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Running head: HIPPOCAMPAL THETA ACTIVITY AND TRACE CONDITIONING

Hippocampal theta-band activity and trace eyeblink conditioning in rabbits

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Abstract

We aimed to examine the relationship between hippocampal theta activity and trace eyeblink conditioning. Hippocampal electrophysiological local field potentials were recorded before, during, and after conditioning or explicitly unpaired training sessions in adult male New Zealand White rabbits. As expected, a high relative power of theta activity (theta ratio) in the hippocampus predicted faster acquisition of the conditioned response during trace conditioning, but, contrary to previous results obtained using the delay paradigm, only in the initial stage of learning. The presentation of the conditioned stimulus overall elicited an increase in the hippocampal theta ratio. The theta ratio decreased in the unpaired group as a function of training, remained high throughout conditioning in the fast learners and rapidly increased in the slow learners initially showing a low theta ratio. Our results indicate a reciprocal connection between the hippocampal oscillatory activity and associative learning. The hippocampal theta ratio seems to reflect changes and differences in the subjects' alertness and responsiveness to external stimuli, which affect the rate of learning, and are in turn affected by both conditioning and unpaired training.

Keywords: Hippocampus, theta, classical conditioning, rabbit, local field potential

Introduction

The memory trace of the conditioned response (CR) acquired during classical eyeblink conditioning (Gormezano, Schneiderman, Deaux, & Fuentes, 1962) is thought to reside in the cerebellum (in humans see, Cheng Disterhoft, Power, Ellis, & Desmond, 2008; Gerwig, Kolb, & Timmann, 2007; in animals, Christian & Thompson, 2005; McCormick, Lavond, Clark, Kettner, Rising, & Thompson, 1981; for a review see Thompson, 2005). The hippocampus is not required for learning when the most simple (delay) form of eyeblink conditioning is applied (Schmaltz & Theios, 1972), but becomes increasingly important if the relations between the conditioning stimuli are more complex, like in trace conditioning (Berger & Orr, 1983; Moyer, Deyo, Disterhoft, 1990; Solomon, Vander Schaaf, Thompson, & Weisz, 1986) or when an unusually long inter-stimulus interval is used in delay conditioning (Beylin, Gandhi, Wood, Talk, Matzel, & Shors, 2001). During trace conditioning, the hippocampus is thought to be involved in the regulation of the adaptive amplitude-time course of the behavioral CR (Berger, Alger, Thompson, 1976; for a review see Berger, Berry, & Thompson, 1986), and to participate in 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).

Theta (3-8 Hz) oscillations controlled by neuronal networks in the medial septum-diagonal band of Broca, the entorhinal cortex and the hippocampus (for a review see Buzsáki, 2002) dominate the hippocampal local field potentials (LFPs) in the awake animal. Compelling evidence for the association of hippocampal theta oscillation with learning was provided by Berry and Thompson (1978), who first showed that the amount of spontaneous hippocampal theta activity before training predicts the learning rate during subsequent delay eyeblink conditioning in rabbits (see also Nokia, Penttonen, Korhonen, & Wikgren, 2008; for

reviews see Berry & Seager, 2001; Berry, Seager, Asaka, & Borgnis, 2000). Training with trials contingent upon high levels of hippocampal theta facilitates learning, especially early in conditioning (Asaka, Mauldin, Griffin, Seager, Shurell, & Berry, 2005; Berry & Swain, 1989; Griffin, Asaka, Darling, & Berry, 2004; Seager, Johnson, Chabot, Asaka, & Berry, 2002). Moreover, disrupting the functioning of the hippocampus and the associated theta oscillations is more detrimental to eyeblink conditioning than lesioning the hippocampus (Allen, Padilla, & Gluck, 2002; Asaka, Griffin, & Berry, 2002; Berry & Thompson, 1979; Salvatierra & Berry, 1989; Solomon, Solomon, Schaaf, & Perry, 1983). It seems that ongoing oscillatory activity in the hippocampus reflects the animal's behavioral and motivational state, determining, for example, the optimal or detrimental conditions for the subsequent acquisition of a conditioned response.

In this experiment we studied the relation between hippocampal theta activity and trace eyeblink conditioning in rabbits. As opposed to Griffin et al. (2004) who manipulated the theta condition of the training paradigm, in our current study, we explored a linear correlation between two continuous data sets (hippocampal theta ratio and learning rate), and not the effect of a manipulation of the training paradigm. In addition to the effects of hippocampal theta activity on learning rate, we also explored the impact of both trace eyeblink conditioning and unpaired training on the magnitude of the hippocampal theta activity at large, a question not approached before. Hippocampal LFPs were recorded before, during, and after each training session, and the relative power of theta activity (theta ratio) was derived. Recordings from multiple time periods were analyzed because the hippocampal theta activity is generally thought to reflect the state of awareness or openness to external stimulation, and this behavioral/motivational state is possibly altered by the presentation of the conditioning stimuli. The learning rate was quantified both in early (trials to 5th CR, Thompson, Berry, Rinaldi, & Berger, 1979) and late (trials to asymptote) phases of learning

as defined by Prokasy (1984). We anticipated that the hippocampal theta ratio recorded before any training is connected to learning rate especially in the early phase of trace eyeblink conditioning (effect of theta-contingent conditioning: Griffin et al., 2004). We also expected to observe differing changes in the hippocampal theta ratio as a function of both training and learning.

