Hippocampal theta (3-8 Hz) activity during classical eyeblink conditioning in rabbits

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Abstract

In 1978, Berry and Thompson showed that the amount of theta (3-8 Hz) activity in the spontaneous hippocampal EEG predicted learning rate in subsequent eyeblink conditioning in rabbits. More recently, the absence of theta activity during the training trial has been shown to have a detrimental effect on learning rate. Here, we aimed to further explore the relationship between theta activity and classical eyeblink conditioning by determining how the relative power of hippocampal theta activity [theta / (theta + delta) ratio] changes during both unpaired control and paired training phases. We found that animals with a higher hippocampal theta ratio immediately before conditioning learned faster and also that in these animals the theta ratio was higher throughout both experimental phases. In fact, while the hippocampal theta ratio remained stable in the fast learners as a function of training, it decreased in the slow learners already during unpaired training. In addition, the presence of hippocampal theta activity enhanced the hippocampal model of the conditioned response (CR) and seemed to be beneficial for CR performance in terms of peak latency during conditioning, but did not have any effect when the animals showed asymptotic learning. Together with earlier findings, these results imply that the behavioral state in which hippocampal theta activity is absent is detrimental for learning, and that the behavioral state in which hippocampal theta activity dominates is beneficial for learning, at least before a well-learned state is achieved.

Keywords: hippocampus, EEG, classical conditioning

1. Introduction

Theta oscillations (3-8 Hz neural activity) originating in the medial septum-diagonal band of Broca, the entorhinal cortex and the hippocampus (for a review see Buzsáki, 2002) have been linked to a host of cognitive processes (for reviews see Buzsáki, 2005; Hasselmo, 2005; Lisman, 2005; Vertes, 2005). One of the most tangible pieces of evidence for the association of theta with learning was provided by Berry and Thompson (1978), who showed that the amount of spontaneous hippocampal theta activity before training predicts learning rate during subsequent delay eyeblink conditioning in rabbits (for reviews see Berry, 1982; Berry, Seager, Asaka, & Borgnis, 2000; Berry, Weisz, & Mamounas, 1987). This could suggest that ongoing oscillatory activity in the hippocampus reflects the animal's behavioral (Berry & Thompson, 1978) and motivational (Berry & Swain, 1989) state, determining, for example, the conditions for subsequent acquisition of a conditioned response. Furthermore, administration of conditioning trials in the presence of hippocampal theta activity facilitates the acquisition of the conditioned response (CR), especially in the early phases of learning (Asaka, Mauldin, Griffin, Seager, Shurell, & Berry, 2005; Griffin, Asaka, Darling, & Berry, 2004; Seager, Johnson, Chabot, Asaka, & Berry, 2002), as well as enhances the hippocampal model of the CR seen in multiple-unit activity (MUA) recordings (Berry & Swain, 1989; Griffin et al., 2004). In addition, disruption of the functioning of the hippocampus is more detrimental to eyeblink conditioning than lesioning it (Allen, Padilla, & Gluck, 2002; Asaka, Griffin, & Berry, 2002; Berry & Thompson, 1979; Salvatierra & Berry, 1989; Solomon, Solomon, Schaaf, & Perry, 1983). That is, abnormal functioning of the hippocampus, and parallel perturbation of the theta activity in the hippocampus, can hinder learning.

The memory trace of the CR acquired during eyeblink classical conditioning (Gormezano, Schneiderman, Deaux, & Fuentes, 1962) is thought to be located in the cerebellum and the associated brain stem circuitry, as has been indicated by various lesion and inactivation studies (Christian & Thompson, 2005; Krupa & Thompson, 1997; McCormick, Lavond, Clark, Kettner, Rising, & Thompson, 1981; for a review see Thompson, 2005). Although the hippocampus is not crucial in learning when a simple delay conditioning paradigm is applied (Schmaltz & Theios, 1972), it becomes increasingly important when the relations between the conditioned (CS) and unconditioned stimulus (US) are made more complex (Berger & Orr, 1983; Moyer, Deyo, & Disterhoft, 1990; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). The hippocampus is thought to participate in the regulation of the adaptive amplitude-time course of the behavioral CR (Berger, Alger, & Thompson, 1976; for review see Berger, Berry, & Thompson, 1986) in trace and discrimination-reversal conditioning, and to contribute to the consolidation of the memory trace in the early phases of learning (Kim, Clark, & Thompson., 1995; Takehara, Kawahara, & Kirino, 2003; Takehara, Kawahara, Takatsuki, & Kirino, 2002). However, hippocampal MUA elicited by the conditioning stimuli increases rapidly early during conditioning, and temporally precedes and models the behavioral CR even when a delay paradigm is used (Berger et al., 1976; 1986). In sum, the hippocampus seems to play a modulatory but not a critical role in the acquisition of a conditioned response in delay eyeblink conditioning.

