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Hippocampal theta phase–contingent memory retrieval in delay and trace eyeblink conditioning

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ABSTRACT

Hippocampal theta oscillations (3–12 Hz) play a prominent role in learning. It has been suggested that encoding and retrieval of memories are supported by different phases of the theta cycle. Our previous study on trace eyeblink conditioning in rabbits suggests that the timing of the conditioned stimulus (CS) in relation to theta phase affects encoding but not retrieval of the memory trace. Here, we directly tested the effects of hippocampal theta phase on memory retrieval in two experiments conducted on adult female New Zealand White rabbits. In Experiment 1, animals were trained in trace eyeblink conditioning followed by extinction, and memory retrieval was tested by presenting the CS at troughs and peaks of the theta cycle during different stages of learning. In Experiment 2, animals were trained in delay conditioning either contingent on a high level of theta or at a random neural state. Conditioning was then followed by extinction conducted either at a random state, contingent on theta trough or contingent on theta peak. Our current results indicate that the phase of theta

at CS onset has no effect on the performance of the behavioral learned response at any stage of classical eyeblink conditioning or extinction. In addition, theta-contingent trial presentation does not improve learning during delay eyeblink conditioning. The results are consistent with our earlier findings and suggest that the theta phase alone is not sufficient to affect learning at the behavioral level. It seems that the retrieval of recently acquired memories and consequently performing a learned response is moderated by neural mechanisms other than hippocampal theta.

Keywords: classical conditioning, hippocampus, learning, memory, theta oscillation

1. INTRODUCTION

The hippocampus is a crucial brain structure in learning (Milner, 1972). According to Buzsáki's (1989) two-stage model of learning and memory, the hippocampus exhibits two states related to the encoding and strengthening of memory traces. When exploring its environment and focusing attention on external stimuli, hippocampal electrophysiological activity is dominated by rhythmic slow wave activity, the theta oscillations. Theta oscillations are most prominent in the hippocampal input region, the dentate gyrus (DG) and near the hippocampal fissure (Buzsáki, 2002; for a review, see Buzsáki & Moser, 2013) and are driven by GABAergic neurons of the medial septum (Hangya et al., 2009). When the level of attention lowers and hippocampal theta activity ceases, the output region of the hippocampus, the CA1, shows activation in the form of large amplitude sharp-wave ripples. Ripples are generated when input from the entorhinal cortex (EC) via the perforant path and the DG

reaches the CA3. Within the CA3 autoassociative network, recently activated neuronal assemblies then re-activate (de Almeida, Idiart & Lisman, 2007), and further activate the CA1 pyramidal cells. Pyramidal cells of the CA1 project the signal back to the neocortex and complete the network loop. Thus, in Buzsaki's (1989) model, the role of theta oscillations is suggested to be most crucial during the encoding of new information. However, others also suggest a role for theta in the retrieval of already encoded memories (Hasselmo, Bodelon & Wyble, 2002).

Lesion studies (Zola-Morgan & Squire, 1990; Moyer & Disterhoft, 1990) and pharmacological manipulations (Asaka et al., 2000) indicate that the hippocampus is needed at least in the early stages of learning in eyeblink conditioning (Takehara et al., 2002). Trace eyeblink conditioning is a hippocampus-dependent task (Solomon et al., 1986; Holland & Bouton, 1999) whereas delay eyeblink conditioning can be acquired even in the absence of the whole forebrain (Svensson, Ivarsson & Hesslow, 1997). Yet studies suggest that hippocampal electrophysiological state, namely the level of theta, correlates with learning even during delay eyeblink conditioning (Berry & Thompson, 1978; Nokia et al., 2008; 2012b). According to Berry et al., when training is carried out during theta, animals learn delay eyeblink conditioning faster than do those trained in the absence of or during low levels of hippocampal theta activity (Seager et al., 2002). This is a finding so far not reported elsewhere.

