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

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Abbreviations: tEBCC, trace eyeblink classical conditioning; LFP, local field potential; mPFC, medial prefrontal cortex; CS, conditioned stimulus; US, unconditioned stimulus; CR, conditioned response; ACC, anterior cingulate cortex; LPN, lateral pontine nuclei; EP, evoked potential
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Abstract

Trace eyeblink classical conditioning (tEBCC) can be accelerated by making training trials contingent upon the naturally generated hippocampal 3-7 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+) or absence (T-) of theta, yielding significantly faster behavioral learning in the T+ paired group. The stimulus-elicited hippocampal unit responses were larger and more rhythmic in the T+ paired group. This facilitation of unit responses was complemented by differences in the hippocampal local field potentials (LFP), with the T+ paired group demonstrating more coherent stimulus-evoked theta than T- paired animals and both unpaired groups. mPFC unit responses in the rapid learning T+ 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+ animals, but not in T- 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 pre-trial hippocampal state is related to enhanced neural activity in critical structures of the distributed network supporting the acquisition of tEBCC.

Keywords: anterior cingulate cortex, hippocampus, medial prefrontal cortex, rabbit eyeblink classical conditioning, brain computer interface
Introduction

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, 2000b). 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 airpuff 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
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anterior cingulate cortex (ACC), prelimbic, and infralimbic cortices. Stimulation of the prelimbic 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 prior to 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+), while triggering in its absence (T-) 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 hippocampal state and cerebellar physiology during tEBCC (Hoffman 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
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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 non-physiological
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) who were randomly assigned to paired or unpaired groups and
theta conditions and kept on a 12:12 light-dark cycle with ad libitum access to food and water.
Experiments were conducted during the light phase and performed at approximately the same
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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 1.5mm 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 ~500KΩ (BAK Imp-1) was achieved, typically leaving ~50µm in tip exposure. Electrodes were lowered bilaterally with respect to bregma into the hippocampus (-4.5 AP, ±5.5 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 2.5-3.5mm for CA1 (stratum oriens) and 2.0-3.0mm for mPFC. Once electrodes were in place, they were cemented to skull anchor screws and soldered to a DB-9 connector (Radio Shack) via an insulated wire (previously soldered to the upper portion of the electrode).

**Training**

Following the 5-day recovery period and prior to training, animals were given at least two 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 upon a custom Labview program (Version 5.01) that performed a real-time (640ms sliding window) fast Fourier
transform from a bandpass (1-25 Hz) hippocampal electrode. The program calculated (every 160ms) a ratio of the power spectrum coefficients with 3.5-8.5 Hz power in the numerator and 0.5-3.5 and 8.5-22 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 960ms. 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 100ms 1.0 kHz 80 dB tone was followed by a 100ms 3 psi corneal airpuff to the left eye separated by a 500ms trace interval. Sessions were approximately 90 minutes with a minimum intertrial interval of 60 seconds. 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 airpuff 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 two sessions with a minimum intertrial interval of 30 seconds 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 DB-9 cable with head-mounted FET amplification and a custom made 4-channel bio-amplifier with a gain of 5200 and filtered between 0.1-8000 Hz. Signals were recorded on a Vetter Model 970118 instrumentation recorder based on a Sony (SLV-640HF) VCR. Eyelid responses were transduced with a custom potentiometer and recorded in Labview on a Power Macintosh (7100/80). A 0.5mm movement was considered a response, with a conditioned response (CR) required to occur between 50ms after tone onset and before airpuff onset. Behavioral analyses were performed on the first
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several days of acquisition, on the occurrence of the 5\textsuperscript{th} 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 \(\mu\)A DC current via Grass Stimulator (SD9) and
Grass Constant Current Unit (CCU1A) was passed through each electrode for 10 seconds
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 EtOH for 36-48 hours 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, while
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 Figure 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 Datawave
Technologies’ (Longmont, CO) 16 channel interface using SciWorks and Experimenter software
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for analysis. All data extraction was triggered by a recorded pulse synchronized to tone and
airpuff onset from Labview.

LFPs were filtered 1-200 Hz and sampled at 500 Hz. An event detection algorithm
extracted a 2-second waveform (500ms pre-CS, 1500ms 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 500ms prior to CS onset (baseline), following the CS EP (trace), and following the US EP
(Post US) to reveal stimulus-evoked changes in rhythmic activity between 1-200Hz in 1Hz
increments.

Multiple units were filtered 500-5000 Hz and sampled at 20 kHz. An event detection
algorithm was used to extract 1.05 seconds (150ms pre-CS, 900ms 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 (10ms 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 first 15 bins (pre-CS) from each bin of the session’s average 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 x training group x day (2 x 2 x 2) ANOVAs and post-hoc t-tests were computed using SPSS (version 11.5) and/or Microsoft Excel (2002).

