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Title: **Effects of hippocampal state-contingent trial presentation on hippocampus-dependent non-spatial classical conditioning and extinction**

Abbreviated title: Hippocampal state and learning

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The Abstract has 234 words, the Introduction has 484 words and the Discussion has 1419 words.

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Abstract

Hippocampal local-field potentials are characterized by two mutually exclusive states: one characterized by regular theta oscillations (~4-8 Hz) and the other by irregular sharp-wave ripples. Presenting stimuli during dominant theta oscillations leads to expedited learning, suggesting that theta indexes a state in which encoding is most effective. However, ripple-contingent training also expedites learning, suggesting that any discrete brain state, much like the external context, can affect learning. We trained adult rabbits in trace eyeblink conditioning, a hippocampus-dependent non-spatial task, followed by extinction. Trials were delivered either in the presence or absence of theta or irrespective of hippocampal state. Conditioning in the absence of theta led to more animals learning, although learning was slower compared to a yoked control group. Contrary to expectations, conditioning in the presence of theta did not affect learning. However, extinction was expedited both when it was conducted contingent on theta and when it was conducted in a state contrary to that used to trigger trials during conditioning. Strong phase-locking of hippocampal theta-band responses to the conditioned stimulus early on during conditioning predicted good learning. No such connection was observed during extinction. Our results suggest that any consistent hippocampal oscillatory state can potentially be used to regulate learning. However, the effects depend on the specific state and task at hand. Finally, much like the external environment, the ongoing neural state appears to act as a context for learning and memory retrieval.

Keywords: hippocampus, theta, classical conditioning, extinction

Introduction

Hippocampal local-field potentials are characterized by two mutually exclusive states: highly rhythmic theta (~4-8 Hz, Green and Arduini, 1954; see also Buzsaki and Moser, 2013) and irregular sharp-wave activity, during which ripples (~200 Hz, Chrobak and Buzsaki, 1996) are present. Spontaneous theta oscillations in an awake animal are thought to reflect a state in which labile memory traces of events that are taking place are formed (Buzsaki, 1989), the read-state. Consistently, a high relative power of hippocampal theta activity before training predicts good learning (Berry and Thompson, 1978; Nokia et al., 2012a) and presenting trials contingent on periods of high theta facilitates hippocampus-dependent trace eyeblink classical conditioning (Griffin et al., 2004). Interestingly, presenting conditioning trials contingent with ripples (in the absence of theta) also expedites learning (Nokia et al., 2010). Thus, it is unclear if presenting trials contingent on any consistent neural state would facilitate learning. In addition, good learning is predicted not only by the spontaneously ongoing oscillatory state, but also strong and well phase-locked hippocampal theta-band responses to the conditioned stimulus (Nokia et al., 2010; Nokia et al., 2009; Nokia and Wikgren, 2010). Whether learning is predominantly dependent on the pre-trial hippocampal oscillatory state, which then determines subsequent responses to the conditioned stimulus, or if the responses themselves determine learning, is not clear.

Most studies on the role of hippocampal oscillatory activity in learning have overlooked learning modifications to already acquired associations. Discriminatory reversal learning does not seem to correlate with hippocampal theta-band responses to conditioning stimuli (Nokia and Wikgren, 2010), but presenting extinction trials contingent on hippocampal ripples hinders extinction (Nokia et al., 2010). To our knowledge, whether hippocampal theta is connected to simple extinction has not been studied. It is also not known

whether an internal state characterized by consistent hippocampal oscillations could, much like the external environment, work as a context for learning (Godden and Baddeley, 1975).

To address the issues raised above, we trained adult female New Zealand White rabbits in trace eyeblink conditioning using a 500-ms trace interval. Training trials were timed to occur when endogenous hippocampal theta was either prominent (T+) or absent (T-). Animals in the respective yoked control (YC) groups received training trials irrespective of their brain activity. For animals that acquired a robust conditioned response, conditioning was followed by extinction. For this, half the animals in the T+ group were switched to the T- group and vice versa. We expected better phase-locked hippocampal theta-band responses to the conditioned stimulus and better and faster conditioning in both the T+ and the T- groups compared to animals trained irrespective of their neural state. In addition, we assumed a predictive correlation between well phase-locked theta-band responses to the conditioned stimulus and good learning. Finally, we hypothesized that extinction would be facilitated in both the T+ group and in animals that were trained contingent on one hippocampal state and extinguished in a different state (T+/T- or T-/T+).

Materials and Methods

Subjects

The subjects were 36 adult female New Zealand White rabbits (Lidköpings kaninfarm, Sweden) aged ~4 months and weighing ~2.7 kg at the time of surgery. The rabbits were housed in individual cages on the premises of the animal research unit 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 am. All procedures were conducted during the light portion of the cycle. All the

experimental procedures were implemented in accordance with directive 2010/63/EU of the European Parliament and of the Council on the care and use of animals for research purposes.