As already mentioned, a close connection between theta activity and learning rate in classical eyeblink conditioning has been established (Berry & Thompson, 1978; for reviews see Berry, 1982; Berry et al., 2000; Berry et al., 1987; Berry & Seager, 2001). Less well known is how hippocampal theta activity itself is altered as a function of both unpaired training and the

conditioning process (Berry, 1982; Berry et al., 2000; Berry et al., 1987). We aimed to investigate how the relative power of spontaneous hippocampal theta activity changes during unpaired training followed by conditioning, an interesting aspect not approached in previous studies. In addition, we aimed to verify the result of Berry and Thompson (1978), who first showed a relation between the pre-training level of spontaneous hippocampal theta activity and learning rate during delay eyeblink conditioning in rabbits. Also, we sought to determine how the relative power of hippocampal theta activity during the pre-CS period affects the properties of the following behavioral CR and the hippocampal neural model of the CR (Berger et al., 1976; Griffin et al., 2004). For the purposes of this study, a hippocampal electroencephalogram (EEG) from a 1-s pre-stimulus period was recorded during each trial, and the power of theta (3-8 Hz) activity in relation to the combined power of delta (below 3 Hz) and theta activity – hereafter termed the hippocampal theta ratio – was calculated. The relation of the hippocampal theta ratio to CR acquisition rate, CR performance and the hippocampal correlate of the behavioral CR shown as increases in MUA (Berger et al., 1976) were analyzed. It was expected that a high hippocampal theta ratio would be associated with faster learning (Berry & Thompson, 1978), more adaptive CRs, and a stronger hippocampal model of the behavioral CR (Griffin et al., 2004). It was also hypothesized that the hippocampal theta ratio would not change during unpaired training, and that conditioning would reduce possible differences in the hippocampal theta ratio between slow and fast learners (Berry, 1982; Berry et al., 1987).

2. Methods and Materials

2.1. Subjects

The subjects were 10 adult female New Zealand albino rabbits aged ~4 months and weighing ~3.7 kg at the time of surgery. The rabbits were housed in individual metal 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 6.00 am. All procedures were conducted during the light portion of the cycle. All the experimental procedures were implemented in accordance with the European Communities Council Directive (86/609/EEC, http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31986L0609:EN:HTML) on the care and use of animals for research purposes.

2.2. Surgery

The rabbits were anesthetized with an i.m. injection of ketamine-xylazine cocktail [Ketaminol vet, (Intervet International B.V., Boxmeer, Netherland), 50 mg/ml, 5.6 ml; Narcoxyl vet, (Intervet International B.V.), 20 mg/ml, 2.2 ml; physiological saline, 2.2 ml]. An injection of 1 ml/kg was given before surgery and then 1 ml every 15-20 minutes. Eyedrops (Oftan, Santen Oy, Tampere, Finland) were used to prevent the eyes from drying. At the beginning of the surgery, the rabbit was placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA) with the bregma 1.5 mm higher than the lambda. A longitudinal incision was made to the scalp and four stainless-steel anchoring screws (5 mm anterior and 5 mm lateral to the bregma; 13 mm posterior and 5 mm lateral to the bregma) were attached to the skull. The screws were connected together and they served as a reference measuring point for the electrophysiological recordings.

