Eyeblink conditioning contingent on hippocampal theta enhances hippocampal and medial prefrontal responses

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Submitted 20 September 2010; accepted in final form 23 February 2011

Darling RD, Takatsuki K, Griffin AL, Berry SD. Eyeblink conditioning contingent on hippocampal theta enhances hippocampal and medial prefrontal responses. J Neurophysiol 105: 2213–2224, 2011. First published February 23, 2011; doi:10.1152/jn.00801.2010.—Trace eyeblink classical conditioning (tEBCC) can be accelerated by making training trials contingent on the naturally generated hippocampal 3- to 7-Hz theta rhythm. However, it is not well-understood how the presence (or absence) of theta affects stimulus-driven changes within the hippocampus and how it correlates with patterns of neural activity in other essential trace conditioning structures, such as the medial prefrontal cortex (mPFC). In the present study, a brain-computer interface delivered paired or unpaired conditioning trials to rabbits during the explicit presence (T+/H11001) or absence (T−/H11002) of theta, yielding significantly faster behavioral learning in the T+/H11001-paired group. The stimulus-elicited hippocampal unit responses were larger and more rhythmic in the T+/H11001-paired group. This facilitation of unit responses was complemented by differences in the hippocampal local field potentials (LFP), with the T+/H11001-paired group demonstrating more coherent stimulus-evoked theta than T−/H11002-paired animals and both unpaired groups. mPFC unit responses in the rapid learning T+/H11001-paired group displayed a clear inhibitory/excitatory sequential pattern of response to the tone that was not seen in any other group. Furthermore, sustained mPFC unit excitation continued through the trace interval in T+/H11001 animals but not in T−/H11002 animals. Thus theta-contingent training is accompanied by 1) acceleration in behavioral learning, 2) enhancement of the hippocampal unit and LFP responses, and 3) enhancement of mPFC unit responses. Together, these data provide evidence that pretrial hippocampal state is related to enhanced neural activity in critical structures of the distributed network supporting the acquisition of tEBCC.

anterior cingulate cortex; hippocampus; medial prefrontal cortex; rabbit eyeblink classical conditioning; brain computer interface

Eyeblink classical conditioning (EBCC) in rodents, lagomorphs, and humans has provided crucial insights into the neurobiology of associative learning (Christian and Thompson 2003; Woodruff-Pak and Steinmetz 2000a,b). Major findings include an essential role of the cerebellum in all forms of EBCC including delay conditioning, in which there is temporal contiguity of the conditioned stimulus (CS; typically a tone) and unconditioned stimulus (US; shock or air puff to the eye) (McCormick and Thompson 1984). When the basic paradigm is modified so that there is a stimulus-free “trace” period between CS and US (tEBCC), the integrity of both the hippocampus and medial prefrontal cortex (mPFC) becomes necessary in addition to the cerebellum (Kalmback et al. 2009; Moyer et al. 1990; Solomon et al. 1986; Weible et al. 2000). How the interaction between these structures becomes crucial in learning the association between the trace conditioning stimuli and facilitates the underlying neural plasticity in all essential tEBCC areas (including the cerebellum) has not yet been adequately explained. As detailed below, there are known cellular correlates of tEBCC in the hippocampus, mPFC, cerebellum, and elsewhere, but there is relatively little information on how these responses are related, especially when learning is optimized or impaired.

Hippocampal CA1 pyramidal cells increase firing rates in response to the EBCC stimuli early in training, suggesting an involvement in detecting the CS-US contingency (Berger et al. 1983; McEchron and Disterhoft 1997). The output of CA1 does not project directly to the cerebellum, but indirect routes may include the mPFC that projects to lateral pontine nuclei (LPN). This link provides a possible means for mPFC influence on cerebellar plasticity during tEBCC since the LPN is in the putative CS pathway to the cerebellum (Arikuni and Ban 1978; Buchanan et al. 1994; Weible et al. 2007). The rabbit mPFC consists of subregions including the anterior cingulate cortex (ACC) and prelaminar and infralaminar cortices. Stimulation of the prelaminar field of mPFC elicits evoked potentials (EPs) in the cerebellar cortex, which demonstrates a functional connectivity between these two structures (Watson et al. 2009). Single-neuron recordings from caudal regions of ACC during tEBCC revealed learning-related activity (Weible et al. 2003). This activity included a period of inhibition followed by sustained excitation in response to the tone, which was interpreted as an enhancement of the signal-to-noise ratio, possibly leading to increased salience of the tone CS.