A computational model by Hasselmo, Bodelón and Wyble (2002) proposes that not only the presence or absence of hippocampal theta might be important but that the phase of the theta cycle determines the optimal time windows for hippocampal encoding and retrieval of memories. First, the model suggests that encoding associations in memory is most effective when synaptic output from the EC to the hippocampus is strong. Second, the model postulates

that retrieval of the learned associations is most effective when the EC input is weak. The fluctuation in the input from the EC to the hippocampus is congruent with the hippocampal fissure theta phase, the peak of theta corresponding to low EC input. Presenting the CS at the peak or trough also affected learning rate, since acquisition of conditioned response (CR) was slower when the CS onset coincided with the theta peak. However, in contradiction to the hypothesis, our results suggested that in well-learned animals the behavioral performance of the CR is not dependent on the theta phase regardless of a clear effect on neural responses (Nokia et al., 2015). However, a possibility remains that an effect might have been seen if memory retrieval had been probed at different stages of learning. Lesion studies suggest that the hippocampal contribution to eyeblink conditioning is strongest early in learning (Takehara et al., 2002).

The studies reported here were conducted to further test if hippocampal theta phase has an effect on the retrieval of a recently acquired memory trace and the consequent performance of a learned response in various stages of the acquisition process as well as during extinction training. It might well be that although no behavioral effect was detected in our previous study on well-trained animals (Nokia et al., 2015), there could be an effect when the memory trace is still in the labile state early in training (Takehara et al., 2002). This notion was addressed in Experiment 1. In line with the model of Hasselmo et al. (2002), our hypothesis was that retrieval would be more efficient if the CS was presented at fissure theta peak. In addition to simple acquisition of a learned response, in Experiment 1 we also studied if the phase of theta moderates the conditioned responses during different stages of extinction training. Again, more efficient retrieval of the previously acquired memory trace manifested as a conditioned response was expected if the CS was presented at fissure theta peak. In Experiment 2, we first sought to confirm the results of the study by Seager et al. (2002) on theta-contingent delay conditioning and then addressed the question of whether extinction of

the learned response is also affected by theta phase. To do so, we first trained rabbits in delay eyeblink conditioning either during theta or regardless of neural state. We expected animals trained during theta to learn faster and/or better than those trained at random (Seager et al., 2002). Then, in well-trained animals we conducted extinction either by presenting the CS alone randomly or always at the theta trough or at the theta peak. Compared to animals trained at a random state, we expected to see impeded extinction learning in animals trained contingent on fissure theta peak (Nokia et al., 2015).

2. MATERIALS AND METHODS

2.1 Subjects

The subjects were 29 (Experiment 1, 13 rabbits; Experiment 2, 16 rabbits) adult female New Zealand White rabbits (Lidköpings kaninfarm, Sweden) weighing approximately 2.8 kg at the time of surgery. The rabbits were housed in individual cages at the Laboratory center of the University of Jyväskylä. Food and water were freely available, and room temperature and humidity were controlled. The rabbits were maintained on a 12/12-hour light/dark cycle, with lights on at 8:00 a.m. All experiments were carried out during the light part of the cycle. All the experimental procedures, care and handling were executed in accordance with Directive 2010/63/EU of the European Parliament and of Council on the protection of animals used for scientific purposes. Animal handling was performed only by trained personnel and the rabbits

were introduced to human contact and handling for a sufficient amount of time before the surgery.

2.2 Surgery

Before the surgery, rabbits were treated with subcutaneous injections (s.c.) of an anti-inflammatory drug (50 mg/mL carprofen [Rimadyl vet, Pfizer Inc. Animal Health], dose: 0.1 ml/kg) and with 2 ml of an analgesic drug (0.3 mg/ml buprenorphine [Temgesic, Schering-Plough Europe] diluted with 0.9 ml of 0.9% NaCl) to moderate acute pain after surgery. The rabbits were anesthetized with an intramuscular injection (i.m.) of ketamine–xylazine cocktail (7.8 mL of 50 mg/ml Ketaminol vet [Intervet International B.V.] mixed with 2.8 ml of 20 mg/ml Narcoxyl vet [Intervet International B.V.]). A dose of 0.8 ml/kg of the cocktail was injected i.m. before surgery. During surgery, additional doses of either the cocktail or ketamine alone were injected subcutaneously approximately every 20–30 min or as needed. Before the surgery, the rabbit's fur was shaved from the top of the head. Then, the rabbit was positioned in a stereotaxic instrument (Kopf Instruments) with the bregma 1.5 mm higher than the lambda. Eye gel was inserted into the rabbit's eyes. At this point, 2.0 ml of lidocaine (10 mg/ml Lidocain [Orion pharma]) was injected s.c. in the area of surgery before making the opening incision.