Surgery

Under general anesthesia, four monopolar recording electrodes made of Teflon-insulated stainless steel wire (bare diameter 125 μm) mounted inside 27-gauge hypodermic stainless steel tubing were chronically implanted into the left dorsal hippocampus 5 mm posterior, 4-7 mm lateral and 6.5-7.4 mm below the bregma, aiming at the hippocampal fissure and dentate gyrus (see (Nokia et al., 2012b) for details on surgery). Four stainless-steel anchoring screws were attached to the skull. The screws were connected in pairs and served as ground and reference for the local-field potential (LFPs). The electrodes were attached to a pin connector and the whole construction cemented in place with dental acrylic. At least one week was allowed for post-surgical recovery.

Conditioning procedure

Prior to the experiments, the rabbits were placed (~20 minutes) in a Plexiglas restraining box located in a ventilated, electrically insulated, and sound-attenuated conditioning chamber to familiarize them with the experimental situation, and to ensure the functioning of the implanted electrodes. Thereafter, experimental sessions were conducted once per day on consecutive days, excluding weekends.

The conditioned stimulus (CS) was a 4-kHz, 85-dB, 200-ms tone and the unconditioned stimulus (US) was a 100-ms corneal airpuff (0.35 bar source pressure, 64 dB sound pressure level) delivered through a nozzle (inner diameter 2 mm) placed approximately 1 cm away from the eye. A fan located inside the conditioning chamber created a steady background noise (65 dB). LabView (National Instruments, Austin, TX, USA) and E-Prime software

(Psychology Software Tools Inc., Pittsburgh, PA, USA) were used to control the presentation of stimuli.

First, an unpaired session was conducted to obtain baseline measures. Both the CS and the US were presented alone 60 times, in random order and irrespective of the hippocampal state, with an inter-trial interval (ITI) of 15 to 25 seconds. Next, the rabbits were pseudo-randomly assigned to three groups: rabbits in the T+ group (n = 11) received training trials contingent on high hippocampal theta activity; rabbits in the T- group (n = 10) received trials when hippocampal theta activity was low; and rabbits in the yoked control group (n = 15) were trained paired with either a T+ (n = 8) or a T- (n = 7) rabbit and thus received trials irrespective of their neural state. All rabbits were trained in trace eyeblink conditioning. Each session consisted of 60 paired trials where a 500-ms silent trace period separated the CS from the following US. The minimum ITI was set to 15 s and varied according to the hippocampal oscillatory state of the T+/T- animal. Conditioning was ceased when the animal reached the predetermined learning criterion of ≥60% conditioned responses in at least 2/3 consecutive training sessions. A minimum of 10 and a maximum of 16 conditioning sessions were conducted. If an animal reached the criterion of ≥60% CRs in 2/3 sessions within the minimum number of 10 sessions but performance was lower than 60% in session 10, conditioning was continued until the animal again attained the 60% criterion.

All animals that had reached the ≥60% criterion during at least one conditioning session were subjected to 10 sessions of extinction training identical to the conditioning except that the US was now omitted. For extinction, the rabbits in the T- and T+ groups were re-assigned so that half the animals remained in the same group and half switched from T+ to T- or vice versa. The animals in the YC group remained in that group also for extinction.

Triggering trials based on hippocampal theta ratio. The LFP signal from one hippocampal electrode was monitored online in consecutive, non-overlapping, 1-s sweeps.

From each sweep, the Fast Fourier Transform was calculated with a resolution of 1 Hz. The theta ratio was calculated as the ratio between the power of the signal at 3.5-8.5 Hz divided by the power of the signal at 1-8.5 Hz (theta / [delta + theta]). For animals in the T+ group, trials were presented when the theta ratio was >85%. For animals in the T- group, trials were presented when the theta ratio was <35%. Note that occasionally epochs of theta/no-theta that fell in the middle of two 1-s sweeps sampled by Labview, and thus it was not necessarily always the first epoch of theta/no-theta (after the 15-s minimum ITI) that triggered a trial. If needed, the threshold for triggering was adjusted individually to an even more stringent value (for example 90% instead of 85 % for an animal in the T+ group), so that the state in which an animal was trained was one of an extremely high (T+) or low (T-) theta ratio in relation to that animal's general theta level.

