- entorhinal cortex gamma synchrony was found to gate the input to CA1 [4,32,33]; Yamamoto and colleagues[25] found transient gamma synchronization is associated to successful working memory execution while the gamma synchrony specifically occurred around the junction of T-maze. In consistent with these findings, in current study we also observed gamma synchrony within the brain circuit constituted by mPFC, RE and HPC. However, in our task the gamma synchrony events could be detected at various spatial positions on the crossword maze, suggesting a more complicated event distribution in a complex spatial environment.

Besides the gamma synchrony, the gamma-theta coupling between different brain regions are also demonstrated to contribute to memory processing[4,30,32,34,35]. While the gammy synchrony links different brain regions and enable the precise information processing between brain areas in a precise time window, the gamma-theta coupling could provide a mechanism for further organizing different gamma oscillations [26,27,36]. In our study, we combined the gamma synchrony and cross-frequency coupling analysis together. By applying the gamma synchrony event as trigger, we found significant difference between RE gamma-HPC theta coupling preceding the correct and incorrect decisions. This may be correlated to the uncertainty of the animals and reflect the efficiency of memory retrieval. The differential HGS event-triggered RE-HPC coupling was also observed when animals were on correct and incorrect maze segments. For both PR and HR HGS events, the event-triggered phase-amplitude coupling showed stronger RE high gamma- HPC theta coupling during the period when the animals deviated from correct trajectory. This enhanced RE high gamma-HPC theta coupling may be due to a stronger memory demanding. When animals made errors and navigated to incorrect maze segments, to return to the correct trajectory, re-localization and retrieval of previously obtained memory would be needed. This processing may need an extra memory effort and a copy of consolidated memory may transmitted from mPFC via RE to HPC, in the end combine with the information representing current spatial environment.

The gamma synchrony event type dependent RE-HPC cross frequency coupling

For both PR and HP HGS events, an event type (Co and Nonco event) dependent phase-amplitude coupling between RE and HPC was also found in current study. In both mPFC-RE and HPC-RE pair, the RE-HPC coupling around the Co event is dramatically stronger than it around the Nonco event. As mentioned above, the gamma synchrony may indicate that the two brain regions are in a state which can make cross-talking more efficient. The co-occurred gamma synchrony may further indicate a network state that all nodes of the circuit are activated and the information transfer is possible. Stronger gamma-theta coupling between the RE (gamma) and HPC (theta) occurring around Co event further supports the idea that the synchronization within the entire circuit could facilitate communication within functional network[26,27,32].

When animals deviated from the correct trajectory and PR event happened individually (Non-co event), comparing to Co event, the event-triggered phase-amplitude coupling showed a comparable phase-locking between RE high frequency oscillation and HPC theta. This high frequency oscillation could be remnants of local spike activities in RE. It has been shown that the local fast gamma oscillation is related to the synchronized firing of local neurons[35]. The mPFC-RE synchrony could reflect the synchronous firing of both mPFC and RE neurons, thus the high frequency oscillation in LFP could be remnants of local spike activities. Therefore, when both mPFC and RE synchronized in gamma range, there could be synchronous RE spikes phase-locking to hippocampal theta and the remnants of RE neuron firing also locked to HPC theta phase.

Interestingly, when only HPC-RE synchrony was detected, neither high gamma nor high frequency oscillation in RE showed strong phase-locking to HPC theta as it was in Co event group. These findings seem to be in line with the idea that the gamma synchrony and gamma-theta coupling could contribute to the information flow between different brain areas. In our study, we hypothesized that the information from mPFC would be transmitted to HPC via RE when there was high memory demanding. If the high gamma synchronization was only observed between RE and HPC, which indicated the first step of information flowing in the circuit (from mPFC to RE) was missing, the absence of RE-HPC cross frequency coupling could be expected. In contrast, when all the nodes (mPFC, RE and HPC) of the circuit were activated and synchronized, all structures of the network were recruited in a time window could ‘talk’ to each other, the memory related information could first flow from mPFC to RE, then transmitted from RE to HPC. Thus, the differentiation between phase-amplitude coupling triggered by Co and Non-co events further revealed the way that gamma oscillations contributes to the transmission of memory related information in the network formed by mPFC, RE and HPC.