A longitudinal incision was made to the scalp and a local anesthetic (2 g of lidocaine-hydrochloride Xylocain [AstraZeneca]) was administered to the wound. Eight holes for electrodes were drilled into the skull along with four holes for the anchoring screws (5 mm anterior and 5 mm lateral to the bregma; 13 mm posterior and 5 mm lateral to the bregma). Two of the posterior and anterior screws were connected together and served as a reference and the ground, respectively, in the electrophysiological recordings. Eight monopolar recording electrodes (Teflon-insulated stainless steel wire; 0.125 mm uninsulated diameter

[A-M Systems]) were chronically implanted into the left dorsal hippocampus, aiming four electrodes to the CA1 (4 mm posterior, 3.5–6.5 mm laterally from the bregma; electrode tip depth from the bregma 6–8 mm) and four above the hippocampal fissure (5 mm posterior, 4–7 mm laterally from the bregma; electrode tip depth 6.2–8.5 mm below the bregma). Wires, skull screws, preamplifier interface, one mounting screw for an air puff mount, and the incision area were cemented with dental acrylic. To prevent nausea after surgery, metoclopramide (0.1 ml/kg, concentration 5mg/ml; Primperan [Sanofi Winthrop Industrie]) was administered s.c. and the rabbit was returned to her home cage wrapped in a towel. Recovery was monitored and the rabbits were medicated with analgesic (buprenorphine [Temgesic, Schering-Plough Europe] diluted with 0.9 ml of 0.9% NaCl) 4 hours after surgery and then every 8 hours for the next 44 hours.

2.3 Experimental procedure

The experimental procedures are illustrated in Figure 1. After one week of recovery from surgery, the animals were accustomed to a plexiglas restraining box without restraining and overall behavior was monitored. On the second day, restrained animals were habituated to the recording chamber and 30 minutes of spontaneous hippocampal local-field potentials (LFPs) were recorded. LFPs and electromyography (EMG, please see section 2.4 below) from the right eye were recorded 5 minutes prior to, during and 1 minute after each session. The inter-trial interval always varied randomly between 30 and 60 s. LabVIEW (National Instruments) was used to monitor neural activity and blinking online, to execute the experimental procedures and to present stimuli. The percentage of learned responses performed by each animal was analyzed after every session using MATLAB (The MathWorks Inc.). Please see section 2.4 for further information.

2.3.1 Experiment 1. Trace eyeblink conditioning, memory retrieval testing and extinction

During the first training session, 60 tone-alone (40-ms, 5-kHz, 75-dB tone) trials were presented regardless of neural state. In addition to hippocampal LFPs, EMG from the right eye was also recorded to determine the frequency of spontaneous eyeblinks elicited by the tone later used as a CS.

Trace eyeblink conditioning was carried out with the tone specified above as the CS and a 100-ms air puff (0.35 bar source pressure) to the right eye as an unconditioned stimulus (US). A trace period of 660 ms was used. A total of 60 training trials were presented during each session, regardless of neural state and in the absence of spontaneous blinking.

Memory retrieval was tested at two stages of acquisition, first when the animal performed a learned response at a rate of 30% and second when learned responding took place during 60% of the trials. Test sessions were conducted within the same day after reaching the set criterion. During each test session, the CS was presented alone to limit the test to memory retrieval. For CS presentation to take place, robust theta oscillations had to be present for at least one second (theta ratio > 80%). The CS onset was timed either to the peak (20 trials) or the trough (20 trials) of the hippocampal fissure theta oscillation, in a random fashion and with an ITI of at least 30 s. On the next day, trace eyeblink conditioning continued as usual.

Animals were trained with trace eyeblink classical conditioning (CC) until they made at least 60% learned responses during two consecutive sessions or up to 20 sessions. Animals that reached the two-session criterion or made more than 60% learned responses during at least one session were considered to have learned the task. In these animals, extinction training was then started. Sixty CS-alone trials per session were presented irrespective of the neural state of the hippocampus. These sessions were carried out until learned responses took place during less than 30% of the trials or up to 10 sessions. The effect of theta phase on memory retrieval

was tested as before on two occasions during extinction training. First when learned responding dropped below 60 % and last when (or if) it dropped below 30 %.