Recordings and data analyses

Bipolar electromyogram (EMG) was recorded using stainless steel wire hooks placed around the upper and lower eyelids for the duration of the training session. To acquire neural measures, a low-noise pre-amplifier (MPA8I, MultiChannel Systems, Reutlingen, Germany) was directly attached to the electrode coupler anchored with dental acrylic to the rabbit's head. A flexible, insulated cable was used to connect the animal to the amplifier (Axon Cyberamp 380, Molecular Devices Corporation (MDC), Union City, CA, USA). All signals were recorded with AxoScope (MDC) software and digitized (Digidata 1322A, MDC) using a 2.02 kHz sampling rate. Before digitization, the LFPs were band-pass-filtered between .1–400 Hz, and the EMG was filtered between 30-400 Hz. MATLAB (The MathWorks Inc., Natick, MA, USA) and SPSS (SPSS Inc., Chicago, IL, USA) were used for offline data analysis.

Baseline theta. Data from the 20-minute habituation session was analyzed off-line to obtain baseline characteristics of hippocampal theta-band activity. A sliding window of 1 s in 200 ms steps was used, and the Fast Fourier Transform was calculated for each epoch. From the data, the duration and inter-event interval was determined for the T+ (theta ratio > 85%) and T- (theta ratio < 35%) epochs. To verify that our Labview routine used for triggering trials had worked as intended, we also calculated pre-trial theta ratios off-line for the first 10 conditioning sessions.

Eyeblinks. The EMG signal was high-pass filtered off-line (>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 500-ms pre-CS period (MEANpre). Also determined was the mean of the standard deviation of the EMG activity during the 500-ms pre-CS period (SDpre). Eyeblinks were defined as EMG activity exceeding a threshold of [MEANpre + 4*SDpre] for at least 10 ms. Trials with eyeblinks during the 100-ms period immediately preceding CS onset were rejected. Eyeblinks during the latter half (250-ms period) of the 500-ms trace period were counted as conditioned responses. The learning criterion was considered to be met when the subject performed ≥60% CRs during at least one conditioning session. Learning rate was quantified as the number of conditioning sessions needed to reach the 60% criterion. We also measured learning rate as the number of trials needed to reach the traditional "8 conditioned responses on 9 consecutive trials" criterion and the number of trials needed to perform the 5th CR.

Phase-locked hippocampal theta-band (4-8 Hz) responses. To assess the temporal accuracy of theta-band responses to the conditioning stimuli, a phase-locking value (PLV) (Palva et al., 2005) was calculated. 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 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 blocks of two sessions.

Statistical analyses.

Repeated measures analysis of variance (ANOVA), with training blocks of two sessions (5) as a within-subjects factor and group as a between-subject factor, was used to analyze changes across training. Whenever a significant interaction of block and group was evident, separate repeated measures ANOVAs were conducted for each group, using blocks as a within-subjects factor. Greenhouse-Geisser corrected p-values were used when the sphericity assumption was violated according to Mauchly's test. One-way ANOVA was used in group comparisons when single variables were tested. Bonferroni corrected p-values were used for post-hoc comparisons. The Chi-square test was used to compare the proportions of animals that learned in each group. Pearson correlation coefficient was used to analyze the connection between hippocampal phase-locked theta-band responses to the CS and learning. Finally, paired samples t-tests were used to analyze differences within subjects.

Histology

Rabbits were anesthetized with an i.m. injection of ketamine-xylazine cocktail and then overdosed with an i.v. injection of pentobarbital (Mebunat vet, Orion-Yhtymä Oyj, Espoo, Finland). Next, the brain was perfused with physiological saline followed by 9% formalin

solution through the ascending aorta. The locations of the electrode tips were marked by passing a DC current (200 µA, 20 s) 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-tip locations were determined with the help of a microscope.

Results

Histology and baseline hippocampal theta activity

One electrode per animal was selected for offline analyses based on the tip location and signal quality (see Fig. 1). Twenty-nine out of 36 animals had correctly placed electrodes in the hippocampus.

The average theta ratio (%) as well as the duration, interval, proportion (of all studied epochs) and theta ratio of high and low theta epochs during the 20-minute baseline recording were quantified and compared among the two experimental and two yoked control groups. One-way ANOVAs revealed no differences between any of the groups in any of the baseline measures, $F [3, 28] = 0.25 - 1.24$, ns.. The average theta ratio was 67% with a standard error of mean (SEM) of ± 1.0 percentage unit. The animals were in a state characterized by a high hippocampal theta ratio (T+, theta ratio > 85%) for, on average, 29.71% (± 1.86 percentage units) of the time. Epochs of high theta lasted, on average, for 1.57 ± 0.03 s and occurred, on average, 2.70 ± 0.08 s apart (onset-to-onset interval). The animals were in a state characterized by a low hippocampal theta ratio (T-, theta ratio < 35%) for, on average, 10.18% (± 0.63 percentage units) of the time. Epochs of low theta lasted, on average, 1.25 ± 0.01 s and occurred, on average, 4.95 ± 0.37 s apart. Paired samples t-tests confirmed that T+

epochs were more frequent (onset-to-onset interval: $t(28) = 5.35$, $p < .001$) and lasted longer (duration: $t(28) = 8.13$, $p < .001$) than T- epochs.