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Main Figures

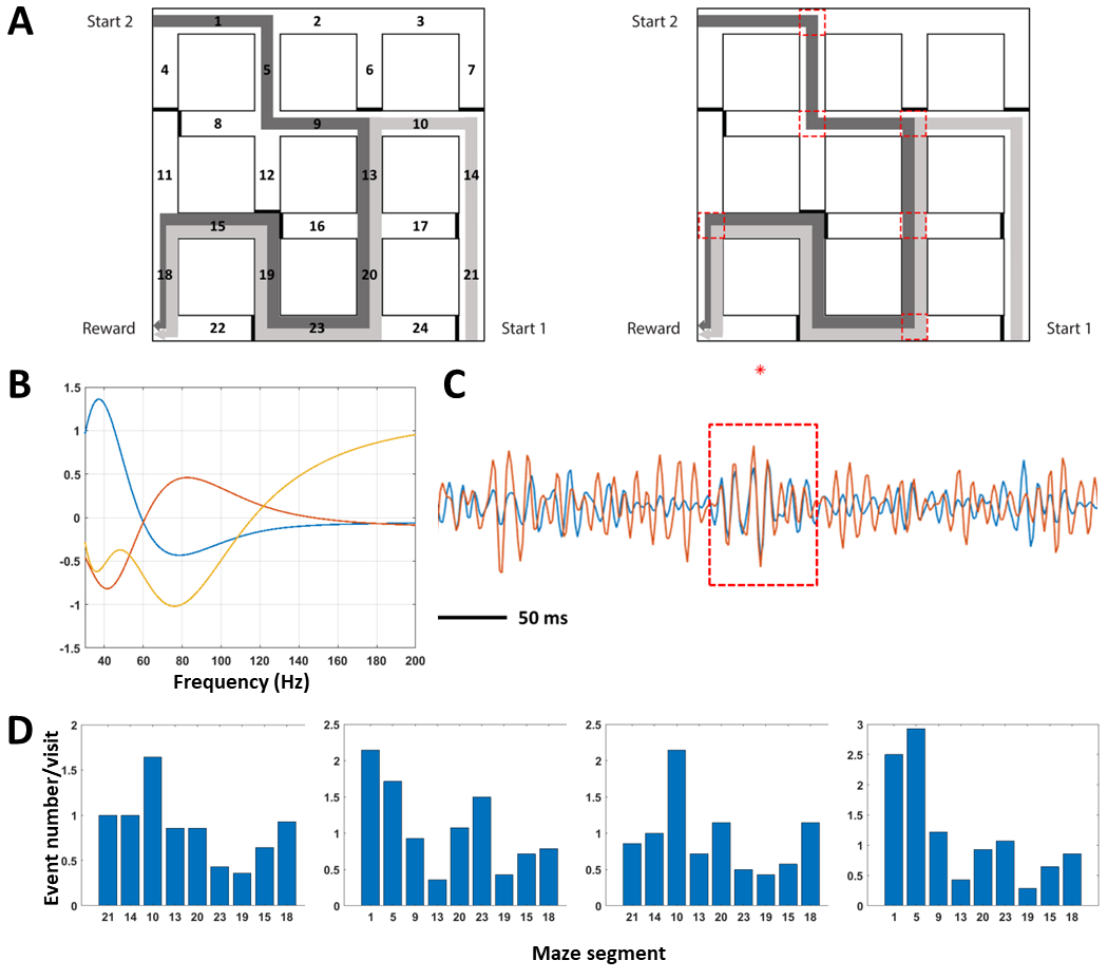


Figure 1. The HGS event distribution over maze segments.
(A) Left: number for each maze segment. The gray and dark gray represent the correct trajectories correspond to different start points. Right: choice points. The red dashed squares marks the choice points where the animal needs to make decision between multiple choices. **(B)** The independent RE spectral component which nested in HPC single theta cycles **(C)** Example of detected HGS event. The red dashed square indicates an HR HGS event, the orange and blue traces represent the bandpass filtered HPC and RE high gamma signal. **(D)** The averaged HGS event over segments of correct trials. From the left to right panels: the mean PR HGS event distribution over the segment of S1 trials; mean PR HGS event distribution over the segment of S2 trials; mean HR HGS event distribution over the segment of S1 trials; mean HR HGS event distribution over the segment of S2 trials (n = 6 rats).