2.3.2 Experiment 2: Delay conditioning and extinction

Delay eyeblink conditioning was carried out with a tone-CS (800 ms, 5 kHz, 75dB) and a 100-ms air puff (0.35 bar source pressure) to the right eye as a US. The US was presented starting at 700 ms from CS onset and thus overlapping and co-terminating with the CS. Sixty trials per session were presented. For eight animals, conditioning was conducted in a random neural state and for the other half when theta was dominant in the hippocampus (the theta ratio had to be over 80% during a 1-s pre-trial period, please see section 2.4). Training trials were only presented when the animal was not already blinking. Four of the sixteen animals were used to pilot the experiment. They were trained for six sessions regardless of learning rate. Two of the rabbits were trained in a random neural state and two when theta was prominent in the hippocampus. Delay conditioning was re-modelled due to poor extinction rates (see Results) for the remaining 12 animals. Now, delay conditioning was terminated as soon as an animal performed > 80% learned responses during two consecutive conditioning sessions.

After delay conditioning, 12 sessions of extinction training with 60 trials per session were conducted. The CS was either presented to a random neural state (n = 5), or the onset of the CS was timed to the peak (n = 6) or the trough (n = 5) of the hippocampal fissure theta oscillation.

2.4 Recordings and data analysis

Bipolar EMG from the trained eye was recorded using stainless steel wire-hooks placed around the right upper and lower eyelids for the duration of the training and test sessions. The raw EMG signal was conveyed to a filter-amplifier (A-M Systems Model 2100), amplified

1000x and band-pass filtered from 100 to 500 Hz. For neural recordings, a ten-fold amplification was executed with a preamplifier (MPA8I, MultiChannel Systems [MCS]) attached to the electrode connector in the rabbit's head. Then the signal was band-pass filtered (1–5000 Hz) with a 64-channel filter amplifier (MCS). Last, the signal was digitized at a rate of 20 kHz, low-pass filtered (500 Hz) and downsampled at a rate of 2 kHz with a MCS USB-ME64 System (MC_Rack software). SPSS (IBM) and MATLAB (MathWorks) were used for offline data analysis.

2.4.1 Eyeblinks

The EMG signal was high-pass filtered offline (> 100 Hz) and Hilbert-transformed. An envelope curve following the peaks of the signal was calculated. Baseline EMG activity was defined for each animal and session as the mean of the peak EMG amplitude during a 250-ms pre-CS period (MEANpre). In addition, the mean of the standard deviation of the EMG activity during the 500-ms pre-CS period (SDpre) was determined. Eyeblinks were defined as EMG activity exceeding a threshold of [MEANpre + 7 × SDpre] for at least 10 ms. Trials with eyeblinks during the 100-ms period immediately preceding CS onset were rejected. Eyeblinks during the last 250 ms of the trace period were counted as conditioned responses.

2.4.2 Phase-locking of hippocampal theta-band (4–8 Hz) responses

To assess the temporal accuracy of the theta-band responses to the conditioning stimuli, a phase-locking value (PLV) (Palva et al., 2005) was calculated. PLV is a measure of temporal consistency of a signal from trial to trial, a higher value meaning a higher probability for the signal to be in the same phase at a given time point after the stimulus onset. In terms of neuronal activity, high phase-locking to an event means that the responding of groups of neurons evoked by the external stimulus–reflected in LFP– is temporally regular and predictable. Predictable firing of groups of neurons within a brain structure can be viewed as a

precursor of forming neuronal assemblies across brain structures. Synchrony of neuronal firing can enhance communication between anatomically connected brain regions (Fries, 2005).

The PLV is based on amplitude-normalized phase information and is thus resistant to changes or differences in signal amplitude. This allows comparable measures to be obtained from data recorded over time in multiple subjects. The hippocampal LFP data were first band-pass filtered between 4 and 8 Hz. Then, a Hilbert transform was run on the signal to obtain the phase information, and the amplitude of the transformed signal normalized to 1 by dividing each data point by its absolute value. Finally, the PLV was obtained by averaging over 60 trials (one session) and taking the absolute value of the mean. The PLV varies between 0 and 1, 0 indicating no phase-locking and 1 indicating perfect phase-locking. For statistical analyses, the mean of the PLV during the CS and subsequent trace-period (700 ms) was derived and averaged over one session.