To verify that our Labview routine had worked as intended, we calculated pre-trial theta ratios offline for the first 10 conditioning sessions. The average theta ratio was 87% ± 0.2 percentage units in the T+ group (n = 11) and 62% ± 4.0 percentage units in the respective yoked control group (n = 3). The average theta ratio was 22% ± 1.5 percentage units in the T- group (n = 11) and 57% ± 1.7 percentage units in the respective yoked control group (n = 5). Examples of episodes with a high and low hippocampal theta ratio that would trigger training trials in the two experimental groups are depicted in Fig. 1B. The power spectra for the 1-s pre-trial periods in the T- and T+ groups are presented in figure 1C.

Inter-trial interval variation across training and between groups

During conditioning (all sessions, max 16) the mean (± SEM) ITI was 34.4 ± 1.3 seconds in the T+ group (YC: 34.1 ± 1.7 seconds) and 35.2 ± 1.1 seconds in the T- group (YC: 35.9 ± 1.3 seconds). Further examination of the first 10 sessions of training (5 blocks of 2 sessions) with repeated measures ANOVA revealed an interaction of conditioning block (5) and group (4) ($F[12, 128] = 2.774$, $p = .014$). Namely, the ITI decreased across training in the T- group from 40.1 ± 2.4 seconds to 33.8 ± 1.1 seconds (repeated measures ANOVA, block: $F[4, 36] = 6.30$, $p = .001$) and in its respective YC group from 41.8 ± 3.1 seconds to 33.8 ± 1.5 seconds ($F[4, 24] = 7.37$, $p = .001$). In the T+ and its respective YC groups, the ITI remained stable across conditioning ($F[4, 40] = .082$, ns. and $F[4, 28] = 0.48$, ns., respectively). Group differences in ITI were present during the first two blocks of training (one-way ANOVAs: $F[3, 35] = 4.75/3.73$, $p = .008/.021$, respectively) after which the ITI in the T- and its respective yoked control group reached the level of the ITI in the T+ and its respective yoked control group (one-way ANOVAs, blocks 3-5: $F[3, 35] = 0.06$-0.21, ns.). To eliminate the

effect of the differing ITI, learning and hippocampal responses during conditioning were compared between the T- and its respective YC groups and between the T+ and its respective YC groups. To further examine the effects of ITI on learning, we compared learning in the two yoked control groups: As a result of a longer ITI early in conditioning, a greater proportion of animals (4/7 vs. 2/8) reached the 60% learning criterion (Chi-Square test: X^2 (1, N = 7) = 3.86, p = .050). In addition, a longer ITI early in conditioning resulted in faster learning in animals that eventually learned (independent samples t-test on sessions to criterion: t (4) = 2.88, p = .045). Reaching the 60%-criterion took on average 14.5 ± 1.5 sessions in the T+ YC group and 7.5 ± 1.5 sessions in the T- YC group.

During extinction (all sessions, 10) the ITI was on average 32.6 ± 1.2 seconds in the T+ group (YC: 32.0 ± 0.8 seconds) and 36.3 ± 2.5 seconds in the T- group (YC: 33.3 ± 1.8 seconds). During extinction, no changes across training or differences between groups in the ITI (repeated measures ANOVA, interaction: F [12, 60] = 0.77, ns., block: F [4, 60] = 1.56, ns., group: F [3, 15] = 0.85, ns.) occurred. Hence, the animals in the YC groups were combined into a single control group (n = 6, average ITI 32.9 ± 1.2 seconds) and comparisons were made between the T- (n = 7), T+ (n = 6) and the YC (n = 6) groups.

To study the effects of the consistency of the pre-trial neural state between conditioning and extinction on extinction, the data were divided into two groups: Same (T+/T+ or T-/T-) and Different (T+/T- or T-/T+). The average ITI was 34.9 ± 1.4 seconds in the group Same (n = 7) and 34.2 ± 3.0 seconds in the group Different (n = 6). Again, no changes across the extinction training or differences between the 3 groups (Same, Different, YC) in ITI (repeated measures ANOVA, interaction: F [8, 64] = 0.45, ns., block: F [4, 64] = 1.55, ns., group: F [2, 16] = 0.28, ns.) occurred. Taken together, possible differences between the groups (T+ vs. T- vs. YC and Same vs. Different vs. YC) in extinction could not be due to differences in ITI.