Investigations of the (3–7 Hz) theta rhythm in the hippocampal local field potential (LFP) of immobile rabbits have revealed that its presence (measured before a conditioning session) can predict faster learning (Berry and Thompson 1978; Nokia et al. 2008). Theta has also been shown to improve behavioral learning when a brain-computer interface is used to trigger each conditioning trial in its presence (T+ group), whereas triggering in its absence (T− group) impairs learning (Asaka et al. 2005; Griffin et al. 2004; Hoffmann and Berry 2009; Seager et al. 2002). During the tone and trace periods, the hippocampal unit profiles were shown to diverge after the first day, with excitation in the T+ group and suppression in the T− group (Griffin et al. 2004). Stimulus-evoked LFPs in the cerebellar cortex and interpositus nucleus demonstrated increased theta coherence and precise phase-locking to hippocampal LFPs in the T+ group, suggesting a strong relationship between hip-
pocampal state and cerebellar physiology during tEBCC (Hoffmann and Berry 2009). The impact of these disparate theta states on forebrain structures (such as mPFC) during tEBCC is unknown, although several studies have found that mPFC single units are phasically related to hippocampal theta and, in many situations, are more clearly related to hippocampal theta than to the LFP in the mPFC (Hartwich et al. 2009; Hyman et al. 2005; Paz et al. 2008; Siapas et al. 2005; Sirota et al. 2008; Young and McNaughton 2009). This raises the possibility that theta in the hippocampus may be a key to understanding the temporal dynamics of neural mechanisms underlying EBCC throughout the brain.

Our brain-computer interface permits a relatively natural ebb and flow of oscillatory patterns yet limits training trials to a very specific brain state based on the presence or absence of the hippocampal theta rhythm. This technique bypasses many of the technical limitations of lesion and artificial stimulation studies that can produce long-lasting, often nonphysiological modifications of a specific brain region or neurochemical system (e.g., Scarlett et al. 2003) with, typically, unreported effects on interconnected structures.

Here, we report that theta-contingent triggering of tEBCC: 1) replicates prior demonstrations of accelerated behavioral learning early in training; 2) enhances coherent stimulus-evoked rhythmicity of the hippocampal LFP; 3) extends the finding of hippocampal unit responses to include augmented rhythmicity during the trace interval; and 4) produces significant hippocampus state-related differences in conditioned unit responses within the mPFC. These findings provide important support in awake, behaving animals, for the hypothesis that natural physiological fluctuations in hippocampal theta state may serve to coordinate neurobiological responses that underlie plasticity in essential tEBCC structures such as the hippocampus and mPFC.

**MATERIALS AND METHODS**

**Subjects.** Subjects were 23 New Zealand White rabbits (*Oryctolagus cuniculus*) from Myrtle’s Rabbitry (Thompson Station, TN) that were randomly assigned to paired or unpaired groups and theta conditions and kept on a 12:12-h light-dark cycle with ad libitum access to food and water. Experiments were conducted during the light phase and performed at approximately the same time, 7 days a week. All procedures involving animals were approved by the Miami University Institutional Animal Care and Use Committee.

**Surgery.** Animals were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), secured in a custom stereotaxic apparatus (Josef Biela Engineering), and situated with bregma 1.5 mm dorsal to lambda. Stainless steel insect pins (size 00; Carolina Biological Supplies) were insulated by repeatedly dipping into Epoxylite and curing in an oven. Electrode tips were shaved of insulation using a scalpel blade under a stereoscope until an impedance of ~500 KΩ (BAK IMP-1) was achieved, typically leaving ~50 μm in tip exposure. Electrodes were lowered bilaterally with respect to bregma into the hippocampus [+4.5 anteroposterior (AP), ±5.5 mediolateral (ML)] and unilaterally into the mPFC corresponding to caudal portions of the ACC (±1.0 AP, ±0.75 ML). Dorsal/ventral locations were determined by monitoring electrophysiological characteristics (with respect to cortical ground screw) as well as stereotaxic coordinates but were generally 2.5–3.5 mm for CA1 (stratum oriens) and 2.0–3.0 mm for mPFC. Once electrodes were in place, they were cemented to skull anchor screws and soldered to a DB9 connector (Radio Shack) via an insulated wire (previously soldered to the upper portion of the electrode).