Four monopolar recording electrodes made of Teflon-insulated stainless steel wire (bare diameter 125 μm, tip length ~200 μm) mounted inside a 27-gauge hypodermic stainless steel tubing were chronically implanted into the right hippocampus (for details, see Korhonen, 1991) 5 mm posterior and 4-7 mm lateral to the bregma. During implantation, EEG and MUA were monitored to define the preferred depth of the electrode (bregma-12 mm + 2.5-5.5 mm). Finally, the electrodes were attached to two pin connectors and the whole construction cemented in place with dental acrylic.

In the final stage of surgery, a nylon loop was sutured into, but not through, the nictitating membrane (NM) of the rabbit's right eye to be used in measuring its movements during training. Oftan Obucain (Santen Oy) eyedrops were used for local anesthesia if needed. Local anesthesia was also used if the NM loop needed to be replaced during the experiment. A subcutaneous injection of analgesic solution (1 ml; 0.1 ml of Temgesic 0.3 mg/ml [Schering-Plough Europe, Brussels, Belgium], diluted in 1 ml of 0.9% NaCl) was given at the end of surgery and, if needed, 8 hours later. At least one week was allowed for post-surgical recovery.

2.3. Conditioning procedure

Prior to the experiments, the rabbits were placed (for approximately 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 functioning of the implanted electrodes. Thereafter, experimental sessions were conducted one per day on consecutive days.

The CS was a 1 kHz, 85-dB, 350-ms tone and the US was a 100-ms corneal airpuff (6.5 psi source pressure, sound pressure level 64 dB) delivered through a nozzle (inner diameter 2 mm)

placed approximately 1 cm away from the eye. A fan located inside the conditioning chamber behind the rabbit created a steady background noise of approximately 65 dB. E-Prime software (Psychology Software Tools Inc., Pittsburgh, PA, USA) was used to control the presentation of stimuli.

To obtain control measures, 5 explicitly unpaired treatment sessions were conducted before conditioning. Unpaired sessions consisted of 70 CS-alone and 70 US-alone trials presented in a random order with an intertrial interval (ITI) averaging out at 20 s (range 15-25 s). Conditioning sessions consisted of 80 trials: 60 conditioning, 10 CS-alone, and 10 US-alone trials were presented in a pseudorandom order with an average ITI of 40 s (range 30-50 s). During the conditioning trials, CS onset preceded US onset by 250 ms, and the two stimuli coterminated. A robust CR (see below) in 8 out of 9 consecutive conditioning or CS-alone trials had to be present to meet the conditioning criterion. All rabbits were conditioned to the criterion (minimum 5 sessions) plus one session.

2.4. Recordings and data-analysis

In order to measure NM movement, the nylon loop attached to the rabbit's NM during surgery was linked to the swivel arm of a minitorque potentiometer by means of a rigid stainless steel hook. The movements of the NM were converted to voltage by the potentiometer where 1 mm equaled 1 V.

To acquire neural measures, low-noise pre-amplifiers were 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 amplifiers (Axon Cyberamp 380, Molecular Devices Corporation, Union City, CA, USA). The data were recorded with AxoScope (Molecular Devices

Corporation) software and digitized (Digidata 1322A, Molecular Devices Corporation) using a 10.42 kHz sampling rate. Before digitization the EEG was band-pass-filtered between 1–200 Hz, and the MUA was filtered between 500-4000 Hz.

Clampfit (Molecular Devices Corporation), MATLAB (The MathWorks Inc., Natick, MA, USA) and SPSS (SPSS Inc., Chicago, IL, USA) were used for the data analysis. Only CS-alone and paired trials were included in the analyses. Trials with NM movement exceeding 0.5 mm in amplitude during the 100 ms period immediately preceding CS onset were rejected. All NM movements exceeding 0.5 mm in amplitude during the 250-ms period immediately following CS onset were counted as conditioned responses. The learning criterion was considered to be met when the subject performed a CR on 8 out of 9 consecutive paired or CS-alone trials. Learning rate was defined as the number of conditioning trials needed to reach learning criterion (trials-to-criterion, TTC). On the basis of our experience of learning rates (in our laboratory, a long term median learning rate has been about 210 TTC) the animals were divided into two groups: slow ($TTC \geq 210$) and fast learners ($TTC < 210$). The threshold was set to 210 trials, so as to reliably separate fast and slow learners before examining the mean TTC in the experimental population. Repeated measures analysis of variance (ANOVA) was used in analyzing changes in CR percentage across training, and independent samples t-test in analyzing differences between slow and fast learners.