Training in the explicit absence of hippocampal theta leads to slower but better learning of trace eyeblink conditioning

The behavioral results of trace eyeblink conditioning and extinction are summarized in figure 2. Note that for conditioning, data were analyzed in two parts: T- vs. its respective YC group and T+ vs. its respective YC group. This was done to avoid confounding effects of differences in the ITI (see preceding section on ITI). Note also that different animals had a different number of conditioning sessions (between 10 and 16) due to individual differences in learning speed. Hence, data from only the first 10 sessions (blocks 1-5) was subjected to repeated measures ANOVA and learning outcome was primarily assessed based on the number of animals that reached the learning criterion.

Conditioning led to increased blinking in response to the tone-CS in both the T+ and the T+ yoked control group (repeated measures ANOVA, block: $F [4, 68] = 3.75$, $p = .024$; group: $F [1, 17] = 0.19$, ns.; interaction: $F [4, 68] = 0.80$, ns.) (see Figure 2A for individual learning curves). The same was true for the T- and the T- yoked control groups (block: $F [4, 60] = 13.53$, $p < .001$; group: $F [1, 15] = 1.59$, ns.; interaction: $F [4, 60] = 1.96$, ns.) (see Figure 2B for individual learning curves). Eventually, within a maximum of 16 conditioning sessions, 4/11 animals in the T+ group and 2/8 animals in the respective yoked control group reached the pre-determined learning criterion of at least 60% conditioned responses within at least one conditioning session (Figure 2C, left panel). The proportion of animals that learned in the T+ group did not differ from that expected on the basis of the proportion of animals that learned in the respective yoked control group (Chi-Square test: $X^2 (1, N = 11) = 0.76$, ns.). In the T- group, 9/10 animals learned the task compared to 4/7 in the respective YC group (Chi-Square test: $X^2 (1, N = 10) = 4.41$, $p = .036$, see Figure 2C, right panel). To summarize, the proportion of animals that learned in the T- group was significantly higher than the corresponding proportion in its respective yoked control group.

When the number of conditioning sessions needed to reach the 60% learning criterion was compared between the T+ and the respective YC groups, no significant difference emerged (one-way ANOVA, F [1, 4] = 4.70, ns./p < .1) (see Figure 2D, left panel). The same analysis for the T- and the respective yoked control groups indicated that animals that reached the learning criterion took significantly longer to do so in the T- group compared to the respective YC group (one-way ANOVA: F [1, 11] = 5.67, p = .036) (see Figure 2D, right panel).

We also analyzed learning rate using the traditional 8/9 criterion (see Methods). According to this criterion, 90% of animals in T- group learned, compared to 71% in the respective yoked control group (Chi-Square test: X^2 (1, N = 10) = 1.69, ns.). In the T+ and corresponding YC groups, 46% compared to 63% of animals learned, respectively (Chi-Square test: X^2 (1, N = 11) = 3.21, ns.). In terms of learning rate, the number of trials to criterion was on average 442 ± 56 in the T- group compared to 246 ± 43 in the corresponding yoked control group (one-way ANOVA: F [1, 12] = 5.67, p = .035). The mean number of trials to criterion in the T+ group was 479 ± 87 compared to 539 ± 70 in the corresponding yoked control group (one-way ANOVA: F [1, 8] = 0.29, ns.).

Further, we studied the number of trials needed to perform the 5th conditioned response. There was no difference in this measure between the T+ and its yoked control group (61 ± 10 vs. 50 ± 9 trials, respectively, one-way ANOVA: F [1, 17] = 0.59, ns.) nor between the T- and its yoked control group (54 ± 10 vs. 45 ± 9 trials, F [1, 15] = 0.38, ns.).

Training contingent on theta or contingent on a hippocampal state opposite to that used for conditioning, facilitated extinction

Altogether, 19 animals learned trace eyeblink conditioning. These animals were further trained in extinction. For extinction, rabbits initially conditioned contingent upon either a

high or low hippocampal theta ratio were re-divided into groups so that half the animals remained in the same group (Same, n = 7) and half were switched from the T+ to the T- group or vice versa (Different, n = 6). Animals in the yoked control group remained in that group also for extinction (n = 6). Altogether, 6 animals were trained in extinction contingent on a high hippocampal theta ratio (T+) and 7 animals were trained contingent on a low hippocampal theta ratio (T-). Contrary to conditioning, during extinction training the inter-trial intervals were stable and comparable in all groups (see section above), hence the data from all the yoked control animals were pooled and comparisons then made between T+, T- and YC groups and between the Same, Different and YC groups.

First, we wanted to ensure that any differences in extinction were not due to differences in the outcome of conditioning. One-way ANOVA conducted on the average CR percentage during the last block of conditioning indicated that the animals in the T-, T+ and YC groups that had reached the learning criterion had indeed learned to a comparable degree ($F [2, 16] = 0.84$, ns.) (see Fig. 2E). The same was true when animals in the T+ and T- groups were grouped based on the consistency of the training paradigm during conditioning vs. extinction: the animals in the Same, Different and YC groups also showed similar levels of conditioned responding at the end of conditioning (one-way ANOVA, last block, $F [2, 16] = 1.38$, ns.). Next, for the purpose of illustration and statistical testing, conditioned responding (%) during extinction was averaged over two consecutive sessions to form 5 blocks.