2.5 Statistical analyses

Repeated-measures analysis of variance (ANOVA), with training sessions or blocks of two sessions as a within-subjects factor and group as a between-subject factor, was used to analyze changes across training. For post-hoc comparisons, Bonferroni correction *p* values are reported. Paired *t* tests were used to analyze differences within subjects regarding the memory retrieval and phase-locking in either the peak or the trough of the theta. All of the *t* tests passed normality tests (Shapiro-Wilk).

2.6 Histology

Rabbits were anesthetized with an i.m. injection of a ketamine-xylazine cocktail. The rabbit's left ear was shaved and it was then overdosed with pentobarbital (Mebunat vet, Orion-

Yhtyma Oyj) by injecting intravenously (i.v.) a large vein in the ear. Then, the brain was perfused with physiological saline followed by 9% formalin solution through the ascending aorta. The locations of the eight electrode tips were marked by passing a DC current (0.2 mA, for 10 seconds) through them. The brain was then removed and stored in formalin for several days. The brain was coronally sectioned with a vibratome into 60-µm-thick slices. The slices were attached to gelatinized slides, dried, and stained with Prussian blue and cresyl violet. The electrode locations were determined with the help of a microscope.

3. RESULTS

Histological examinations confirmed that the recording electrodes for theta were in or near the hippocampal fissure, as intended, in 27 animals (see Figure 2A). Two rabbits with broken electrodes were used in the Random group in Experiment 2.

3.1 Experiment 1: Phase contingent trial onset affects neural but not behavioral responses in trace eyeblink conditioning

All training sessions included, 11 out of 13 animals learned to blink in response to the CS in over 30% of training trials per session. However, only seven out of thirteen rabbits reached the criterion of more than 60% learned responses. These seven animals went through extinction training and 5 of them met the extinction criterion, that is, they made less than 30% learned responses at the end of extinction training. These learning results meant that memory retrieval tests to study the effects of theta phase on neural and behavioral responses to the CS

were conducted first on 11 animals (early acquisition, > 30% learned responses), then on seven animals (successful acquisition, > 60%), then on seven animals (early extinction, < 60%) and finally on five animals (successful extinction < 30%).

Hippocampal responses and conditioned responses to the CS onset taking place at the peak vs. the trough of the theta oscillation in hippocampal fissure was tested with paired samples *t* tests. The results are summarized in Figure 3. Phase-locked hippocampal theta-band responses to the CS (see Nokia et al., 2015) were moderated by the phase of the theta early in acquisition when animals performed > 30% learned responses, $t(10) = 4.98$, $p < 0.001$, $d = 0.25$. Namely, phase-locking was higher when the CS onset was timed to the fissure theta trough compared to when it was timed to the theta peak. This was the case also early in extinction training, < 60 % learned responses, $t(6) = 3.82$, $p = 0.009$, $d = 0.17$, and after successful extinction, < 30% learned responses, $t(4) = 4.91$, $p = 0.008$, $d = 0.32$. After successful acquisition of the learned response (> 60% learned responses), phase-locking of hippocampal theta-band responses to the CS was similar regardless of whether the CS onset was at the peak or the trough of the theta cycle, $t(6) = 1.47$, $p = 0.192$. The performance of the conditioned response was not moderated by the phase of the fissure theta cycle at any stage of acquisition or extinction of the learned response; paired samples *t* tests: > 30% learned responses: $t(10) = 1.10$, $p = 0.296$; > 60% learned responses: $t(6) = 1.45$, $p = 0.199$; < 60% learned responses: $t(6) = 0.97$, $p = 0.370$; < 30% learned responses: $t(4) = 0.17$, $p = 0.876$.

3.2 Experiment 2: The presence of theta did not affect learning in delay conditioning and theta phase did not affect extinction

As seen in Figure 4c, the degree of phase-locking of hippocampal theta-band responses to the CS did not differ between the Theta ($n = 8$) and Random group ($n = 6$) and also did not change during the delay conditioning. See Figure 4a; interaction of group and session: $F(3,$

36) = 0.64, p = 0.592; main effect of session: F(3, 36) = 0.91, p = 0.446; main effect of group F(1, 12) = 1.48, p = 0.247. In addition, the proportion of learned responses increased across the first four sessions of delay eyeblink conditioning equally in both groups, rabbits trained irrespective of neural state (Random, n = 8) and those trained in the presence of theta (Theta, n = 8). See Figure 4c; repeated measures ANOVA, interaction of group and session: F(3, 42) = 0.82, p = 0.493; main effect of session: F(3, 42) = 67.86, p < 0.001; main effect of group F(1, 14) = 0.07, p = 0.800. That is, there was no difference between groups in hippocampal responses to the CS, and hence no effect of theta on delay eyeblink conditioning.