**Training.** Following the 5-day recovery period and before training, animals were given at least 2 sessions of adaptation. Animals were secured in a custom-built Plexiglas restrainer box and placed in an electrically shielded and sound attenuating chamber. Training procedures involved paired or unpaired trials in either the presence (T+) or absence (T−) of hippocampal theta. Specifically, the initiation of each theta-dependent trial was contingent on a custom LabVIEW program (version 5.01) that performed a real-time (640-ms sliding window) fast Fourier transform (FFT) from a band-pass (1–25 Hz) hippocampal electrode. The program calculated (every 160 ms) a ratio of the power spectrum coefficients with 3.5- to 8.5-Hz power in the numerator and 0.5- to 3.5- and 8.5- to 22-Hz power in the denominator. Trials were initiated when the ratio exceeded 1.0 (3 consecutive times) for T+ animals and below 0.3 (3 consecutive times) for T− animals so the total pretrial period for theta detection is 960 ms. The theta triggering paradigm has been previously described in detail (Asaka et al. 2005; Griffin et al. 2004; Hoffmann and Berry 2009; Seager et al. 2002). For paired trials, a 100-ms, 1.0-kHz, 80-dB tone was followed by a 100-ms, 3-psi corneal air puff to the left eye separated by a 500-ms trace interval. Sessions were ~90 min with a minimum...
intertrial interval of 60 s. Paired animals were trained at least until they performed 8 conditioned responses out of 9 consecutive trials (8/9 CRs), which is a common level of stable performance. Unpaired animals were presented with identical stimuli but were pseudorandomly presented (restricted to 8 tone-alone and 8 air puff-alone trials in each 16-trial block with no more than 2 like trials in succession) and were never paired. Unpaired animals were given at least 2 sessions with a minimum intertrial interval of 30 s to equalize the number of stimuli presented during a session to paired animals (see RESULTS for comparison).

The signals were monitored through a custom-built DB9 cable with head-mounted field effect transistor (FET) amplification and a custom-made four-channel bioamplifier with a gain of 5,200 and filtered between 0.1 and 8,000 Hz. Signals were recorded on a Vetter Model 970118 instrumentation recorder based on a Sony (SLV-640HF) video cassette recorder (VCR). Eyelid responses were transduced with a custom potentiometer and recorded in LabVIEW on a Power Macintosh (7100/80). A 0.5-mm movement was considered a response, with a CR required to occur between 50 ms after tone onset and before air puff onset. Behavioral analyses were performed on the first several days of acquisition, on the occurrence of the 5th CR (an indicator of contingency detection) and on the first occurrence of 8/9 CRs (stable performance).

**Histology.** Following training procedures, rabbits were given an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). A 200-μA direct current (DC) via Grass Stimulator (SD9) and Grass Constant Current Unit (CCU1A) was passed through each electrode for 10 s followed by an intravenous injection of pentobarbital (0.2205 mg/kg) to the marginal ear vein. Animals were perfused intracardially with a 0.9% saline solution followed by a 10% formalin solution. The brains were removed and immersed in formalin solution for at least 7 days followed by a 20% solution of ethanol for 36–48 h before being frozen.

Coronal sections were taken via a Minotome cryostat through each electrode location and embedded on gelatin-coated slides. Prussian blue with safranin counterstain was used to mark the displaced iron from the stainless steel electrode tip (Fig. 1). Animals whose histology showed incorrect hippocampal electrode placements were excluded from all analyses, whereas animals with incorrect mPFC electrode placement were only excluded from corresponding PFC analyses. Four animals had hippocampal placements in more ventral locations toward the hippocampal fissure (e.g., lacunosum moleculare). The hippocampal LFP recordings for these animals were inverted for LFP analyses (refer to Fig. 8 in Bragin et al. 1995 for explanation of reversal of LFP polarity across the pyramidal cell layer).

**Neural analysis.** All neural activity was filtered with a Krohn-Hite (3700) filter and input into a DataWave Technologies (Longmont, CO) 16-channel interface using SciWorks and Experimenter software for analysis. All data extraction was triggered by a recorded pulse synchronized to tone and air puff onset from LabVIEW.

LFPs were filtered 1–200 Hz and sampled at 500 Hz. An event detection algorithm extracted a 2-s waveform (500 ms pre-CS, 1,500 ms post-CS). Extracted waveforms from each trial were scaled (without changing time or frequency domain parameters) to avoid variation in signal size due to unrelated phenomenon such as differences in electrode tip impedance. The waveforms were then averaged across a session and then across animals within theta and treatment groups to create a daily waveform average for each group and structure. A custom Excel (Microsoft) macro computed an FFT on each scaled hippocampal and mPFC trial for the 500 ms before CS onset (baseline), following the CS EP (trace), and following the US EP (post-US) to reveal stimulus-evoked changes in rhythmic activity between 1 and 200 Hz in 1-Hz increments.