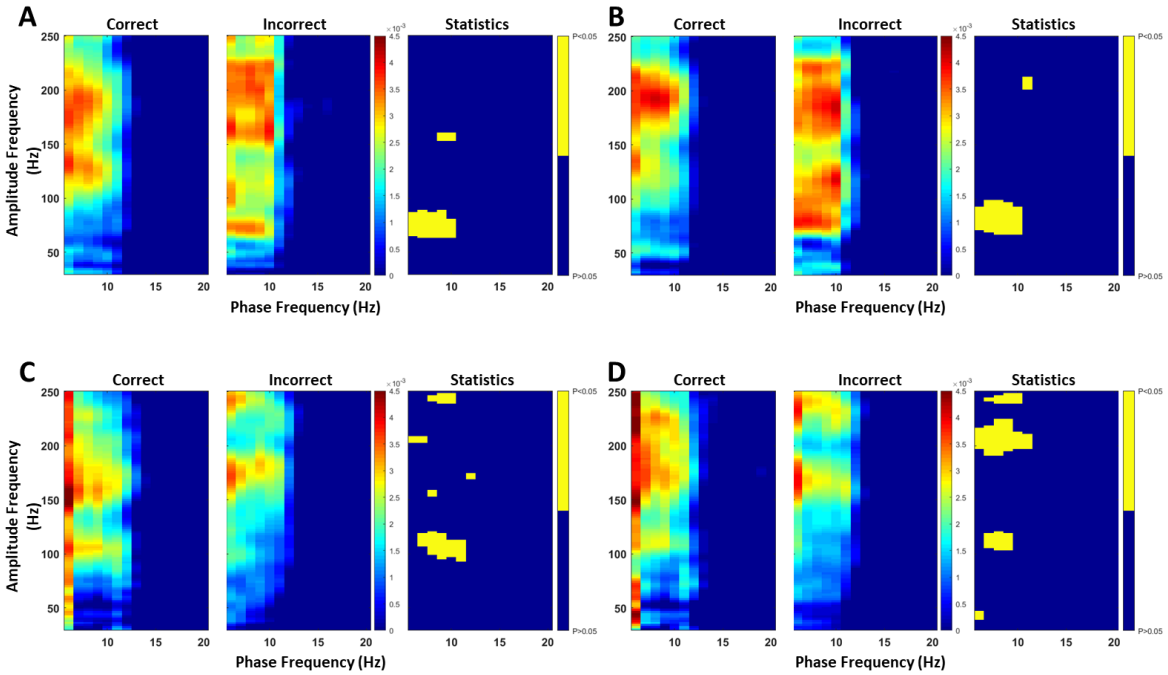


Figure 2. The HGS event-triggered phase-amplitude couplings precede correct and incorrect decisions of the first choice point.

(A) Left and Middle: the averaged phase-amplitude comodulogram around the PR HGS events which occurred before the correct and incorrect decisions of the first choice point (in both S1 and S2 trials). The colorbar at the right represents the modulation index. Positive values indicates the statistically significant (P<0.05) phase-amplitude coupling between RE and HPC (see Materials and Methods). Right: the statistical matrix, each yellow pixel indicates a significant difference between the modulation indexes of specific frequency-pair in correct and incorrect group, while the blue pixel represents nonsignificant difference. **(B)** Left and Middle: the averaged phase-amplitude comodulogram around the HR HGS events which occurred before the correct and incorrect decisions of the first choice point (in both S1 and S2 trials). As in (A), the Positive values indicates the statistically significant (P<0.05) phase-amplitude coupling between RE and HPC. Right: the statistical result of the comparison between correct and incorrect group. **(C)** and **(D)**: The same as in (A) and (B), respectively, but on day3.