To determine the relative power of hippocampal theta activity during the 1-s prestimulus period, Fast Fourier Transform was run on the prestimulus period EEG using a Hamming window. Next the power of the 3.2–8.3 Hz activity (theta) was divided by that of the 1-8.3 Hz (delta + theta) activity, yielding a measure of the relative power of hippocampal theta activity, the hippocampal theta ratio. Delta + theta frequencies were used as the sole reference for theta, first,

because in absolute power delta and theta frequencies are fairly comparable and, second, because the absolute power of the higher frequencies (8 Hz <) is considerably smaller than that of theta. The hippocampal theta ratio from CS-alone and paired trials (a total of max. 70 trials per session) was then averaged by subjects and sessions and plotted with TTC, and Pearson correlation coefficients calculated. Changes in hippocampal theta ratio across training were examined with repeated measures ANOVA, and independent samples t-tests were used in analyzing differences between fast and slow learners.

To assess the impact of hippocampal theta activity on CR performance and related hippocampal MUA, two sessions from each animal were selected for analysis: one with a CR percentage of approximately 50, representing the intermediate learning state (ILS), and the one with the CR percentage closest to 100, representing the well-learned state (WLS). The selection of these particular sessions is explained by the fairly high number of trials showing a CR needed to make comparisons between averages recorded during theta and non-theta trials. A comparison of averages would not have been possible, although interesting, in the truly early learning state since the data would only have included around 5-10 trials with a CR. A trial was classified as a non-theta trial if the hippocampal theta ratio was less than 40%. If the theta ratio was 80% or more, the trial was classified as a theta trial. The criterion values of 40% and 80% were based on the total average across training of the hippocampal theta ratio (~60%) +/- one standard deviation (~20 percentage units). An equal number of theta and non-theta trials showing a CR were selected for analysis. If there were no theta or no non-theta trials showing a CR, the subject was excluded from further analysis. From the MUA recordings the spike frequency exceeding a preset amplitude threshold was counted off-line per 10 ms bin. The threshold was set at the level of about 20 spikes/s when no stimulation was given. For statistical testing, MUA from the 10 bins

immediately preceding the US-onset (CR-period) was standardized by subtracting the mean of the 10 bins immediately preceding CS-onset (preCS-period) and then dividing by the standard deviation of the mean of the preCS-period activity. In addition, CR peak amplitude and latency, and standardized CR-period MUA were averaged across subject and trial type (theta vs. non-theta) and compared with paired samples t-test.

2.5. Histology

After the experiments the 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 by putting physiological saline followed by 10% formalin through the ascending aorta. The locations of the electrode tips were marked by passing a DC current (300 μA, 20 s) through it. The brain was then removed and stored in 10% formalin solution for approximately two weeks. The brains were then frozen and coronally sectioned with a microtome into 100-μm-thick slices. Between sections, the frozen brain was photographed with a digital camera. The slices were attached to gelatinized slides and later stained with Prussian blue and cresyl violet. The electrode-tip locations were determined from the digital photographs and stained slides with the help of a microscope and stereotaxic atlases (Bures, Petran, & Zachar, 1967; Lavond & Steinmetz, 2003).

3. Results

3.1. Histological results

All the rabbits had at least one correctly placed recording electrode in the hippocampus. For the analyses, one electrode per animal from hippocampal region CA1 was selected on the basis of

the location and signal properties (see Fig. 1). Theta oscillation is most powerful near the hippocampal fissure, in the stratum lacunosum-moleculare layer of CA1 (Buzsáki, 2002).