Multiple units were filtered 500–5,000 Hz and sampled at 20 kHz. An event detection algorithm was used to extract 1.05 s (150 ms pre-CS, 900 ms post-CS) of extracellular multiple-unit signals from noise (multiple-unit spikes were required to be at least 2.5 times the level of noise) by placing a window discriminator above the filtered signal. A peristimulus histogram (10-ms bins) was created for each trial and averaged over the session, creating an average daily histogram of 105 averaged bins. Standard scores were calculated for each session by subtracting the mean of the 1st 15 bins (pre-CS) from each bin of the average of the session and dividing by the pre-CS standard deviation. These standard scores were then summed across periods of interest and averaged across animals to create a daily period average for each group and structure. Summing was used to correspond to past literature in which total CS period activity or total US period activity was presented. Statistically, this is a simple transform of the mean, yielding identical inferential significance. Theta group × training day (2 × 2 × 2) ANOVAs and post hoc t-tests were computed using SPSS (version 11.5) and/or Microsoft Excel (2002).

Fig. 2. Behavioral analyses of paired animals demonstrated accelerated learning in theta-present (T⁺; n = 8) compared with theta-absent (T⁻) animals (n = 6). Cumulative conditioned responses (CRs) (A) and percentage CRs (B) for all animals over the 1st 4 days of conditioning are shown. Number of days (C) and trials (D) for animals to reach the early learning criterion of the 5th CR revealed that T⁺ animals detected the conditioned stimulus (CS)-unconditioned stimulus contingency earlier than T⁻ animals. Error bars represent SE.
Behavior. T+ animals reached the early learning criterion of the 5th CR in fewer trials [105.13 (SD 42.97)], t(12) = −3.12, P < 0.01 and fewer days [2.63 (SD 1.06)], t(9) = −2.77, P = 0.01 compared with T− animals [trial 173.17 (SD 38.38); day 4.50 (SD 1.38)], signifying an earlier detection of the CS-US contingency. Unlike previous theta-triggering studies, no significant differences in number of trials or days to the asymptotic learning criterion of 8 CRs out of 9 consecutive trials (8/9 CRs) were found between T+ [trial 270.25 (SD 112.27); day 6.5 (SD 2.56)] and T− [trial 284.83 (SD 63.30); day 7.0 (SD 1.79)] animals, trial t(11) = 0.31, P = 0.38; day t(12) = 0.43, P = 0.34. Ruling out potential learning rate differences due to differences in the number of trials or average intertrial intervals between theta groups, we observed that T+− [49.63 (SD 12.94)] and T−− [47.71 (SD 12.91)] paired animals received statistically the same number of trials per session as measured by averaging over the 1st 4 days of conditioning, t(50) = 0.549, P = 0.585. Figure 2 illustrates the different early learning trajectories in terms of cumulative [F(1,12) = 1.99, P = 0.18] and percentage [F(1,12) = 1.91, P = 0.19] CRs over the 1st 4 days of conditioning and the number of trials and days to the 5th CR.

Hippocampal LFPs. Time-locked EPs in the hippocampus were elicited by both stimuli in all four groups (T+−paired, T+−unpaired, and T−−unpaired), similar to what has been described previously in animals given paired conditioning (Hoffmann and Berry 2009). Figure 3 illustrates the differences between groups in the averaged hippocampal waveforms across trials. Note the enhanced amplitude and coherent rhythmicity in the T+− waveforms, especially in response to the US in the T+−group. These EPs preceded a reset of ongoing theta in both T+− groups, seen in the averaged waveforms for paired and unpaired animals, and elicited minimally coherent theta in the T−− groups. As evident in the averaged waveform, the coherent poststimulus theta rhythmicity was most robust in the T+−group. Further waveform analysis revealed larger increases in 6- to 8-Hz spectral frequencies after both stimuli in the T+− groups (7–8 Hz in T+−unpaired) compared with both T−− groups (Fig. 4). In general, the results of the spectral analyses demonstrated a suppressed or unchanging response to stimuli in the 3- to 5- and 9- to 12-Hz ranges for all groups, with the most robust 6- to 8-Hz theta increase in T+−paired animals. Within groups, the EPs and frequency spectra did not differ over conditioning days.