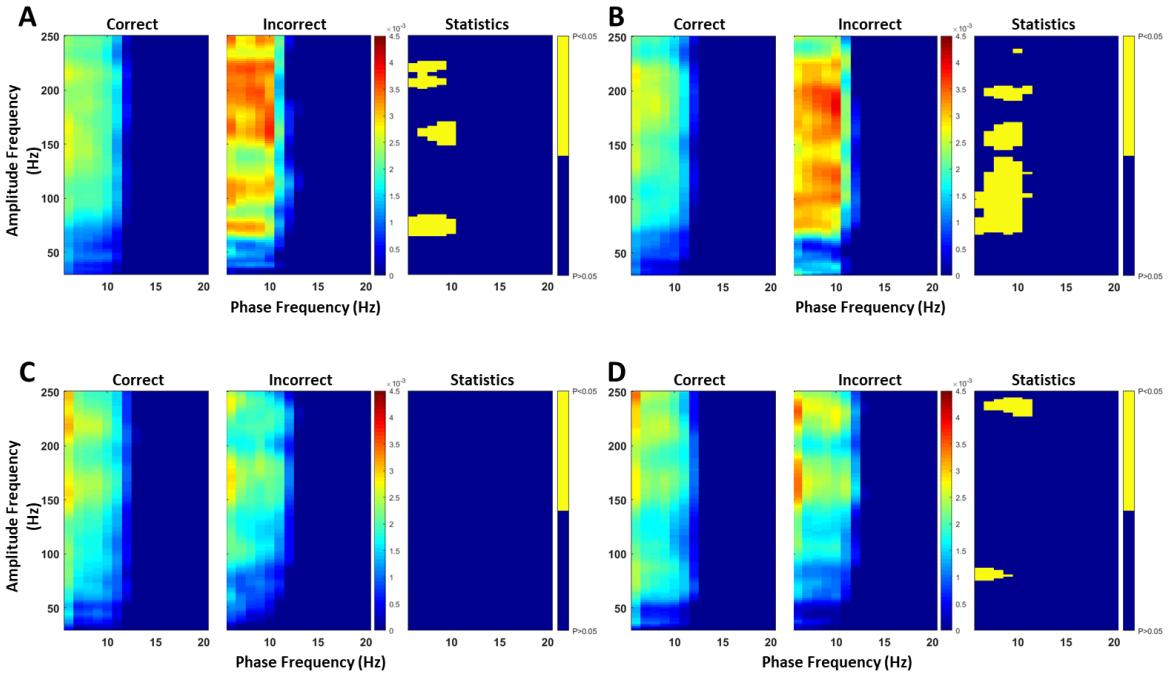


Figure 3. The HGS event-triggered phase-amplitude couplings precede correct and incorrect decisions of all choice points.

(A) Left and Middle: the averaged phase-amplitude comodulogram around the PR HGS events which occurred before the correct and incorrect decisions for all the choice points in both S1 and S2 trials. Right: the statistical result between correct and incorrect group. **(B)** Left and Middle: the averaged phase-amplitude comodulogram around the HR HGS events which occurred before the correct and incorrect decisions for all the choice points in both S1 and S2 trials. Right: the statistical result of the comparison between the correct and incorrect group. **(C)** and **(D)**: The same as in (A) and (B), respectively, but on day3.