3.2. Behavioral results

No change in conditioned responding was seen during unpaired training (Fig. 2A). All rabbits acquired a robust CR as a consequence of conditioning [F (5, 45) = 36.20, $p < .001$]. An average of 187 conditioning trials was needed to reach the criterion (TTC, min = 86, max = 311, SD = 84.21). A predetermined threshold of 210 TTC was used to divide the animals into slow and fast learners. TTC mean was 270.50 and standard deviation 38.60 in the slow learner group and 131.50 and 50.94 in the fast learner group (see Fig. 2A). In the well-learned state, the CR percentages were virtually equal in fast and slow learners (M = 86.9, SD = 9.5 vs. M = 88.8, SD = 4.7, respectively) implying that the groups eventually learned to the same extent but at different rates.

3.3. Correlations between the hippocampal theta ratio and learning rate

To test the effect of unpaired treatment on the hippocampal theta ratio in fast and slow learners, a repeated measures ANOVA (5 unpaired sessions as levels) was conducted for fast and slow learner groups separately: As a consequence of unpaired training the hippocampal theta ratio was reduced in slow learners [F (4, 12) = 4.78, $p < .05$], whereas in fast learners no change occurred. Thus, at the end of unpaired training, the hippocampal theta ratio was higher in the fast than slow learners [t (8) = 2.84, $p < .05$] (Figs. 2B and 3A). Also a repeated measures ANOVA using unpaired sessions (5) as levels and group (fast vs. slow learners) as a fixed factor was conducted to further examine the differential change in the theta ratio in fast and slow learners across unpaired training. The results revealed a nearly significant main effect of session on the

hippocampal theta ratio [F (4, 32) = 2.43 p < .1; Linear F (1, 8) = 8.065, p < .05], but no interaction between session and group (Fig. 2B). The hippocampal theta ratio recorded during the last unpaired session varied from 51% to 71% (M = 63%, SD = 5 percentage units), and was negatively correlated with TTC [r = -.681, p < .05], indicating a faster learning rate during subsequent conditioning in the case of a higher hippocampal theta ratio at the end of unpaired training (Fig. 3A).

At the end of conditioning (well-learned) the hippocampal theta ratio varied between 46% and 71 % (M = 62%, SD = 8 percentage units), and correlated with learning rate [r = -.676, p < .05] (see Figs. 2B and 3B). Conditioning resulted in no significant change in the hippocampal theta ratio either in slow or in fast learners (Fig. 2B). However, fast learners showed a higher hippocampal theta ratio compared to slow learners even after the CR had been acquired, [t (8) = 4.50, p < .01] (Fig. 3B). A further correlation between the theta ratio before conditioning (5^{th} unpaired session) and in the well-learned state [N = 10, r = .777, p < .01] showed that the theta ratio had relatively high consistency over the conditioning training, i.e. the change in the theta ratio during conditioning was minimal. Even further, we calculated a partial correlation between the theta ratio in the well-learned state and learning rate, controlling for the effect of the theta ratio recorded before conditioning (5^{th} unpaired session) [df = 7, r = -.317, p > .4]. Most of the correlation between the theta ratio in the well-learned state and learning rate is explained by the theta ratio recorded before conditioning. However, it must be noted that the correlation coefficient is negative (-.317), suggesting a parallel connection between the change in the theta ratio during conditioning and learning rate, as in the previous analyses, i.e. a reduced theta ratio is connected to slower learning and vice versa.

The overall change in the hippocampal theta ratio induced by training was derived by subtracting the theta ratio obtained during the first unpaired session from the theta ratio obtained in the well-learned state (session with the highest CR percentage). This change in the hippocampal theta ratio varied between -12 and +8 percentage units (M = -3 percentage units, SD = 7 percentage units). In slow learners the hippocampal theta ratio dropped as a consequence of training (M = -9 percentage units, SD = 5 percentage units), whereas in fast learners it remained roughly the same (M = +1 percentage units, SD = 3 percentage units; see Figs. 2B and 3C). The difference between fast and slow learners was significant [t (8) = 3.60, p < .01] (Fig. 3C).