Hippocampal multiple units. The hippocampal multiple-unit responses were enhanced in the T+−group compared with all other groups. This finding paralleled previous observations of T+−group enhancement using the theta-triggered paradigm during tEBCC (Griffin et al. 2004), and unlike previous investigations, we report for the first time unit observations of T+−paired animals (Fig. 5). After dividing the 500-ms trace period into two 250-ms periods, a 2 × 2 × 2 (theta group × training group × day) ANOVA of the late-trace period (250 ms) revealed main effects of theta group, F(1,19) = 3.58, P = 0.07 and of conditioning group (paired vs. unpaired), F(1,19) = 10.1, P = 0.01. A subsequent post hoc test revealed that the T+−paired group had significantly greater late-trace excitation (P ≤ 0.03) than all other groups (Fig. 6A). This enhanced excitatory response in T+−paired animals was maintained (compared with T−−paired animals) through the early learning phase (5th CR) and stable CR performance (8/9 CRs; P ≤ 0.04; Fig. 6B). An autocorrelation of the T+−paired averaged-unit histogram on the day of 5th CR revealed ~6.25-Hz (peak at 160 ms) rhythm but showed no obvious periodicity in T−−paired animals (Fig. 6C). This larger
and more rhythmic hippocampal unit firing is complementary to the more coherent 6- to 8-Hz hippocampal LFPs during the trace period in T⁺-paired animals. Together, these findings support the interpretation that theta state is a significant facilitator of increased hippocampal plasticity early in tEBCC training.

mPFC LFPs. Similar to hippocampal LFPs, mPFC EPs were elicited by both stimuli in all four groups (Fig. 7). However, unlike hippocampal LFPs, theta activity in the mPFC was not generated in response to the presentation of the conditioning stimuli for any of the groups. In fact, the EPs created a much slower (2 Hz) response that was not dependent on theta group or training group. The EPs and subsequent LFPs did not change substantially over conditioning days.

mPFC multiple units. A noticeable sequential pattern of inhibition followed by excitation was seen in response to the tone in the T⁻-paired group only (Fig. 8). A 2 × 2 × 2 (theta group × training group × day) ANOVA was performed to test for a difference in this CS-elicited response, quantified by the difference in the 80-ms post-CS-onset period of excitation from the previous 80-ms period of inhibition. This test revealed a significant theta group × training group × day interaction $F(1,13) = 5.07, P = 0.04$ during the 2nd half (250 ms) of the trace interval, similar to what had been demonstrated with the hippocampal multiple units (Figs. 5, 6, and 9B). The T⁻ groups had excitatory responses during the late-trace interval, whereas the T⁺ groups actually showed suppression below their baseline, and their US-elicited responses appeared somewhat smaller. By day 2, T⁺-paired animals tended to display larger excitatory responses to the US than T⁻-paired animals (and both unpaired groups), which was maintained throughout conditioning ($P < 0.08$; Fig. 9C). Taken together, these findings show that trials given during hippocampal theta (T⁺) resulted in a qualitatively different mPFC firing response profile, including a sequential inhibitory/excitatory CS-elicited response, sustained firing leading up to the US (trace period), and a marginally larger US response.

**DISCUSSION**

Behavior. Theta triggering has been shown repeatedly to improve acquisition rates of EBCC (Asaka et al. 2005; Griffin et al. 2004; Hoffmann and Berry 2009; Seager et al. 2002). In the present study, the greater cumulative and percentage CRs in the T⁺ group during the first 4 days of conditioning, along with statistically fewer trials and days to the 5th CR, provided further evidence for the behavioral benefit of theta
triggering early in training. The 5th CR criterion has traditionally been used to mark the end of stage one of the two-phase model of learning during which the animal detects the CS-US contingency before organizing adaptive behavioral responding (Prokasy 1972). Therefore, the presence of theta appears to be positively correlated with the animal’s ability to detect the CS-US relationship, which is especially important when the stimulus parameters are temporally stretched, such as in the trace paradigm. To our knowledge, this is one of the few experimental techniques to use locally recorded and naturally fluctuating brain patterns to accelerate behavioral learning, thus motivating the exploration of its effects on memory systems throughout the brain.

**Hippocampus.** The current study was an important demonstration of the dissimilar effects of theta triggering on hippocampal LFPs in paired and unpaired animals. The conditioning stimuli generated EPs that reset ongoing theta in the T\(^+\) groups and elicited weakly coherent theta in the T\(^-\) groups. In most of the EBCC literature (in which hippocampal theta state is unknown and/or uncontrolled), it could be assumed that trials are presented in variable theta states that would sometimes produce a reset of ongoing theta while at other times require an abrupt initiation of theta. This heterogeneity in pretrial theta state could help explain the intermediate behavioral learning rates reported throughout the EBCC literature and, importantly, our previously reported time-yoked (theta variable) controls whose behavior statistically falls between T\(^+\)/H11001 and T\(^-\)/H11002 animals (Griffin et al. 2004; Seager et al. 2002).