Extinction led to decreased blinking in response to the tone-CS alone in the T+, the T- and the YC group (repeated measures ANOVA, block: $F [4, 64] = 17.21$, $p < .001$; interaction: $F [8, 64] = 1.60$, ns.) (see Fig. 2E). A significant effect of group was also observed: $F [2, 16] = 4.06$, $p = .037$. Post-hoc comparisons between groups indicated that the T+ group showed superior extinction compared to the yoked control group (Bonferroni-

corrected p = .048). No difference emerged between either the T- and T+ or between the T- and the yoked control group.

Next, we explored the effects of the consistency of the internal training context between conditioning and extinction training on learning extinction. Repeated measures ANOVA revealed an interaction of group (Same vs. Different vs. YC) and extinction block (5): F [8, 64] = 2.26, P = .034 (see Fig. 2F). Separate repeated measures ANOVAs conducted for each group indicated that conditioned responding decreased as a function of extinction (5 blocks) in the yoked control group (F [4, 20] = 14.83, p < .001) and in the group trained in the same state it had been conditioned in (group Same: F [4, 24] = 8.41, p < .001) whereas no change took place in the group conditioned in one state and trained in extinction in another state (group Different: F [4, 20] = 1.74, ns.). One-way ANOVAs further revealed that learned responding differed between the groups during the first (F [2, 16] = 4.74, p = .024) and second (F [2, 16] = 3.94, p = .041) blocks of extinction. Specifically, conditioned responding was lower in the Different group compared to the YC group during the first two blocks of extinction (p = .022/.038). In other words, animals that had been switched from T+ to T- or from T- to T+ training between conditioning and extinction very rapidly ceased responding to the tone-CS.

Phase-locked hippocampal theta-band (4-8 Hz) responses to the conditioned stimulus predicted learning during trace eyeblink conditioning

Phase-locking of hippocampal theta-band responses to the conditioned stimulus measured using the phase-locking value (PLV, see Methods above) was compared between groups and across training (T+ vs. its YC group and T- vs. its YC group for conditioning and T+ vs. T- vs. YC for extinction). Repeated measures ANOVAs did not reveal any significant differences between groups or changes across training during either conditioning or

extinction (data and exact statistics not shown). Hence, data from all groups was pooled to facilitate more straightforward study of the connection between phase-locked theta-band responses and learning. However, repeated measures ANOVAs did not reveal any significant differences between groups or changes across training when phase-locked theta-band responses to the CS elicited during conditioning were compared between animals that learned (n = 17) vs. animals that did not learn (n = 12) (data and exact statistics not shown).

Next, correlations were calculated between hippocampal responses to the CS and learning. Well phase-locked hippocampal theta-band responses to the conditioned stimulus early on during trace eyeblink conditioning (1st block) predicted good learning measured as the percentage of behavioral learned responses elicited (blocks 3-5, r = .396-.429, p = .020-.034, n = 29). To better quantify learning in the middle of conditioning, the average CR percentage during the two middle sessions (rounding down, if needed) was calculated for each animal. This measure correlated significantly with the theta-band phase-locking value to the CS recorded during the 1st conditioning block: r = .400, p = .032 (Fig. 3A). The phase-locking of hippocampal theta-band responses to the CS during the 1st conditioning block also predicted learned responding at the end of conditioning (Fig. 3B), although the connection was weaker: r = .340, ns./p < .1. As one animal (in the T+ group) had very well phase-locked theta-band responses yet did not learn, it was classified as an outlier. When removed from the data, the correlation between hippocampal responses and learning at the end of conditioning also became significant, r = .483, p = .009, n = 28. No significant correlations were found between the phase-locked hippocampal theta-band responses to the CS and measures of learning rate (session to 60% CRs/ TTC/ Trials to 5th CR: n = 17/ 20/ 29, r = -.34/ -20/ 27, ns.).

We also calculated the correlations between the phase-locked hippocampal theta-band responses to the CS early in the extinction training (1st block) and conditioned responding

throughout extinction (5 blocks), and found no significant correlations (n = 16, r = .044-.254, ns.).