Method

Subjects

The subjects were 31 adult male New Zealand White rabbits (Harlan Netherlands, B.V., Horst, Netherlands) aged ~4 months and weighing ~2.7 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.

Surgery

Subcutaneous injections of an analgesic solution (2 ml; 0.1 ml of buprenorfine 0.3 mg/ml [Temgesic, Schering-Plough Europe, Brussels, Belgium], diluted in 1 ml of 0.9% NaCl) and of an anti-inflammatory drug (0.1 ml/kg; carprofen 50 mg/ml [Rimadyl vet, Pfizer Inc. Animal Health, Espoo, Finland]) was given 30 min preceding the onset of the 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, 7.8 ml;

Narcoxyl vet, [Intervet International B.V.], 20 mg/ml, 2.8 ml). An injection of 0.8 ml/kg of the cocktail was given before surgery and additional 0.8 ml-doses of either the cocktail or ketamine approximately every 20 minutes s.c.. 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 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; 13/10 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.

Two or three monopolar recording electrodes made of Teflon-insulated stainless steel wire (bare diameter 125 μm , tip length $\sim 200 \mu\text{m}$) 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 4-6 mm lateral to the bregma. During implantation, LFPs were monitored to define the preferred depth of the electrode (bregma - 6.5-7.2 mm). Finally, the electrodes were attached to a pin connector and the whole construction cemented in place with dental acrylic.

Analgesic (buprenofine, see above for details) was administered every 8 hours for the next 24-48 hours depending on the recovery rate of the animal. A 0.1 ml/kg s.c. injection of metoclopramide (5 mg/ml; Primperan [Sanofi Winthrop Industrie, Quétigny, France]) was given 8 hours after the surgery to facilitate normal feeding and drinking. At least one week was allowed for post-surgical recovery.

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 the functioning of the implanted electrodes. Thereafter, experimental sessions were conducted once per day on consecutive days.

The conditioned stimulus (CS) was a 2 kHz, 85-dB, 200-ms tone and the unconditioned stimulus (US) was a 100-ms corneal airpuff (0.35 bar 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.

The rabbits were randomly assigned to control ($n = 12$) and experimental ($n = 19$) groups. Both received 10 daily sessions of either trace eyeblink conditioning (experimental) or explicitly unpaired training (control). The 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). The 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 700 ms thus creating a 500-ms trace period.

Recordings and data-analysis

Eyeblinks were measured 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 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, Union City, CA, USA). Both the neural data and the bipolar electromyogram (EMG) were recorded with AxoScope (Molecular Devices Corporation) software and digitized (Digidata 1322A, Molecular Devices Corporation) using a 10.26 kHz sampling rate. Before digitization, the LFPs were band-pass-filtered between .1–4000 Hz, and the EMG was filtered between 30-300 Hz.

Clampfit (Molecular Devices Corporation), MATLAB (The MathWorks Inc., Natick, MA, USA) and SPSS (SPSS Inc., Chicago, IL, USA) were used for the data analyses. CS-alone and paired trials were included in the analyses. The EMG signal was further high-pass filtered over 100 Hz and Hilbert-transformed. Following this, an envelope curve following the peaks of the signal was calculated using the real and imaginary parts of the Hilbert transformation. The baseline EMG activity was defined for each animal and session as the mean of the maximum EMG amplitude during a 500-ms pre-CS period (MAXpre). 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 $[\text{MAXpre} + 4 * \text{SDpre}]$. The trials showing eyeblinks during the 100 ms period immediately preceding CS onset were rejected. Eyeblinks during the 500-ms trace period were counted as conditioned responses. For rabbits in the experimental group, the learning criterion of asymptotic performance was considered to be met when the subject performed a CR on 8 out of 9 consecutive paired or CS-alone trials. The learning rate was defined firstly as the number of conditioning trials needed to perform the 5th CR, and secondly, as the number of conditioning trials needed to reach asymptotic performance. On the basis of the number of trials needed to reach the 5th CR ($M \pm \text{SEM}$), the subjects were classified as fast or slow learners.

Theta ratio. To first determine the characteristics of spontaneous hippocampal theta activity, the 5-min continuous recording conducted prior to the first training session was examined: a sliding window of 500 ms in 50 ms steps was used, and the Fast Fourier Transform (FFT) was calculated with a resolution of 0.5 Hz. The theta ratio was calculated at each time window ($\text{theta} / [\text{delta} + \text{theta}]$). Delta and theta frequencies were used as the sole reference for theta, firstly, because in absolute power, delta and theta frequencies are fairly comparable and, secondly, because the absolute power of the higher frequencies ($8 \text{ Hz} <$) is considerably smaller than that of theta. A theta ratio of 80% was used as a cut-off point when determining the high-theta periods. The duration and dominant frequency within the theta band (3-8 Hz) were determined for each detected high-theta period.