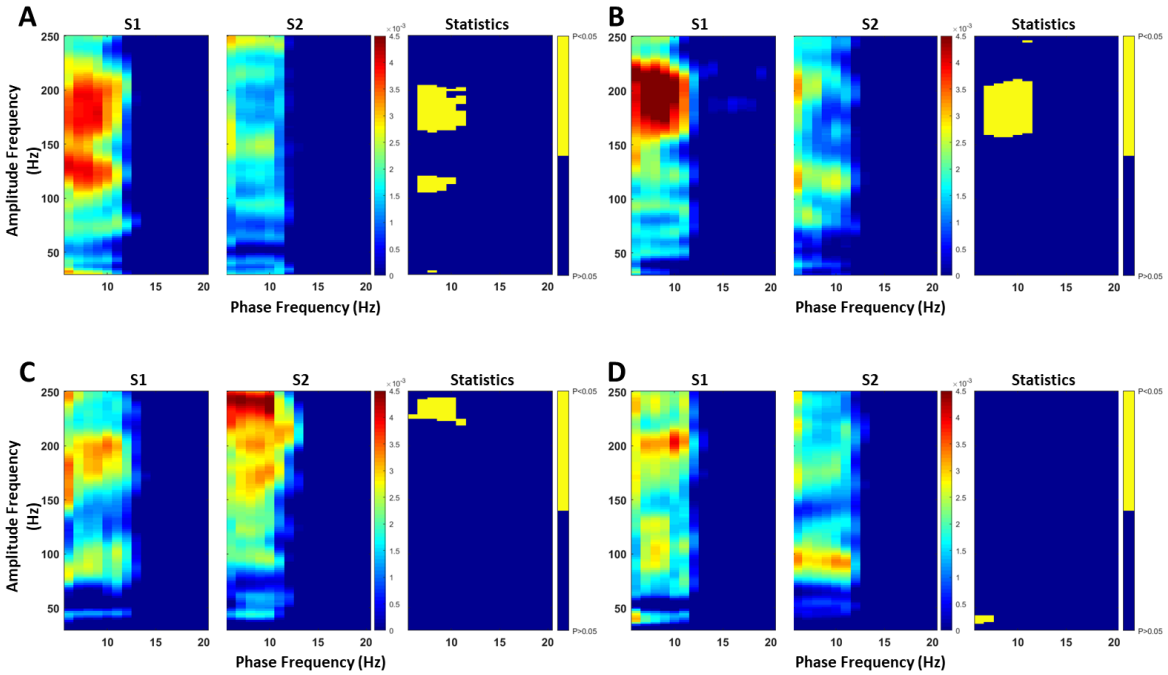


Figure 4. The HGS event-triggered phase-amplitude couplings preceding the correct entering to maze segment13.

(A) Left and Middle: the averaged phase-amplitude comodulogram around the PR HGS events detected before the correct entering to maze segment13 in S1 and S2 trial, separately. Right: the statistical result of the comparison between S1 and S2 trials. **(B)** Left and Middle: the same as the left and middle panel in (A), but the phase-amplitude comodulogram is triggered with PR HGS event. Right: the statistical result of the comparison between S1 and S2 trials. **(C)** and **(D)**: The same as in (A) and (B), respectively, but on day3.