The ability of various sensory stimuli to evoke a transition from nontheta to theta in animals has been documented (Green and Arduini 1954). However, here the coherence of poststimulus theta was more pronounced in T\(^+\) animals and most robust in the faster-learning T\(^+\)-paired group (Figs. 3 and 4). This suggests a more tightly coupled response from animals that were already generating theta before the stimulus occurred (T\(^+\)) and better yet whether the event acquires behavioral

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Fig. 5. Hippocampal multiple-unit histograms for paired animals (day of 5th CR) and unpaired animals (day 1). The T\(^+\)-paired group demonstrated greater excitation in response to the tone compared with all other groups. See Fig. 6 for quantitative analysis. Histograms are in 10-ms bins with 150-ms baseline and 900 ms thereafter. Arrows denote tone and air puff onset. T\(^+\)-paired \(n = 8\); T\(^-\)-paired \(n = 8\); T\(^-\)-unpaired \(n = 4\); T\(^-\)-unpaired \(n = 3\).
significance through associative learning (paired group). Such phase resets in oscillatory processes are important components in models of coordinated distributed systems (Buzsaki 2006; Hasselmo et al. 2002; Womelsdorf et al. 2007) and may be one mechanism for the enhanced unit responses and the corresponding faster learning in T+ animals (Griffin et al. 2004).

Theta reset following stimulus presentation has been demonstrated in a variety of situations and species, but the precise conditions that enhance poststimulus theta coherence and learning-related plasticity in the hippocampus and extrahippocampal structures needs to be explored further (Adey and Walter 1963; Buzsaki et al. 1979; Mormann et al. 2005; Tesche and Karhu 2000). Our finding of more coherent theta following the presentation of associative EBCC stimuli (compared with unpaired stimuli) in our T+ group is similar to the discovery of theta reset in rats performing a continuous conditional discrimination task but not during simple sensory discrimination (Griffin et al. 2004).

Theta reset following stimulus presentation has been demonstrated in a variety of situations and species, but the precise conditions that enhance poststimulus theta coherence and learning-related plasticity in the hippocampus and extrahippocampal structures needs to be explored further (Adey and Walter 1963; Buzsaki et al. 1979; Mormann et al. 2005; Tesche and Karhu 2000). Our finding of more coherent theta following the presentation of associative EBCC stimuli (compared with unpaired stimuli) in our T+ group is similar to the discovery of theta reset in rats performing a continuous conditional discrimination task but not during simple sensory discrimination (Givens 1996; Williams and Givens 2003). The important distinction between those two paradigms is the necessity of holding the characteristics of the previous stimulus in memory during the continuous conditional discrimination, implying that the reset may be involved in cognitive processes such as working memory. The stimulus-evoked reset of theta during their working memory task also was shown to provide optimal conditions for long-term potentiation measured in the awake and behaving animal, suggesting an enhancement of neural plasticity (McCartney et al. 2004). Even though our paradigm does not contain typical working memory components, it is possible that the noncontiguous arrangement of and/or temporally stretched stimuli during eEBCC may tap into a working memory component by requiring the animal to maintain a CS representation or inhibit a response through the stimulus-free trace period. Such similar theta reset phenomena in working memory tasks and eEBCC may facilitate investigations and theoretical interpretations of the underlying cognitive processes involved in both.

Because the T+ groups displayed more coherent theta following the tone (Figs. 3 and 4), our T+ -paired animals may have a more consistent US arrival time with respect to theta phase. Specifically, highly consistent US arrival on the depolarizing phase of theta waves could be a mechanism for early behavior modification and enhanced hippocampal plasticity in T+ -paired animals. This is compatible with the proposal that ideal theta phases (and synaptic locations) optimize retrieval vs. encoding of information within the hippocampal formation (Hasselmo et al. 2002; Hyman et al. 2003; Wyble et al. 2000). In addition, because both T- groups displayed less theta coherence and a suppression of unit activity following the tone, the absence of pretrial theta may be especially detrimental to the development of hippocampal plasticity and may be responsible for delayed behavioral acquisition. A more detailed understanding of the benefits of theta-contingent EBCC may be discovered by incorporating phase-locked triggering in which trials are initiated only on certain phases of hippocampal theta