The overall hippocampal theta ratio was determined for both pre- and post-session continuous recordings, as well as for the 1-s sweeps preceding and following the presentation of the CS alone (10 trials per session per animal). A Hamming window of 8192 samples in length with a 50% overlap was used in calculating the FFT. In the control group, the CS-alone trial selection was based on the running number of the CS presentations during conditioning sessions, i.e. the running number of the selected trials was the same in both the experimental and the control group. The hippocampal theta ratio was determined from a 1-s time period following the CS-onset by 300 ms, ensuring that the possible event-related potential was excluded from the period under analysis.

Statistical analyses. In addition to comparisons between the conditioning and the control group, differences between fast learners and non-learners were also studied. Repeated measures analysis of variance (ANOVA) with training blocks of two sessions as a within-subjects factor (5), and group (unpaired vs. conditioning or fast vs. slow learners) as a between-subject factor, was used in analyzing changes across the training. Whenever a significant interaction of block and group was evident, separate repeated measures ANOVAs

were conducted for each group using blocks (5) as a within-subjects factor. Greenhouse-Geisser correction was used for the correction of p -values when necessary. Independent samples t-test was used in comparing the groups block by block. In the experimental group, correlations (Pearson) were calculated between learning rate measures and the hippocampal theta ratio. In order to study the differences in theta ratios determined for pre- vs. post session and pre- vs. post-CS recordings, paired samples t-tests were conducted.

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 (200 μ A, 20 s) through it. The brain was then removed and stored in 10% formalin + 10% sucrose solution for approximately one week. The brain was then frozen and coronally sectioned with a microtome into 100- μ m-thick slices. The slices were attached to gelatinized slides and later stained with Prussian blue and cresyl violet. The electrode-tip locations were determined from the stained slides with the help of a microscope and stereotaxic atlases (Bures, Petran, & Zachar, 1967; Lavond & Steinmetz, 2003).

Results

Histology

Theta oscillation is most powerful near the hippocampal fissure, in the stratum lacunosum-moleculare layer of the CA1, and weakest in the pyramidal layer of the CA1 (Bragin, Jandó, Nádasdy, Hetke, Wise, & Buzsáki, 1995; Buzsáki, 2002). From the stratum lacunosum-moleculare layer of the CA1 to the hilus of the dentate gyrus, the hippocampal

theta oscillation has roughly the same order of magnitude. Thus, one electrode per animal close to the hippocampal fissure in the CA1 ($n = 4$) or the molecular layer, granule layer ($n = 18$) and hilus ($n = 9$) of the dentate gyrus was selected on the basis of the location and signal quality (see Fig. 1).

Behavioral results

The group averages of conditioned responding across training are shown in Fig. 2. Responding to the tone-CS increased as a function of training (block: $F [4, 116] = 22.38, p < .001$; group: $F [1, 29] = 8.55, p < .01$; block x group: $F [4, 116] = 3.02, p < .05$), in both the conditioning ($F [4, 72] = 22.98, p < .001$) and in the unpaired control group ($F [4, 44] = 5.60, p < .05$) (see Figure 2A). Some subjects in the unpaired control group showed sensitization and increased behavioral responding to the tone towards the end of the training, but block-by-block comparisons confirmed that subjects in the conditioning group showed more CRs from the second training block onwards ($t (29) = 2.40 - 3.53, p < .05/.01$). In the conditioning group, the mean number of trials needed to reach the 5th CR was 35, with a standard error of mean (SEM) of 6 (min = 7, max = 114). The average number of trials needed to reach the asymptotic learning criterion was 243 with a SEM of 34 (min = 72, max = 550). Three out of the 19 subjects in the conditioning group did not reach the learning criterion within the 10 training sessions. On the basis of the number of trials needed to reach the 5th CR, 9 subjects were classified as fast learners and 4 as slow learners (Figure 2B). Fast learners showed more CRs during the first training block ($t (11) = 2.46, p < .05$), but later in learning the CR acquisition rates were equal (block: $F [4, 44] = 16.19, p < .001$; group: *ns.*; block x group: *ns.*) (see Figure 2B).

The hippocampal theta ratio was connected to learning rate early in trace eyeblink conditioning

The theta ratio recorded before the first training session (baseline) varied between 30 and 82% (mean 54%, SEM 3 percentage units). There were no differences in spontaneous theta ratios recorded from near the hippocampal fissure in the CA1 (n = 3, mean 58%, SEM 5 percentage units) or the molecular or granule layer (n = 10, mean 58%, SEM 4 percentage units) or hilus (n = 6, mean 45%, SEM 6 percentage units), One-Way ANOVA, $F [2, 16] = 2.24, p > .05$. The LFPs showed a theta ratio higher than 80% on average half of the time (mean 49%, SEM 3 percentage units) during the baseline-recording. During periods of high theta ratio, the dominant theta frequency was, on average, 5.70 Hz (SEM 0.05 Hz). The individual periods of high theta ratio had a mean duration of 1.12 s (SEM 0.07 s). Approximately 30.80 (SEM 1.36) separate high-theta periods occurred per minute, with an inter-event interval of approx. 2.08 s (SEM 0.07 s).