After delay conditioning, all rabbits were trained in extinction. For five animals, the CS alone was always presented at theta trough (Trough) and for six animals it was always presented at theta peak (Peak). For the remaining five animals, the CS was presented irrespective of the neural state (Random). Phase-locked hippocampal theta-band responses to the CS were different between the trough, the peak, and the random group during extinction training (repeated measures ANOVA, interaction of group and session: F(10, 55) = 1.26, p = 0.28; main effect of session: F(5, 55) = 0.86, p = 0.517; main effect of group F(2, 11) = 9.32, p < 0.004; see Figure 4b). Specifically, the phase-locking value (PLV) was higher, when the CS onset was timed to the fissure theta trough (n = 5) compared to when it was timed to the theta peak (n = 6) or when the CS was presented in a random neural state (n = 3). In the beginning of extinction (Block 1, first two sessions) phase-locked hippocampal theta-band responses to the CS observed in the Trough group were statistically different from those observed in the Peak group, F(1, 9) = 5.63, p = 0.04, but did not differ from those observed in the Random group, F(1, 6) = 4.26, p = 0.09. During subsequent extinction training (Block 2 and onwards), hippocampal theta-band responses in the Trough group were statistically significantly better phase-locked to the CS compared to responses in the Peak group, F(1, 9) = 5.87–15.70, p < 0.05–0.005, and in the Random group, F(1, 6) = 6.13–15.31, p < 0.05. Despite the difference

in neural responding, behavioral learned responses decreased equally in all of the groups during extinction training; repeated measures ANOVA, interaction of group and session: $F(10, 65) = 1.08$, $p = 0.392$; main effect of session: $F(5, 65) = 5.79$, $p = 0.004$; main effect of group $F(1, 13) = 3.52$, $p = 0.060$). Note that none of the animals accomplished full extinction during the 12 sessions that were conducted (see Figure 4d).

4. DISCUSSION

Our main aim in the two experiments reported here was to find out if the behavioral memory trace of a recently formed association would be more readily retrieved if the conditioned stimulus was presented at the peak of the hippocampal fissure theta oscillation. The peak of fissure theta represents a moment in time during which input from the EC to the hippocampus is suppressed, and during which the hippocampus is assumed to be in an optimal state for memory retrieval (Hasselmo et al., 2002). We found that the behavioral performance of a learned response was not moderated by hippocampal theta phase either early or late in learning trace eyeblink conditioning (Experiment 1) or during extinction learning (both experiments). However, hippocampal responses to the conditioned stimulus were overall temporally more uniformly organized, that is, better synchronized, when the CS was presented to the hippocampal fissure theta trough compared to when it was presented to the theta peak (both experiments). Thus, it seems that the two phases, peak and trough, of the theta oscillation represent differential microstates of the hippocampus, leading to differential

responses at the level of the hippocampus, but this is not sufficient to exert an effect on retrieving the conditioned response acquired during eyeblink conditioning.

Our results indicating no effect of theta phase on memory retrieval across conditioning and extinction are consistent with our earlier findings in well-learned animals (Nokia et al., 2015) and suggest that retrieval of recently acquired memories and consequently performing a learned response is moderated by neural mechanisms other than hippocampal theta. Earlier attempts to link the model by Hasselmo and colleagues (2002) to behavioral data have produced opposing results: Hasselmo (2005) explains the results of M'Harzi et al. (1987) with his model of memory retrieval. In a study by M'Harzi et al. (1987), rats were trained in a T-maze task before and after fornix lesions. The assumption in Hasselmo (2005) was that the lesion could damage the properties of the theta rhythm and cause long-term potentiation in the CA3 auto-associative network and in the synapses between the CA3 and CA1 pyramidal cells. Indeed, at the behavioral level, after the fornix lesion, rats had problems learning a new reward location in the maze. That is, as would be guessed based on the computational model (Hasselmo et al., 2002), encoding to memory was impaired and retrieving old memories was strengthened when input from the EC to the hippocampus was eliminated (M'Harzi et al., 1987). Thus, evidence exists both for and against the computational model of Hasselmo et al. (2002).