3.4. Effects of the pre-CS period theta activity on CR properties and hippocampal MUA

One animal was excluded from analyses due to an insufficient number of theta trials. CR peak latencies were shorter during hippocampal theta trials (M = 228.6 ms, SD = 14.5 ms) than non-theta trials (M = 238.4 ms, SD = 9.0 ms) during intermediate learning state, [t (8) = 3.09, p < .05] (see Figs. 4A, B). Also, the standardized hippocampal CR-period MUA was greater during theta trials (M = 0.79, SD = 0.48) than non-theta trials (M = 0.36, SD = 0.56) in the intermediate learning state [t (8) = 2.55, p < .05] (Fig. 4C, D). No such differences were found when the hippocampal non-theta and theta trials in the well-learned state were compared. No differences in CR amplitudes were found between the hippocampal non-theta and theta trials in either phase of learning.

4. Discussion

Previously, a connection has been shown to exist between the oscillatory state of the hippocampus and learning rate during delay eyeblink conditioning in rabbits (Berry &

Thompson, 1978; for review see Berry & Seager, 2001). Our aim was to verify the result of Berry and Thompson (1978) and to investigate how possible changes in the hippocampal theta ratio [theta / (delta + theta)] develop during both unpaired training and subsequent delay eyeblink conditioning. We also aimed to examine how the hippocampal theta ratio recorded during the pre-CS period affects the following behavioral CR and the hippocampal neural model of the CR. Our results showed, that the hippocampal theta activity is related to learning rate even when unpaired training precedes delay eyeblink conditioning, that the hippocampal theta ratio decreases in the slow learners to-be during unpaired training, and that during ongoing learning, a high hippocampal theta ratio preceding the CS onset magnifies the hippocampal neural model of the CR and results in shorter CR peak latency.

As expected, our results showed that the hippocampal theta ratio recorded before conditioning was associated with learning rate. This is in agreement with previous studies (Berry & Thompson, 1978) indicating an association between the behavioral/motivational state of the animal, as reflected in the hippocampal theta ratio, and learning rate in delay eyeblink conditioning. In addition, according to our results, this association seems to hold even when an unpaired control treatment precedes conditioning. Although all the animals in our experiment eventually acquired the CR, a lower hippocampal theta ratio was associated with slower learning. A possible explanation of this would be that hippocampal theta activity is especially important for learning the CS-US contingency which, according to Prokasy (1984; 1987), occurs during the earliest phase of conditioning, before the emergence of behavioral CRs. This is borne out by the fact that whereas slow learners took longer than fast learners to start showing CRs, the following CR acquisition and shaping (Prokasy, 1984; 1987) proceeded at a rate compatible with that of the fast learners.

Contrary to our hypothesis and previous results (Berry, 1982; Berry et al., 1987), the hippocampal theta ratio decreased in slow learners already as a consequence of unpaired training, while remaining stable in fast learners throughout both experimental phases, thus resulting in differentiation of the hippocampal EEG frequency distributions between fast and slow learners across training. According to Berry (1982), as a consequence of conditioning the hippocampal EEG frequency distributions in fast, medium, and slow learners became more similar. The differences between the results of the present experiment and those of Berry (1982) might partially be explained by differences in the duration (2 min continuous vs. 1 s per trial) and timing (before and after training vs. within training) of the EEG samples used to determine hippocampal theta. Alternatively, the decrease in hippocampal theta ratio in the slow learners to-be induced by the unpaired training might reflect latent inhibition (for reviews see Lubow, 1973, 1989; Solomon & Moore, 1975) or learned irrelevance (Allen, Chelius, Masand, Gluck, & Myers, 2002). Previously, in a latent inhibition experiment by Borgnis (Berry et al., 2000; Berry & Seager, 2001), it was shown that slow-wave activity (below theta) in the hippocampus increased as a consequence of unpaired presentations of the CS and the US, resulting in less theta activity. It has been suggested that explicitly unpaired presentations of the CS and the US result in learning that the CS predicts no-event (switching theory, Weiner & Feldon, 1997). It might be that the slow learners to-be in our study were especially prone to such an association which in turn retarded acquisition of the CS-US association in the conditioning phase of the experiment.