The results of correlation analyses are summarized in Table 1. Overall, the hippocampal theta ratio recorded immediately before and after the conditioning session correlated with the rate at which the animals reached the 5th conditioned response: the higher the hippocampal theta ratio, the faster the animal learned. More precisely, significant correlations were found between the pre-session theta ratio in all other sessions except sessions 4 and 6, and trials to 5th CR: $r = -.46 - -.54, p < .05$. Similarly, statistically significant correlations were found between the post-session theta ratio in sessions 1 through 4 and session 6, and trials to 5th CR: $r = -.49 - -.60, p < .05/.01$.

The overall theta ratio recorded immediately preceding and following the presentation of the tone-CS alone also correlated with the rate at which the rabbits reached the 5th CR (see Table 1). Again, the higher the relative power of hippocampal theta activity immediately

preceding and following the tone-CS, the faster the animal learned. Statistically significant correlations were found between pre-CS theta ratio in sessions 1 through 3 and 6 through 8, and trials to 5th CR: $r = -.46 - -.62, p < .05/.01$. Accordingly, statistically significant correlations were also found between the post-CS theta ratio in sessions 1 and 2 and trials to 5th CR: $r = -.68/-.46, p < .01/.05$, respectively.

In addition to the correlations between the absolute theta ratios and the early learning rate, the change (%) in the post-CS theta ratio between the first and last sessions correlated with trials to 5th CR ($r = .60, p < .01$), indicating that an increase in the theta ratio was connected to slower learning early in training. The change in the post-CS theta ratios between the first and last sessions varied between -41 % and +106 % ($M = +5 \%$, SEM 8 percentage units).

No significant correlation was found between the number of trials needed to reach asymptotic CR performance and the hippocampal theta ratio recorded either before or after the session or the CS. Therefore, we also calculated the correlation between the number of trials needed to reach the 5th CR and the number of trials needed to reach asymptotic performance, and found that there was no robust connection: $r = -.27, ns.$. In our previous study, in which the delay-paradigm was used (Nokia et al. 2008), the number of trials needed to reach the 5th CR was not included in the analyses. However, for comparison, we now calculated the correlation between the number of trials needed to reach the 5th CR and the number of trials needed to reach asymptotic performance also during delay eyeblink conditioning using the same data (Nokia et al., 2008). The result showed a significant correlation between learning rate early (trials to 5th CR) and late (trials to asymptote) in conditioning when using the delay-paradigm: $r = .86, p < .01, n = 10$.

The hippocampal theta ratio decreased as a function of unpaired training, remained high in the fast learners and increased in the slow learners

Spontaneous hippocampal theta ratios recorded preceding the training session (Figure 3A) and preceding the tone-CS (Figure 3E) showed no statistically significant changes across conditioning or unpaired training, or differences between the unpaired control group and the conditioning group. Both the theta ratio recorded immediately following the training session (see Figure 3C) as well as following the presentation of the tone-CS alone (see Figure 3G) showed an interaction between training block (5) and group (unpaired vs. conditioning): $F [4, 116] = 3.44, p < .05$, and $F [4, 116] = 3.63, p < .05$, respectively. Separate analyses for the unpaired and conditioning groups revealed that the hippocampal theta ratio remained at a high level throughout conditioning (post-session: $F [4, 72] = 1.79, p > .05$, and post-CS: $F [4, 72] = 1.11, p > .05$) but decreased as a function of unpaired training (post-session: $F [4, 44] = 2.68, p < .05$, and post-CS: $F [4, 44] = 7.50, p < .001$). Block-by-block analyses showed that the hippocampal theta ratio recorded following the training session was higher in the unpaired group than in the conditioning group within the first training block ($t (29) = 2.11, p < .05$), after which the situation was reversed (see Figure 3C). The hippocampal theta ratio recorded in response to the tone-CS alone showed a similar change pattern across training, and was higher in the conditioning group when compared to the unpaired control group during the second and third training blocks ($t (29) = 2.93, p < .01$, and $t (29) = 2.62, p < .05$, respectively) (see Figure 3G).

When fast and slow learners were compared, a consistent group effect was found regarding both the spontaneous theta ratios recorded preceding each training session ($F [1, 11] = 12.34, p < .01$; blocks 1 through 5, $t (11) = 2.67 - 3.95, p < .05/.01$, see Figure 3B) and the spontaneous theta ratios recorded during the session, preceding the presentation of the

tone-CS alone ($F [1, 11] = 19.82, p = .001$, blocks 2 through 5, $t (11) = 2.45 - 4.04, p < .05/.01$, see Figure 3F). The spontaneous hippocampal theta ratio was higher in the fast learners than in the slow learners throughout the training.