Contrary to the prediction based on the model by Hasselmo and colleagues (2002) we could also have hypothesized that more CRs would have been performed when the CS was presented to the hippocampal theta trough. This is because, in our previous study, higher phase-locking of hippocampal theta-band responses and better learning was acquired if the CS was presented to theta trough compared to when it was presented to the theta peak (Nokia et al. 2015). It is also known that high relative hippocampal theta amplitude indicates phase

synchrony of theta oscillations between the hippocampus and the cerebellum (Wikgren et al., 2010), the structure that is critical in CR acquisition and performance in both delay and trace conditioning (Thompson & Steinmetz, 2009). Based on these findings and the assumption that attention is crucial for learning, we have tentatively interpreted the phase-locked theta-band activity in the hippocampus to be a measure of attention targeted towards the CS. However, in our present experiments, even though the CS seemed to elicit more attention when presented at the trough of the theta cycle than when it was presented at the peak, there was no difference in how often the animals produced conditioned responses. A possible explanation for this is the time-limited role of the hippocampus in learning. It might be that at the time we started to test for retrieval of the CR, the animals had already passed the stage of learning where hippocampal contribution is thought to be critical, that is, the phase of 'contingency detection' (Prokasy, 1984). That is, even though phase-locked hippocampal theta might serve as an index of attention, hippocampal processing may not be relevant in memory retrieval after the association between the CS and the US has been established. In support of this, lesion studies suggest that hippocampal contribution to learning eyeblink conditioning is most important early in training, whereas later in memory consolidation the role of the neocortex becomes more crucial (Takehara et al., 2002).

In Experiment 2 we took on another unresolved question, namely whether or not spontaneously occurring hippocampal theta oscillations promote learning. We trained rabbits in delay eyeblink conditioning either during the dominant theta state or during a random neural state. Interestingly, we did not find any effect of theta-contingent training on learning. This is in contrast to the results in Seager et al. (2002), who trained rabbits in delay eyeblink conditioning contingent on theta and in the absence of theta and found faster learning in the group trained contingent on theta. The discrepancy in results could in part be explained by differences in methods between the two studies: Seager et al. (2002) trained rabbits with a

freely varying ITI and used a 350-ms tone-CS co-terminating with a 100-ms air puff. In our study, we used a 30–60 second ITI and an 800-ms tone-CS. Regardless of these differences in ITI and CS duration, we would have expected to see an effect of theta contingent training on learning if theta is beneficial for learning overall. Note that in Experiment 2 we chose not to expose the animals to a CS-alone session (as we usually do). This choice was made in order to minimize methodological differences between our study and that of Seager et al. (2002). Last, in the present experiment, we used female rabbits whereas the sex of the animals was not reported in the Seager et al. (2002) paper. If Seager and colleagues (2002) used males, it could possibly explain the difference in learning as differences in hippocampal theta have been reported between male and female rats (for example, see Maren et al., 1994). Although the differences of hippocampal theta properties between sexes have not been studied in rabbits, the correlation between overall baseline theta power and learning is present in both male (Nokia et al., 2009) and female rabbits (Nokia et al., 2008). Thus, it is possible but perhaps unlikely that sex differences would account for discrepancies between the results of our current experiment and those reported earlier (Seager et al., 2002).

As discussed above, our current results indicate no improvement in learning if eyeblink conditioning is conducted contingent on hippocampal theta. Quite the contrary this is consistent with previous findings from our lab: rabbits trained in a random neural state (Nokia et al., 2015) or in the explicit absence of theta (Nokia et al., 2014) learn better compared to those trained contingent on theta. As already mentioned, hippocampal theta has traditionally been thought to indicate attention or a heightened state of vigilance (Buzsaki, 2002). However, it is not possible to say, for certain, to what is the subject paying attention when theta is observed in the hippocampus. Thus, the spontaneously occurring theta oscillations might actually indicate episodes during which attention is targeted towards aspects of the training situation (for example background noise, lighting) irrelevant for learning the task at

hand. This might explain why theta-contingent training does not enhance learning or memory retrieval. It might also be that in healthy young rabbits enhancing learning is difficult because the animals already perform as well as they can (ceiling effect). A different outcome could be possible when using i.e. elderly rabbits or rabbits with impaired ability to learn.