Our results indicated that a high hippocampal theta ratio during the pre-CS period magnified the neural model of the CR shown in the hippocampal MUA recordings, and facilitated the performance of the behavioral CR during learning, but not after the probability of a CR reached asymptote. Our result showing the amplification of CR-period MUA during theta

trials is in accordance with a previous study by Griffin et al. (2004) concerning the effects of theta-contingent training on hippocampal MUA. Griffin et al. (2004) showed, using a trace paradigm, that compared to non-theta-contingent, theta-contingent training resulted in greater increases in hippocampal MUA within the first three days of conditioning. In addition to the increases in hippocampal MUA, our results showed that a high hippocampal theta ratio during the pre-CS period facilitated the adaptive performance (i.e., peak timing) of the behavioral CR during conditioning. This is in accordance with the notion that the hippocampus is involved in the regulation of the adaptive amplitude-time course of the CR (Berger et al., 1976; 1986). Interestingly, the hippocampal theta ratio was associated with the amplitude of the hippocampal neural model of the CR and CR timing only during ongoing learning, and not after an asymptotic level of conditioned responding had been reached, implying a time-limited role for the hippocampus. This is compatible with previous studies indicating that the hippocampus plays a more significant role during the early phases of learning, when the consolidation of the memory trace is still in progress (Kim et al., 1995; Takehara et al., 2003; Takehara et al., 2002). As our data consisted of short, 1-s sweeps (duration the theta sequence usually exceeds), we could not control for the effect of the duration of the hippocampal theta sequence preceding trial presentation on subsequent CR performance and on the hippocampal model of the CR. This would have been interesting, for it could be that the animal is required to be in the behavioral/motivational state indexed by hippocampal theta activity for a certain period for it to have an effect on behavior.

The absence of normal theta activity in the hippocampus has been shown to be detrimental for both trace (jaw movement: Asaka et al., 2002) and delay (Allen et al., 2002; Berry & Thompson, 1979; Salvatierra & Berry, 1989) conditioning. Likewise, non-theta-contingent

training retards learning in both trace (Griffin et al., 2004) and delay (Seager et al., 2002) eyeblink conditioning. However, theta-contingent training enhances learning only early in trace conditioning (Griffin et al., 2004), and has merely a marginal enhancement effect during delay conditioning (Seager et al., 2002). Here, we showed that the hippocampal theta ratio decreases as a function of explicitly unpaired treatment in the slow learners to-be, i.e., the low relative power of hippocampal theta before conditioning seemed to hinder learning. This could indicate that rather than being a critical precursor of learning in the delay variant of classical eyeblink conditioning, hippocampal theta might be an index of the overall oscillatory state of the brain reflecting the behavioral/motivational state of the animal. Theta activity has traditionally been linked to a multitude of cognitive processes that require and represent an aroused brain state (for reviews see Buzsáki, 2005; Hasselmo, 2005; Lisman, 2005; Vertes, 2005). Slow (3-8 Hz) theta oscillation has been specifically related to alert immobility as opposed to the faster (8-12 Hz) theta usually associated with exploratory behavior (Berry & Seager, 2001; O'Keefe & Nadel, 1978). It is possible that the dominance of theta activity in the brain, indexed by high relative power of theta in the hippocampus, indicates a behavioral and/or motivational state facilitating the animal's active and efficient observation of environmental stimuli and their associations, i.e., the detection of the CS-US contingency early in conditioning (Prokasy, 1984; 1987). This would explain the detrimental effects of disrupting the generation of normal theta activity in the brain on classical eyeblink conditioning (Allen et al., 2002; Asaka et al., 2002; Berry & Thompson, 1979; Salvatierra & Berry, 1989; Solomon et al., 1983). Theta activity might have an even more critical role in trace conditioning compared to delay conditioning since it has been shown in humans that the two learning tasks are differentially dependent on stimulus-contingency awareness (Clark & Squire, 1998). Furthermore, various studies have shown that hippocampal lesions disrupt trace

eyeblink conditioning (Kim et al., 1995; Moyer et al., 1990; Solomon et al., 1986) without having much effect on delay conditioning (Schmaltz & Theios, 1972; Solomon & Moore, 1975; Solomon et al., 1986).