The hippocampal theta ratios recorded after the training session in the fast learners remained at much the same level throughout the conditioning and increased as a function of training in the slow learners (block: $F [4, 44] = 5.14, p < .01$; group: $F [1, 11] = 11.59, p < .01$; block x group: $F [4, 44] = 5.60, p < .01$) (see Figure 3D). The results were confirmed by separate analyses for fast and slow learners, $F [4, 32] = 0.65, p > .05$, and $F [4, 12] = 4.25, p < .05$, respectively. Block by block comparisons showed a statistically significant difference between fast and slow learners during the first 3 blocks of training: $t (11) = 2.86 - 6.01, p < .05/.001$. Additionally, the hippocampal theta ratios recorded immediately after the presentation of the tone-CS alone remained stable across the conditioning in the fast learners and increased rapidly early in the slow learners (block: $F [4, 44] = 5.19, p < .05$; group: $F [1, 11] = 14.32, p < .01$; block x group: $F [4, 44] = 8.82, p < .01$) (see Figure 3H). The results were again confirmed by separate analyses for fast and slow learners, $F [4, 32] = 2.46, p > .05$, and $F [4, 12] = 3.51, p < .05$, respectively. Block-by-block comparisons showed a statistically significant difference between fast and slow learners during blocks 3 and 5: $t (11) = 2.98/3.12, p < .05$, respectively.

Training and the presentation of the conditioned stimulus increased the hippocampal theta ratio

Block-by-block (5) paired-samples t-tests confirmed that the hippocampal theta ratio was consistently higher immediately after the presentation of the tone-CS (Fig. 3G) than during the pre-CS baseline period (Fig. 3E) both in the conditioning group as well as in the unpaired control group ($t (18) = 6.82-10.90, p < .001$, and $t (11) = 4.77-7.96, p < .001$,

respectively). The occurrence of unpaired training (pre- vs. post-session) increased the hippocampal theta ratio only in the beginning of training (blocks 1 and 2, $t(11) = 4.97/2.83$, $p < .001/.05$), while the occurrence of conditioning increased the hippocampal theta ratio during training blocks 2 through 4 ($t(18) = 2.40-4.10$, $p < .05/.001$) (see Figs. 3A and 3C).

Comparisons conducted separately for fast and slow learners (see Figs. 3F and 3H) revealed that in the fast learners the presentation of the CS evoked a robust increase in the hippocampal theta ratio throughout training ($t(8) = 4.85-8.91$, $p < .001$). In the slow learners, the CS began to evoke higher hippocampal theta ratios from the 2nd training block onwards ($t(3) = 3.52-6.27$, $p < .05/.01$). In the fast learners the occurrence of the conditioning session (pre- vs. post-session, Figs. 3B and 3D) increased the hippocampal theta ratio only in the beginning of training (blocks 1 through 3, $t(8) = 2.51-3.05$, $p < .05$). On the contrary, in the slow learners the occurrence of the conditioning session lead to higher hippocampal theta ratio only during the last block of training, $t(3) = 4.72$, $p < .05$.

Discussion

The relative power of hippocampal theta activity has been shown to predict learning rate during subsequent delay eyeblink conditioning (Berry & Thompson, 1978; Nokia et al., 2008), and hippocampal theta -contingent trial presentation has been shown to affect learning rate during trace eyeblink conditioning (Griffin et al., 2004). The present study aimed to extend the findings of Griffin et al. (2004) by using the spontaneous level of hippocampal theta activity to predict learning rate during standard trace eyeblink conditioning, and to further explore the relation of the hippocampal theta ratio and the learning process. The study produced three main findings. Firstly, the baseline hippocampal theta ratio predicted the number of trials needed to accomplish the initial stage of learning (5th CR), but had no predictive power regarding the rate at which asymptotic performance (a CR in 8 out of 9

consecutive trials) was acquired. Secondly, the hippocampal theta ratio measured both immediately after the CS and during a stimulus-free period after the training session decreased as a function of unpaired training, while remaining high in the fast learners and increasing in the slow learners. Thirdly, the presentation of the tone-CS and the occurrence of training as a whole both increased the hippocampal theta ratio.

Hippocampal theta activity is crucial only in the initial part of conditioning, when the CS-US association is being acquired

Our first main result showed that the behavioral/motivational state of the animal reflected by the hippocampal theta ratio predicted the number of trials needed to reach the 5th CR, but not the number of trials needed to reach asymptotic performance. This is in line with previous work on theta-contingent trace eyeblink conditioning from Griffin et al. (2004) who showed that the presence of hippocampal theta activity is especially important in the early phase of learning. As opposed to the Griffin et al. (2004) study, in the current experiment we showed a linear correlation between two continuous data sets (hippocampal theta ratio and trials to 5th CR), and not just a difference depending upon manipulation of the theta state during training. The present results thus provide important support for the theta-contingent conditioning studies, and show that the connection between the hippocampal theta activity and learning rate during trace eyeblink conditioning is strong enough to be seen also without special training arrangements.