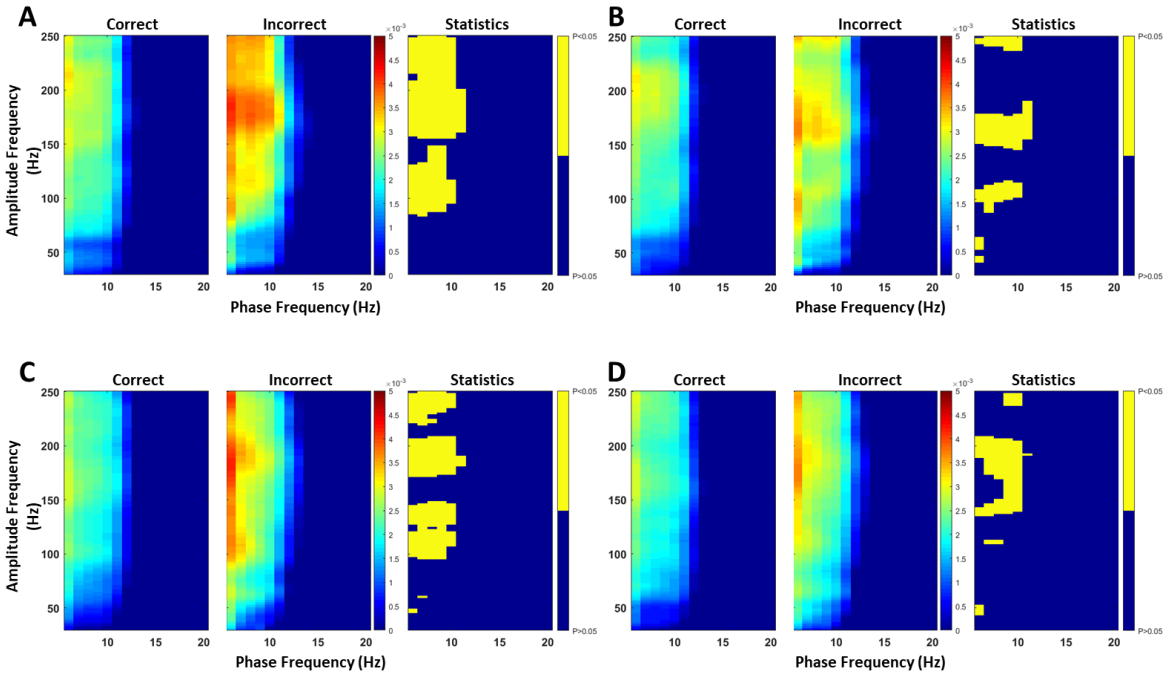


Figure 5. The HGS event-triggered phase-amplitude couplings on correct and incorrect maze segments.

(A) Left and Middle: the averaged phase-amplitude comodulogram triggered by the PR HGS events on the correct (correct groups) and incorrect (incorrect group) maze segments. Right: the statistical result of comparison between correct and incorrect group. **(B)** Left and Middle: the same as the left and middle panel in (A), but with PR HGS event as trigger. Right: the statistical result of the comparison between correct and incorrect group. **(C)** and **(D)**: The same as in (A) and (B), respectively, but on day3.