Discussion

We set out to study how training contingent on a state characterized by the presence versus absence of hippocampal theta activity affects trace eyeblink conditioning and subsequent extinction. Contrary to our hypothesis and previous results (Griffin et al., 2004), training contingent on a high (T+) level of hippocampal theta did not affect trace eyeblink conditioning. Curiously, learning was more probable, yet required more trials, in animals that were trained in the absence of hippocampal theta (T-) compared to their yoked controls. As hypothesized, training contingent on high theta promoted extinction. In addition, like training in one external environment and testing in another results in a decrease in memory retrieval (Godden and Baddeley, 1975), changing the hippocampal state in which the subject was conditioned versus trained in extinction facilitated extinction. Finally, we examined whether the pre-trial oscillatory state determines the subsequent hippocampal response to the conditioned stimulus and thus predicts learning. Contrary to our hypothesis, responses did not differ based on the pre-trial hippocampal oscillatory state. However, as hypothesized, hippocampal theta-band responses reliably predicted learning during conditioning (but not extinction).

We found that although learning is slower, trace eyeblink conditioning leads to a robust learned response more often, if trials are presented in the absence of hippocampal theta (T-). This is in line with previous findings of improved learning when conditioning trials are presented contingent on hippocampal ripples (Nokia et al., 2010) and of slower learning in the absence of theta (Griffin et al., 2004). However, training contingent on the absence of theta enhanced learning only after prolonged training, whereas ripple-contingent training

facilitated the commencement of conditioned responding early in training (Nokia et al., 2010). The state in which ripples occur is clearly more uniform than the non-theta state, a difference that might explain the difference in the timing of the effects. Contrary to our hypothesis, as well as earlier findings on the connection between theta and learning (Griffin et al., 2004; Seager et al., 2002), presenting conditioning trials contingent on dominant hippocampal theta (T+) had no effect on learning outcome in our present experiment. There was a trend ($p < .1$) for animals in the T+ group that did learn to do so faster than their yoked controls, which would fit with previous findings by Griffin et al. (2004). The discrepancy between our results and those in the aforementioned study could be related to methodological differences, such as the definition of the T+ and T- states, the inter-trial interval, or the parameters of the stimuli used for conditioning. It might be, for example, that T+ conditioning only affects learning outcome if the conditioned and/or unconditioned stimulus is presented to the trough of the fissure theta, when input from the entorhinal cortex dominates and long-term potentiation occurs at the CA3-CA1 synapses (Hasselmo et al., 2002). In our experiment, the phase of theta was random at both CS and US onset. Further, the data and analyses presented by Griffin et al. (2004) concentrated on early acquisition, whereas we trained animals up to 16 days and only found a facilitative effect of T- treatment on conditioning very late in training. Thus, it is difficult to make straightforward comparisons between the two studies. Nevertheless, if the transient state in which theta-band activity dominates hippocampal electrophysiological oscillations generally promotes learning, the T+ group in our study should have learned trace eyeblink conditioning significantly better and/or faster.

In our study, T+ training facilitated extinction whereas T- training had no effect on it. This is in line with our previous finding of slower extinction when the conditioned stimulus is presented contingent on hippocampal ripples (Nokia et al., 2010). These results suggest that

pre-trial neural states and resultant behavior during extinction training constitute a continuum, with dominant theta and inhibition of responding at one end, no-theta somewhere in the middle and ripples and heightened responding at the other extreme. This seems plausible, since the state in which ripples occur promotes excitatory responding in the hippocampus (Buzsaki, 1986) whereas CA1 pyramidal cells rarely fire action potentials during periods of dominant theta (Kamondi et al., 1998). Instead of faster learning of the new "CS - no-US" -association, the expedited extinction in the T+ group could be due to a failure in memory retrieval and/or the performance of the conditioned response. Interestingly, in the present experiment, extinction was also facilitated in animals that were initially conditioned contingent on one state and later extinguished contingent on a different state. This is in line with our hypothesis suggesting that the internal neural state can act as a context for learning and memory similar to the external environment: retrieval is known to be deteriorated when memory is tested in an environment different from that where it was acquired (Godden and Baddeley, 1975; Gordon et al., 1981). Of course, state-inconsistent extinction could also just expedite learning that the conditioned stimulus is no longer followed by an unconditioned stimulus. Before any further speculation about these results, the dilemma between expedited learning and a simple retrieval/performance deficit should be addressed in futures studies by incorporating a re-learning or testing phase following extinction training.

In addition to behavior we also analyzed phase-locked hippocampal theta-band (4-8 Hz) responses to the conditioned stimulus. The pre-trial hippocampal state did not have an effect on the responses either during conditioning or during extinction. This is at odds with our hypothesis and the behavioral results reviewed above. It seems that regulating the pre-trial hippocampal state to control learning affected information processing in the hippocampus in a way not detectable with our methods and/or in circuits crucial for learning outside the hippocampus, perhaps for example in the cerebellum or in the medial prefrontal