The results of the present study showing the predictive connection between the hippocampal theta activity and learning rate together with the results of Griffin et al. (2004) have interesting theoretical implications. Generally, trace eyeblink conditioning is thought to require hippocampus-dependent declarative memory of the CS-US association for successful learning: Studies in humans have shown that awareness of the predictive association between

the CS and the US is a prerequisite for learning during differential trace eyeblink conditioning (Clark & Squire, 1998), and facilitates learning during single-cue trace eyeblink conditioning (Manns, Clark, & Squire, 2000) – the paradigm also used in our present experiment and in the study of Griffin et al. (2004). According to Prokasy (1984), the CS-US association is acquired early in conditioning (Phase 1, contingency detection and response selection), before the acquisition of CRs accelerates (Phase 2, response acquisition). Consistently with Prokasy's theory, and with our current results, lesioning the hippocampus impairs conditioned responding after trace eyeblink conditioning only if it is carried out early in the process of memory consolidation (rabbits: Kim et al., 1995; mice: Takehara et al., 2003; rats: Takehara et al., 2002). Furthermore, as stated above, the effects of theta-contingent eyeblink conditioning, during which trials are systematically administered when the hippocampal theta ratio is either high (T+) or low (T-), are strongest in the early phases of learning (Asaka et al., 2005; Griffin et al., 2004; Seager et al., 2002).

Since we observed no connection between the hippocampal theta ratio and the learning rate later in trace eyeblink conditioning (i.e. trials to asymptotic performance), we can assume that other factors contribute to the rate at which the asymptotic level of performance is reached. In addition, since the subjects showing significant differences in the rate at which they reached the 5th CR – fast versus slow learners – showed equal learning rates later in conditioning, it seems that the two learning phases – acquisition of the CS-US association and acquisition of the CR – are independent from each other. This conclusion is also supported by the Griffin et al. (2004) study, in which learning was retarded in the T- -group only in the initial phase of conditioning. In sum, together with earlier findings, our results based on spontaneous brain activity recordings and the use of a standard trace paradigm, support Prokasy's (1984) theory of two distinct phases in learning: the first phase, including the formation of the CS-US association and the selection of the correct response, seems to be

dependent on the hippocampal oscillatory activity, while the second phase of response acquisition is mediated by other brain structures, perhaps the medial prefrontal cortex (Takehara et al., 2003) or the cerebellum (for a review see Thompson, 2005).

The results discussed above, showing a connection between the hippocampal theta ratio and learning rate early in trace eyeblink conditioning, also offer a new perspective on earlier findings showing that the hippocampal theta ratio predicts the rate of learning to asymptote during delay eyeblink conditioning (Berry & Thompson, 1978; Nokia et al., 2008). The difference in results can be explained by the degree of complexity of the CS-US association, which is simpler during delay than trace eyeblink conditioning. As already mentioned, at least in humans, trace conditioning, but not delay conditioning, requires conscious knowledge of the CS-US association (Clark & Squire, 1998). Likewise, trace conditioning is disrupted by hippocampal lesions (Moyer et al., 1990; Solomon et al., 1986), whereas standard delay conditioning is not (Schmaltz & Theios, 1972). Overall, the CS-US association and asymptotic responding is acquired faster and more straightforwardly during delay than trace conditioning. During trace conditioning, learning to asymptote does not progress in the same linear fashion as during delay conditioning, which implies a more complex learning process and increases the variation in learning rates later in conditioning. In fact, as our additional analysis of the previously published data (Nokia et al., 2008) indicates, the number of trials needed to reach the 5th CR correlates strongly with the number of trials to asymptotic learning criterion in delay eyeblink conditioning. However, this correlation does not apply to trace eyeblink conditioning as shown by our current results. It could also be the case that the two phases of learning, contingency detection and response acquisition (Prokasy, 1984), are temporally overlapping processes during delay conditioning, yet sequential processes during trace conditioning.

There is one difference in methods between earlier studies (for example Berry & Thompson, 1978; Griffin et al., 2004; Nokia et al. 2008) and the present experiment that merits further discussion. Whereas in previous experiments recordings have been made from the hippocampal subregion CA1, near the pyramidal cell layer, in the current experiment we recorded hippocampal activity near the hippocampal fissure and in the dentate gyrus. Recordings from the pyramidal layer are supported by the fact that most of the conditioning-related hippocampal plasticity occurs in the pyramidal cells of the CA1 (Berger & Thompson, 1978). However, our decision to record from near the hippocampal fissure and the dentate gyrus is based on the fact, that, within the hippocampus, that is where the theta oscillation is biggest in amplitude (Bragin et al., 1995; Buszáki, 2002). Our choice evidently led to the limitation of not being able to monitor the hippocampal pyramidal cell plasticity, which would have been interesting, too. There is of course also the possibility, although unlikely, that the relative power of the theta activity in the CA1 has a correlation with learning rate different from the correlation obtained in our current experiment using recordings from the fissure/dentate gyrus. This issue merits further attention, and can be addressed in future studies by recording simultaneously from both the CA1 pyramidal layer as well as from the lower subregions of the hippocampus.