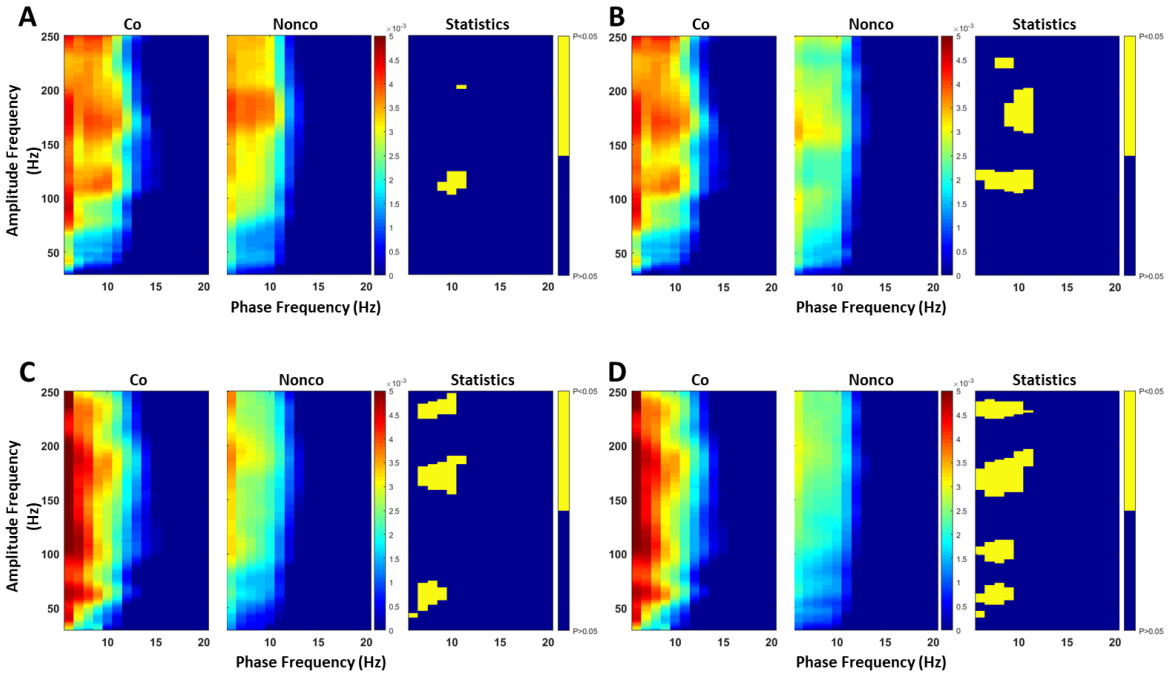
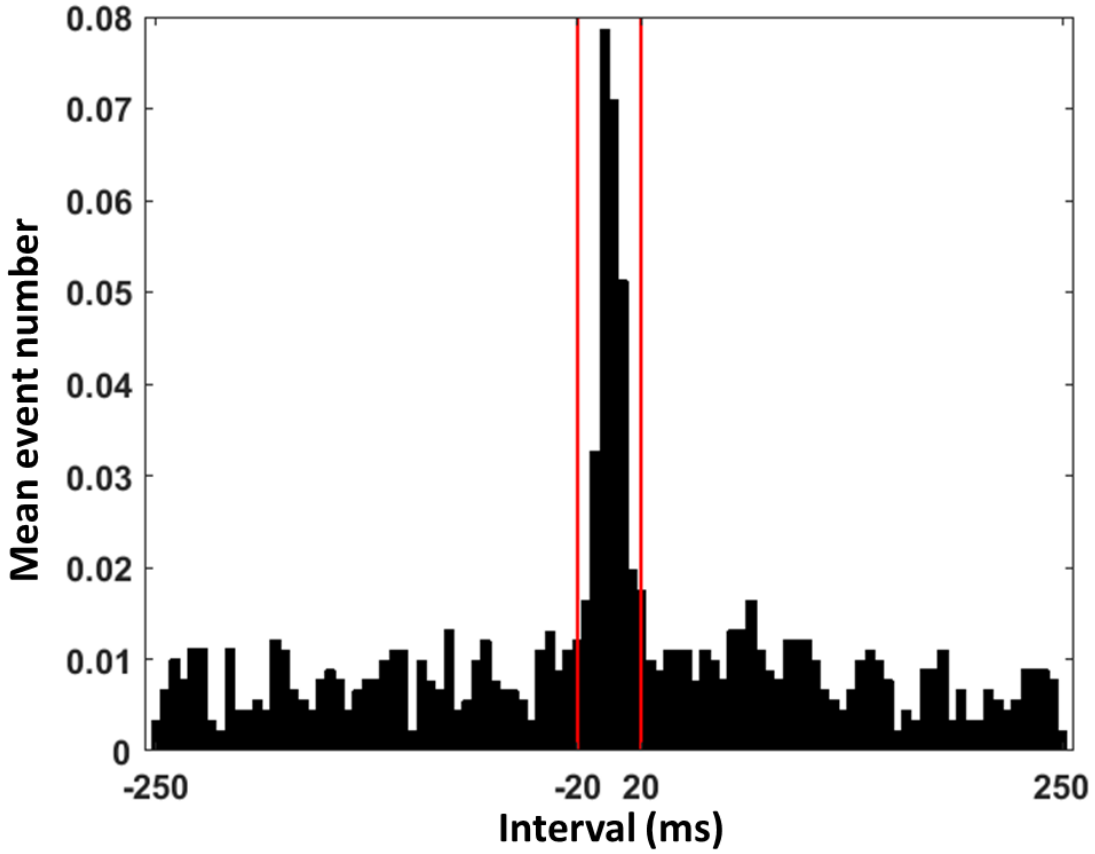


Figure 5. The phase-amplitude couplings triggered by Co and Nonco HGS event, on incorrect maze segments.

(A) Left and Middle: the averaged phase-amplitude comodulogram around the Co and Nonco PR HGS events when rats were on incorrect maze segments. Right: the statistical result of comparison between Co and Nonco group. **(B)** Left and Middle: the same as the left and middle panel in (A), but with PR HGS event as trigger. Right: the statistical result of the comparison between Co and Nonco group. **(C)** and **(D)**: The same as in (A) and (B), respectively, but on day3.



Supplementary Figure 1. Cross-correlogram with PR and HR HGS events, on day5. The vertical red lines mark the time window from -20 to 20 ms.