Last, in both experiments reported here we also conducted extinction training after eyeblink conditioning. We anticipated that rabbits would successfully develop extinction from both trace (Experiment 1) and delay (Experiment 2) trained association. For the delay-conditioned animals this did not happen even after 12 sessions of CS-alone training (720 trials). Correspondingly, Kehoe (2006) succeeded in extinction of well-learned delay-conditioned rabbits by presenting only approximately 60 CS-alone trials. The reason for the poor extinction in our studies is unknown.

Although our results show that at the neuronal level the phase of the hippocampal theta rhythm modulates the neuronal activation to the CS during eyeblink conditioning, further experiments have to be conducted to demonstrate this effect at the behavioral level.

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FIGURE LEGENDS

Figure 1. Outline of the experimental procedures for Experiment 1 (A) and Experiment 2 (B).

A) Animals (*n* = 13) in Experiment 1 were trained in trace eyeblink conditioning (TEBC) with a 40-ms, 5-kHz, 75-dB tone as a conditioned stimulus (CS) and a 100-ms air puff as an unconditioned stimulus (US). The trace period was 660 ms. Examples of an unconditioned response (UR) and a conditioned response (CR) during one conditioning trial are illustrated. After conditioning, rabbits that had learned (*n* = 7) were subjected to extinction training. Memory retention depending on the phase of the hippocampal fissure theta oscillation was tested at four different stages of learning. B) Animals (*n* = 16) in Experiment 2 were trained in delay eyeblink conditioning with an 800-ms tone as a conditioned stimulus (CS). The CS and the US overlapped during the last 100 ms of the tone and co-terminated. Half of the animals were trained in a random neural state (Random, *n* = 8) and half were trained contingent on

hippocampal theta (Theta, $n = 8$). After conditioning, animals were divided into three groups for extinction training: Random ($n = 5$), Theta peak ($n = 6$) and Theta trough ($n = 5$).

Figure 2. In both experiments, hippocampal local-field potentials were recorded from the fissure (A) and displayed characteristic theta oscillations (B).

A) A 60-micron cresyl violet–stained slice of a rabbit hippocampus with the locations of CA1, CA3, dentate gyrus (DG), hippocampal fissure (HF) and pyramidal cell layer (pyr) indicated. The recording electrode tip location is marked with an arrow. B) Example of theta recorded from the fissure.

Figure 3. In Experiment 1 we found no effect of theta phase on memory retrieval at the behavioral level.

The effects of theta phase on memory retrieval were tested at four different stages of learning: when animals performed more than 30% conditioned responses (CRs) during trace eyeblink classical conditioning (CC), when animals performed more than 60% CRs during CC, or when the percentage of CRs dropped below 60% and below 30% during extinction training (EXT). During test sessions, the conditioned stimulus (CS) was presented alone 40 times: 20 times to the trough of theta (Trough) and 20 times to the peak of theta (Peak). The order of the trials was random. A) Overall, hippocampal theta-band (4–8 Hz) responses to the CS were more temporally uniform from trial to trial (indicated by a higher phase-locking value, PLV) if the CS was presented to the hippocampal fissure theta trough compared to theta peak. Asterisks refer to statistically significant differences between Peak and Trough conditions (paired samples t-test): *** $p < .001$, ** $p < .01$. B) Despite the difference in hippocampal responding, there was no difference in the rate of behavioral learned responding (%) between trials presented at theta trough and theta peak.

Figure 4. In Experiment 2 we found no effect of theta-contingent training on learning delay eyeblink conditioning. We also did not find an effect of theta phase–contingent training on extinction learning at the behavioral level, despite more uniform hippocampal responses elicited in the group receiving the conditioned stimulus always at theta trough.

A) Hippocampal theta-band (4–8 Hz) responses to the CS were equally temporally uniform from trial to trial (quantified as the phase-locking value, PLV) in the Theta (n = 6) and Random (n = 8) groups during delay eyeblink conditioning. B) During extinction training theta-band phase-locking was higher in the Trough group (n = 5) compared to the Peak (n = 6) and the Random (n = 3) groups. Asterisks refer to statistically significant differences between groups (Bonferroni-corrected post-hoc comparisons): ** p < .01, * p < .05. C) Theta-contingent training had no effect of learning delay eyeblink conditioning at the behavioral level. D) Theta phase–contingent training had no effect on learning extinction at the behavioral level.