It remains to be resolved whether the detrimental effect of the absence of theta activity on eyeblink conditioning results from deficits of either learning (difficulty in forming an association between the CS and the US), memory (difficulty in retrieving the established association during the CS), or performance (difficulty in acting upon the established association in an adaptive manner, i.e., in performing an optimal CR). Irrespective of which of the preceding options is most valid, it seems that a behavioral/motivational state in which theta activity is absent is detrimental to learning, and that a behavioral/motivational state in which hippocampal theta activity dominates is beneficial for learning, at least early in training.

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Figure legends

Figure 1. The location of recording electrodes in the hippocampus. Four Teflon-insulated stainless steel monopolar recording electrodes were chronically implanted into the right hippocampus 5 mm posterior and 4-7 mm lateral to the bregma. One electrode per animal from the hippocampal region CA1 was selected for the analyses on the basis of location (shown in the figure) and signal properties.

Figure 2. Percentage of conditioned responses (CRs, *A*) and the hippocampal theta ratio (*B*) in fast and slow learners across unpaired training and delay eyeblink conditioning. *A*: No change in CR percentage occurred during unpaired treatment (left). All animals acquired a robust CR by the end of conditioning [F (5, 45) = 36.20, $p < .001$] (right). Based on a predetermined threshold of 210 trials to criterion, the animals were divided into fast (n = 6) and slow (n = 4) learners. No differences in CR percentage were present between slow and fast learners after conditioning (well-learned). *B*: The hippocampal theta ratio, derived from the EEG recorded during the 1-s pre-CS period of each trial [theta / (delta + theta)], was reduced in the slow learners to-be during unpaired training [F (4, 12) = 4.61, $p < .05$], and showed significant differences between fast and slow learners at the end of unpaired training [t (8) = 2.84, $p < .05$] and after conditioning [t (8) = 4.50, $p < .01$] (see also Fig. 3). Vertical lines depict standard error of mean.

Figure 3. The hippocampal theta ratio recorded before (*A*) and after (*B*) delay eyeblink conditioning correlated with learning rate, as did also the overall change in the theta ratio

across training (*C*). *A*: The more hippocampal theta activity there was present immediately before conditioning, the faster a rabbit reached the learning criterion [N = 10, r = -.681, $p < .05$]. At the end of unpaired training, slow learners to-be showed significantly less hippocampal theta than fast learners to-be [t (8) = 2.84, $p < .05$]. *B*: The faster a rabbit learned, the higher the hippocampal theta ratio was after conditioning [N = 10, r = -.676, $p < .05$; slow vs. fast learners t (8) = 4.50, $p < .01$]. *C*: In fast-learning rabbits the hippocampal theta ratio remained roughly the same throughout training, whereas in slow learners it was reduced [t (8) = 3.60, $p < .01$].

Figure 4. A high (> 80%) hippocampal theta ratio immediately preceding CS onset resulted in shorter CR peak latency (*A* and *B*) and increased multiple-unit activity (MUA) during the CR-period (*C* and *D*) when learning was incomplete (intermediate learning state, ILS), but not when the rabbits showed asymptotic learning (well-learned state, WLS). Two sessions from each animal were selected for analysis: One with a CR percentage of ~50% representing an intermediate learning state (ILS), and the one with the CR percentage closest to 100 representing a well-learned state (WLS). Next, trials showing a CR were selected. Based on the hippocampal theta ratio (0-100%) during a 1-s period immediately preceding CS onset, the trials were further divided into theta (> 80%) and non-theta (< 40%) trials. One animal was excluded from the analysis due to an insufficient number of theta trials showing a CR. *A*: CR peak latency was shorter during theta trials in the ILS [t (8) = 3.09, $p < .05$], but not in the WLS. *B*: Grand averages (n = 9) of NM movement during theta and non-theta trials showing a CR in the ILS. *C*: CR-related standardized hippocampal MUA was greater during theta trials in the ILS [t (8) = 2.55, $p < .05$], but not in the WLS. *D*: Grand averages (n = 9) of hippocampal MUA during theta and non-theta trials showing a CR in the ILS. Gray horizontal bars indicate the CR-

period, from which the standardized MUA score in subplot C was obtained. Vertical lines in the bar charts indicate standard error of mean.