Fig. 6. Hippocampal multiple-unit analysis. A: the late-trace period (250 ms) excitation is significantly greater in the T+ -paired group compared with T- -paired animals and both unpaired groups over the 1st 2 days of stimulus presentation. B: T+/T- -paired differences occurred at the earliest stages of learning (5th CR) and persisted through asymptotic responding (8/9 CR). Error bars represent SE. C: autocorrelation of unit histograms during the trace period on the day of 5th CR reveals periodicity ~6.25 Hz (160 ms) in T+ -paired animals but no obvious periodicity in T- -paired animals. Dashed line represents 1st peak at 160 ms. T+ -paired n = 8; T- -paired n = 8; T+ -unpaired n = 4; T- -unpaired n = 3. Avg, average; Std, standard.
mPFC neural activity between T
electrophysiological responses of extrahippocampal forebrain
US is effectively phase-locked to the elicited theta).
accommodate the CS-elicited frequency of theta (so that the
(CS phase-locked) or by varying the interstimulus interval to
accommodate the CS-elicited frequency of theta (so that the
US is effectively phase-locked to the elicited theta).

mPFC. This study is unique in its attempt to investigate the
electrophysiological responses of extrahippocampal forebrain
structures during theta-triggered tEBCC. Any differences seen
in mPFC neural activity between T+ and T− groups correlate
with hippocampal state and thus may reflect how the hip-

pocampus impacts mPFC function. Interestingly, the LFPs
from mPFC in both theta conditions revealed EPs to the
conditioning stimuli and a slow (<2 Hz) evoked (possibly oscillatory) response with no obvious differences between
groups (see Siroti and Buzsaki 2005 for significance of the
slow mPFC oscillation in information transfer). Therefore, the
differences in mPFC multiple-unit firing were not because of
differences in (or rhythmicity of) the LFP within the mPFC but
were more related to the rhythmic hippocampal LFP and,
importantly, the enhanced rhythmicity of multiple-unit firing
(statistically likely to be pyramidal output neurons) in the
hippocampus. This is in agreement with reports that mPFC
single-unit responses are more phasically related to the hip-

pocampal LFP (theta) than to the LFP in the surrounding
mPFC (Hartwich et al. 2009; Sirota et al. 2008).
A prior study of single units in mPFC (caudal ACC; essen-
tially identical to the present study with the exception of
theta-contingent trial presentation) revealed a tone-evoked se-
cquence of inhibition followed by excitation (Weible et al.
2003). This was interpreted as an enhancement of the signal-
to-noise ratio resulting in increased salience of the tone that
predicts air puff in the trained group. Our recordings replicated
this response sequence with multiple units but found it to be
significant only in T+ -paired animals (Figs. 8 and 9), suggest-
ing that the hippocampal theta state may provide a functionally
significant and temporally limited window in which individual
mPFC neurons can amplify the salience of sensory information,
possibly leading to faster learning. The smaller (or even
negative) means of the inhibition/excitation pattern for T−
unpaired and T+ -paired animals suggest a reduction of the
signal-to-noise ratio compared with T+ animals. This could
hinder the T−-paired group’s ability to develop a strong asso-
ciation between the CS and US and may delay (or even prevent)
optimally timed behavioral responses.

The prolonged excitation of the T+ group’s mPFC multiple-
unit activity into the late-trace period provides further support
for the association of faster-learning, pretrial hippocampal
theta and sustained trace-period unit excitation, as this pro-
longed excitation has been documented previously in (non-
theta-triggered) mPFC single units of paired animals only and
in (theta-triggered) hippocampal multiple units of faster-learn-
ing animals (Griffin et al. 2004; Weible et al. 2003). Persistent
firing during trace intervals has been noted in a variety of
forebrain structures (including the hippocampal region and
mPFC) and paradigms (delayed nonmatch to sample, fear
conditioning, and eyelid conditioning) and seems to be
related to the temporal characteristics and familiarity of the
stimuli (Bang and Brown 2009; Griffin et al. 2004; Hasselmo
and Stern 2006; McEchron et al. 2003; Weible et al. 2003).
Interestingly, some of these reports have demonstrated this
persistent activity is reliant on the cholinergic system, which is
a main component in driving the 3- to 7-Hz theta rhythm in the
hippocampus and historically related to attentional processes
(Kramis et al. 1975; Sarter et al. 2005).