cortex (Weiss and Disterhoft, 2011). However, regardless of experimental group, phase-locked hippocampal theta-band responses to the conditioned stimulus predicted learning: the better phase-locked the responses early in training, the more conditioned responses the animal elicited in the middle and end of conditioning. This observation is in line with our hypothesis and with previous results (Nokia et al., 2009; Nokia et al., 2010) indicating higher-amplitude and better phase-locking of hippocampal theta-band responses to the conditioned stimulus early in training in animals that eventually learn trace eyeblink conditioning. The result is not surprising since it was suggested as early as during the 1970s that the phase-locking of hippocampal theta activity in response to a conditioned stimulus accompanies learning its significance (Buzsaki et al., 1979). It could be that events evoking a highly temporally uniform response in the hippocampus from one occasion to another are processed more accurately and efficiently (perhaps throughout the brain), and that this leads to efficient learning. Via oscillatory synchronization (Fries, 2005), temporally regular responses to repeated stimuli could enable (at least in theory) efficient signaling between the brain structures involved in learning about those stimuli (Wikgren et al., 2010; Hoffmann and Berry, 2009) and hence promote plasticity (Hebb, 1949). At the psychological level, our interpretation of hippocampal phase-locked theta-band responses to external stimuli is that they reflect the level of selective attention paid to that specific stimulus: highly phase-locked responses reflect strong attention whereas lack of regularly repeating responses indicates low attention (see also Vinogradova et al., 1993).

Conclusions

Current and previous findings on hippocampal state -contingent conditioning and extinction suggest that the notion of ongoing, spontaneously occurring hippocampal theta oscillations as an all-encompassing promoter of learning is too simplistic. Although an overall high level of

hippocampal theta, possibly indicating a predisposition of an individual to be aroused and attentive toward its surroundings rather than mellow, seems to consistently predict good learning (Berry and Thompson, 1978; Nokia et al., 2009; Nokia et al., 2012a), effects of theta-triggered training depend on the task at hand and are more variable. We suggest that any distinguishable spontaneously generated (hippocampal) oscillatory state can affect learning by setting the stage for excitatory vs. inhibitory responding at the neural and behavioral levels, and perhaps also by serving as an additional cue or context for learning, much like the features of the external environment, at the psychological level. It would seem that states that occur relatively rarely, such as the explicit absence of theta oscillations, might serve as more distinct cues and thus affect learning more than states that take place relatively often, such as the high theta state. However, rather than the pre-event activity, in the context of acquiring a conditioned response, learning is predicted by the temporal regularity of hippocampal responses to the event early in training. We suggest that at the psychological level the temporally regular responses translate onto selective attention.

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Figure legends

Figure 1. Electrodes were placed in the dorsal hippocampus (A) and used to record local-field potentials (LFPs). The examples shown in panel B illustrate periods of high and low levels of hippocampal theta activity. The power spectra presented in panel C further demonstrate differences between groups in pre-trial hippocampal LFPs. A) Electrodes had been correctly placed in the hippocampus in 29 out of 36 animals. The 7 animals without hippocampal recordings were all in the yoked control group. B) Examples of LFPs with high (T+, > 85%) and low (T-, < 35%) theta ratio. C) Power spectra corresponding to the pre-trial periods during conditioning sessions 1-10 illustrate the presence of theta in the T+ group and the absence of theta in the T- group.

Figure 2. Training contingent on the explicit absence of hippocampal theta promoted trace eyeblink conditioning (A-D), whereas training contingent on dominant hippocampal theta facilitated extinction (E). Further, extinction was facilitated when training was conducted in an internal state opposite to the one used to time training trials during the initial conditioning (F). A and C) Animals trained in the presence of theta (T+) and their corresponding yoked control group learned to a similar degree. B and C) Animals trained in the absence of theta (T-) learned more often than animals in the corresponding yoked control group (Chi-Square, $p < .05$). D) Animals that reached the learning criterion took significantly longer to do so in the T- group compared to the respective YC group (One-way ANOVA, $p < .05$). E) Compared to the yoked control group, extinction was facilitated in the group trained in the presence of dominant hippocampal theta (T+). F) Compared to the yoked control group, extinction was also facilitated in animals trained in a neural state different to that used for conditioning (Different). The effect was limited to the first two blocks of extinction. In E and F, asterisks refer to repeated measures ANOVAs (aligned with the x-axis) and one-way ANOVAs (above or below data points): ***

$p < .001$ and * $p < .05$. In F, line number 1 indicated results of repeated measures ANOVA with 3 groups and 5 blocks and line number 2 indicated separate repeated measures ANOVAs with 5 blocks for each group.

Figure 3. Hippocampal phase-locked theta-band responses to the conditioned stimulus (CS) recorded early in training predicted learned responding in the middle (A) and at the end (B) of trace eyeblink classical conditioning. Pearson's correlation coefficients were calculated, ** $p < .01$ and * $p < .05$. Dotted lines depict linear regression. In B, results are presented including (black) and excluding (red) the outlier marked with a red triangle.