An interesting point for future work will also be to study the effects of hippocampal theta activity on learning rate during delay eyeblink conditioning when an unusually long inter-stimulus interval is applied. It has been shown that hippocampal lesions impair CR acquisition during delay conditioning if the ISI is 1400 ms (Beylin et al., 2001), which implies that the hippocampal theta oscillation might have a crucial part also in learning during this more difficult delay conditioning paradigm.

Hippocampal theta activity reflects both individual differences as well as changes related to the attentional demands of the situation in the behavioral/motivational state of the subjects

The second main result of our study indicated that the hippocampal theta ratio decreases as a function of explicitly unpaired training, remains high in the fast learners and increases in the slow learners as a function of conditioning. In addition, our results showed that the occurrence of the training session, and especially the presentation of the tone-CS increases the hippocampal theta ratio. Thus, not only does the behavioral/motivational state reflected by the theta ratio predict the learning rate in the early phase of trace eyeblink conditioning, but also, trace eyeblink conditioning as well as unpaired training both evoke changes in the behavioral/motivational state of the subjects as reflected by the changes in the relative power of hippocampal theta activity.

Our results showed that the CS evokes a robust increase in the hippocampal theta ratio in both the conditioning and the control groups throughout training, which implies to a general phenomenon possibly related to an automatic shift of attention towards any sufficiently strong external stimulus. The rapid decrease in the hippocampal theta ratio recorded after the session and after the tone-CS early in training as a function of unpaired training could be explained by a decrease in the attention directed towards the conditioning stimuli, as the subjects found that the stimuli had no predictive value in relation to each other, and that they could not avoid the aversive effects of the US. Similarly, compared to a baseline situation, the occurrence of the unpaired training session induces a shift to a more alert state only in the beginning of training, which is again compatible with the view of less attention directed towards the stimuli as training continues.

The fact that in the fast learners, the hippocampal theta ratio remains equally high throughout conditioning, whereas in the slow learners the hippocampal theta ratio recorded after the CS and after the training session is initially low but increases as conditioning proceeds could also be related to the attentional demands set by the conditioning situation. The fact that in the slow learners the CS only starts to evoke the immediate increase in the hippocampal theta ratio from the second training block onwards together with the finding that they also show lower spontaneous hippocampal theta ratios overall (see above), implies that these subjects are in an attenuated state of attention, at least in the beginning of training. The conditioning session was shown to evoke an increase in the hippocampal theta ratio in the fast learners in the beginning and middle phases of learning, whereas in the slow learners no increase was visible until the last training block. It could be that the fast learners shift to a more alert behavioral/motivational state during ongoing learning, but as they reach a phase of stable conditioned responding, the performance of the CR becomes automatic, and the conditioning situation no longer requires their full attention. All in all, the fast learners remained at a state of high hippocampal theta activity throughout training. On the other hand, slow learners seemed less affected by the conditioning session as a whole, which is probably only another example of their lower state of alertness overall.

Previously, a connection has been shown to exist between the *change* in the relative power of hippocampal theta activity across training and the learning rate early in delay eyeblink conditioning (Thompson et al., 1979): The faster the animals learned, the greater the increase in the electroencephalogram frequency (i.e. away from a synchronized theta-state). Conversely, the slower the animals learned, the greater the shift towards a synchronized theta-state. Thompson et al. (1979) interpreted their results to support the general notion of the inverted-U relation between arousal and performance. Successful learning during trace conditioning most likely requires an optimal (i.e. not too low but not too high either) state of

arousal throughout training, which would explain why, in our current study, subjects showing a high hippocampal theta ratio to begin with (faster learners) remained in that state throughout conditioning and the ones showing a low theta ratio in the beginning (slow learners) shifted to a more synchronized theta-state as conditioning proceeded.

Our results showing a high hippocampal theta ratio in the fast learners across training and an increase in the slow learners early in training could also be related to the increase in hippocampal multiple-unit activity during theta-contingent trace eyeblink conditioning reported earlier by Griffin et al. (2004). Griffin et al. (2004) showed that trace eyeblink conditioning accelerated the hippocampal firing rate recorded after the presentation of the tone-CS early in training if the training trials were presented in the presence of hippocampal theta activity. However, if the training trials were presented in the absence of hippocampal theta activity, hippocampal firing after the presentation of the tone-CS was inhibited. Based on both the results of Griffin et al. (2004) and our current findings, it seems plausible that a high relative power of hippocampal theta activity is needed in order to support hippocampal plasticity indicated by an increase in firing rates of hippocampal neurons during the training trial.

The lack of a significant increase across training in the spontaneous hippocampal theta ratios (recorded preceding the training session, and within each training session, preceding the presentation of the CS), and also the lack of differences between the conditioning and unpaired control group, suggests that trace eyeblink conditioning using a tone-CS and an airpuff-US evokes only transient changes towards a more aroused behavioral/motivational state, and also that the mere exposure to the conditioning environment is not enough to change the animal's behavioral/motivational state towards a more attentive state. The robust differences between the fast and slow learners in pre-training and pre-CS spontaneous

hippocampal theta ratios probably reflect relatively persistent differences in their ways of reacting to novel situations.