It is important to note that the late-trace excitation of
mPFC multiple units in the current study was present in both
the paired and unpaired T+ groups, suggesting that the
effect is not associative. However, the previously reported
excitatory mPFC single-unit responses were shown to gradu-
ally habituate over 6 days of paired conditioning or within
30 trials in the pseudoconditioned group, suggesting its impor-
tance in detecting and adapting behaviorally to rele-
vant environmental stimuli (Weible et al. 2003). In the
present study, late-trace excitation was significantly differ-
ent between T+ - and T−-paired animals until 8 CRs out of
9 consecutive trials (8/9 CRs), which was on day 6.7 ± 0.59
SE when collapsed across theta groups. This is essentially a replication of the habituated late-trace response since neither the $T^+$-nor $T^-$-paired group’s late trace excitatory responses were significantly greater than baseline at this point of conditioning (Figs. 8 and 9). Because $T^+$-unpaired animals also had sustained firing following the tone, it is possible that the presence of hippocampal theta is generally related to enhanced mPFC neuron responses early during stimulus exposure but more so when the tone is behaviorally significant and the firing of hippocampal neurons is highly periodic at theta frequencies. With only 2 days of unpaired conditioning, we do not know whether the $T^+$-unpaired tone response would eventually habituate and at what rate compared with other groups. It is clear, however, that the presence of hippocampal theta is accompanied by a prolonged excitatory mPFC response to a tone.

The LPN, which are a source of mossy-fiber input to the cerebellum, are activated during the presence of a tone CS, and stimulation of this pathway can even serve as the CS in EBCC if it overlaps with the US (which means delay but not trace conditioning) (Aitkin and Boyd 1978; Boyd and Aitkin 1976; McCormick et al. 1983; Steinmetz et al. 1986). Inactivation of the LPN via muscimol in well-trained animals abolished subsequent trace eyeblink CRs but did not affect subsequent delay EBCC using mossy-fiber stimulation as the CS (Kalmback et al. 2009). This suggests that the CS tone that activates the brief mossy-fiber input during the tone period (which is the only prerequisite for the coterminating stimuli in the delay paradigm) is anatomically distinct from the sustained and essential mossy-fiber input that is activated during trace conditioning in the LPN. Since the mPFC projects directly to the LPN, perhaps sustained mPFC excitation (as shown here in the $T^+$-paired group) is a mechanism for prolonged mossy-fiber excitation via...
LPN that is needed to extend the tone-evoked responses and bridge the stimulus-free trace period. This would help explain the necessity of forebrain structures such as the hippocampus and mPFC during trace (but not delay) conditioning and suggests a mechanism for accelerated learning and possibly enhanced plasticity in the cerebellum during theta-triggered conditioning. To summarize, the benefit of theta triggering on mPFC function may be to increase the salience of the tone via the CS-evoked inhibitory/excitatory sequence and to maintain this excitatory response up to (and possibly following) US onset.

**Conclusions.** The results of this study demonstrated significant differences in behavioral, hippocampal, and mPFC responses to tEBCC training that was contingent on the pretrial theta state of the hippocampus. Such results had been hypothesized because theta oscillations are thought to play a significant role in the optimal coordination of a distributed system for tEBCC that involves the hippocampus and mPFC. By triggering trials during discrete periods of hippocampal theta, and without artificial manipulation by lesion or drugs, it was possible to investigate how the brain may be using this oscillatory state to respond adaptively to changing environmental contingencies.

However, it is possible that hippocampal theta is a covariate with another factor that may contribute to these disparate responses. For a more extensive description of the neural codes that participate in memory formation and to help determine a direction of causality in understanding the impact of hippocampal theta, it will be necessary to utilize the theta triggering paradigm while recording LFPs and single units from a variety of interconnected areas, including medial/lateral septum, dorsomedial thalamic nuclei, LPN, cerebellum, and subregions of...
the hippocampus and mPFC. By assessing the electrophysiological responses of neurons throughout the brain, although animals, by design, learn at different rates, we hope to substantiate the widespread impact of hippocampal theta on behavioral memory acquisition and the corresponding neural plasticity.

ACKNOWLEDGMENTS

We thank Lynn Johnson, Loren Hoffmann, Jennifer Muncy, Rajesh Thurai-ratnam, and Paul May for their technical contribution and Allan Pantle for suggestions as a member of the Master of Arts committee for R. D. Darling. This research was submitted to the Department of Psychology at Miami University in partial fulfillment by R. D. Darling for the Master of Arts degree.

GRANTS

K. Takatsuki is the recipient of a Fellowship for Young Scientists from the Japan Society for the Promotion of Science. This work was supported by National Science Foundation Grant IOB-0517575.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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