Conclusions

Our results are in line with the results of previous studies regarding the role of the hippocampus in learning during a complex form of eyeblink conditioning, and verify the predictive connection between the behavioral/motivational state reflected by the hippocampal oscillatory theta activity and learning rate in the initial phase of trace eyeblink conditioning, when the contingency between the conditioned and the unconditioned stimulus is detected. In addition, our results show a mutual connection between training and learning, and the behavioral/motivational state reflected by the hippocampal oscillatory theta activity: The changes in the magnitude of the hippocampal theta activity seem to reflect the changes in the state of the animal induced by the attentional demands of the environment.

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Table 1

The relative power of hippocampal theta activity was connected to learning rate early in conditioning

Hippocampal theta ratio	trials to 5 th conditioned response (n = 19)
baseline (pre- 1 st session)	$r = -.49^*$
pre-session	$r = -.53^*$
post-session	$r = -.49^*$
pre-CS	$r = -.61^{**}$
post-CS	$r = -.51^*$
post-CS change	$r = .60^{**}$

Note. Correlations were calculated using the Pearson correlation coefficient. The spontaneous hippocampal theta ratio recorded preceding the 1st conditioning session (baseline) predicted learning rate early in conditioning. Overall, the higher the hippocampal theta ratio throughout training (pre-session, post-session, pre-CS and post-CS: average over all training sessions), the faster the animal learned early in conditioning. In addition, the difference in the post-CS hippocampal theta ratio between the first and last conditioning sessions (post-CS change) correlated with learning rate early in conditioning: Slower learning early in conditioning was connected to an increase in the theta ratio. No statistically significant correlations between the hippocampal theta ratio and the rate of acquisition late in learning (trials to asymptote) were present.

* $p < .05$, ** $p < .01$

Figure Captions

Figure 1. Recording electrode locations. All of the rabbits had at least one electrode in the hippocampal fissure or in the dentate gyrus/hilus. The open triangles refer to the subjects in the unpaired control group ($n = 12$) and the filled circles to the subjects in the conditioning group ($n = 19$).

Figure 2. Behavioral performance across training. All of the subjects were trained for 10 sessions conducted on consecutive days. **A)** The mean number of paired trials needed to reach the conditioning criterion was 243 equaling the 5th training session (3rd training block). However, three out of 19 rabbits in the conditioning group did not reach the learning criterion at all. Some rabbits in the unpaired control group also started to blink in response to the tone-CS, most probably as a consequence of sensitization. **B)** On the basis of the number of trials needed to reach the 5th CR, 4 subjects in the conditioning group were classified as slow learners and 9 as fast learners. The vertical lines depict the standard error of mean. Line no. 1 refers to the results of repeated measures ANOVA conducted for the whole data (blocks x group), and line no. 2 refers to ANOVAs conducted separately for the unpaired training and conditioning groups. The asterisks depict statistical significance: * $p < .05$, ** $p < .01$, *** $p < .001$. The asterisks between the lines refer to the statistically significant results of the independent samples t-tests between the two groups (unpaired training vs. conditioning, and fast vs. slow learners).

Figure 3. Changes in the relative power of hippocampal theta activity as a function of training. In repeated measures ANOVA blocks of two sessions (5) were used as within subject factors and group (2) as between-subjects factor. Interactions of block and group were further analyzed by conducting repeated measures ANOVA for both groups separately. Differences between groups within each training block were explored using independent

samples t-tests. **A)** and **E)** Spontaneous hippocampal theta ratios recorded preceding each training session and preceding the presentation of the CS did not differ between the unpaired and the conditioning group, and showed no changes across training. **B)** and **F)** Spontaneous hippocampal theta ratios recorded preceding each training session and before the presentation of the CS were higher in the fast learners than in the slow learners throughout training. **C)** The hippocampal theta ratio recorded immediately after the training session was decreased in the unpaired control group and increased in the conditioning group as a function of training. **D)** The hippocampal theta ratio recorded immediately after the training session remained stable in the fast learners and was increased in the slow learners as a function of training. **G)** The hippocampal theta ratio recorded immediately after the presentation of the tone-CS alone was decreased in the unpaired control group and increased in the conditioning group as a function of training. **H)** The hippocampal theta ratio recorded immediately after the presentation of the tone-CS alone remained stable in the fast learners and was increased in the slow learners as a function of training. All subplots: the vertical lines depict the standard error of mean. Line no. 1 refers to the results of repeated measures ANOVA conducted for the whole data (blocks x group), and line no. 2 refers to ANOVAs conducted separately for the unpaired training and conditioning groups, or the slow and fast learners. The asterisks depict statistical significance: * $p < .05$, ** $p < .01$, *** $p < .001$. The asterisks between the lines refer to the statistically significant results of independent samples t-test between the two groups (unpaired training vs. conditioning, and fast vs. slow learners).

- ▽ unpaired training (n = 12)
- conditioning (n = 19)