Results

Behavior

T+ animals reached the early learning criterion of the 5th conditioned response (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 to 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 conditioned responses 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 first four 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 percent (F(1,12) = 1.91, p = 0.19) CRs over the first four 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- paired, T+ unpaired, 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+ paired group. These EPs preceded a reset of ongoing theta in both T+ groups, seen in the averaged waveforms for paired and for unpaired animals, and elicited minimally coherent theta in the T- groups. As evident in the averaged waveform, the coherent post-stimulus theta rhythmicity was most robust in the T+ paired group. Further waveform analysis revealed larger increases in 6-8 Hz spectral frequencies after both stimuli in the T+ groups (7-8 Hz in T+ unpaired) compared to both T- groups (Fig. 4). In general, the results of the spectral analyses demonstrated a suppressed or unchanging response to stimuli in the 3-5 Hz and 9-12 Hz ranges for all groups, with the most robust 6-8 Hz theta increase in T+ paired animals. Within groups, the evoked potentials and frequency spectra did not differ over conditioning days.

The hippocampal multiple unit responses were enhanced in the T+ paired group compared to all other groups. This finding paralleled previous observations of T+ paired group enhancement using the theta-triggered paradigm during tEBCC (Griffin et al. 2004), and unlike previous investigations, we report for the first time unit responses from theta-triggered unpaired animals (Fig. 5). After dividing the 500ms trace period into two 250ms periods, a 2 x 2 x 2 (theta group x training group x day) ANOVA of the late trace period (250ms) 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-hot test revealed that the T+ paired group had significantly greater late trace excitation \( (p \leq 0.03) \) than all other groups (Fig. 6A). This enhanced excitatory response in T+ paired animals was maintained (compared to T- paired
animals) through the early learning phase (5\textsuperscript{th} CR) and stable CR performance (8/9 CRs) ($p \leq 0.04$) (Fig. 6B). An autocorrelation of the T+ paired averaged unit histogram on the day of 5\textsuperscript{th} 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 6-8 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 x 2 x 2 (theta group x training group x day) ANOVA was performed to test for a difference in this CS-elicited response, quantified by the difference in the 80ms post CS-onset period of excitation from the previous 80ms period of inhibition. This test revealed a significant theta group x training group x day interaction $F(1,13) = 6.33$, $p = 0.03$. On day 2, T+ paired animals demonstrated this CS-elicited sequential response more than all other groups regardless of theta or training condition (vs T- paired $t(5) = 2.07, p = 0.05$, vs T+ unpaired $t(5) = 1.94, p = 0.05$ and vs T- unpaired $t(5) = 1.88, p = 0.06$) (Fig. 9A). T+ animals continued to display this sequential response throughout paired conditioning when
compared to T- animals on the day of 5\textsuperscript{th} CR, t(7) = 1.68, \(p = 0.07\), and 8/9 CRs, t(7) = 2.52, \(p = 0.02\).

A 2 x 2 x 2 (theta group x training group x day) ANOVA revealed a significant main effect of theta group, \(F(1,13) = 5.07, p = 0.04\) during the 2\textsuperscript{nd} half (250ms) of the trace interval, similar to what had been demonstrated with the hippocampal multiple units (Figs. 9B and 5,6). 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 (\(p \leq 0.08\)) (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 percent conditioned responses (CRs) in the T+ group during the first four days of conditioning, along with statistically fewer trials and days to the 5\textsuperscript{th} CR, provided further evidence for the behavioral benefit of theta-triggering early in training. The 5\textsuperscript{th} 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 prior to 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.

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 on-going 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 on-going theta, while at other times require an abrupt initiation of theta. This heterogeneity in pre-trial 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+ and T- animals (Griffin et al. 2004; Seager et al. 2002).

The ability of various sensory stimuli to evoke a transition from non-theta to theta in animals has been documented (Green and Arduini 1954). However, here the coherence of post-stimulus theta was more pronounced in T+ animals and most robust in the faster learning T+ paired group (Figs. 3,4). This suggests a more tightly coupled response from animals that were already generating theta before the stimulus occurred (T+), and better yet if the event acquires behavioral significance through associative learning (paired group). Such phase resets in oscillatory processes are important components in models of coordinated distributed systems.
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(Hasselmo et al. 2002; Buzsaki 2006; 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 post-stimulus 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 to 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 LTP 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 non-contiguous arrangement of and/or temporally stretched stimuli during tEBCC 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 tEBCC 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. 3,4), 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 versus 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 pre-trial 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 (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).

This study is unique in its attempt to investigate the electrophysiological responses of extra-hippocampal 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 hippocampus impacts mPFC function. Interestingly, the LFPs from mPFC in both theta conditions revealed EPs to the conditioning stimuli and a slow (<2Hz) evoked (possibly oscillatory) response with no obvious differences between groups (see Sirota 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...
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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 hippocampal LFP (theta) than to the LFP in the surrounding mPFC (Hartich et al. 2009; Sirota et al. 2008).

A prior study of single units in mPFC (caudal ACC) (essentially identical to the present study with the exception of theta contingent trial presentation) revealed a tone-evoked sequence 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 airpuff 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. 8,9), suggesting 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 to T+ animals. This could hinder the T- paired groups’ ability to develop a strong association 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, pre-trial hippocampal theta, and sustained trace period unit excitation, as this prolonged 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 learning animals (Weible et al. 2003; Griffin et al.
Persistent firing during trace intervals has been noted in a variety of forebrain structures (including the hippocampal region and mPFC) and paradigms (delayed non-match to sample, fear conditioning, eyeblink conditioning) and seems to be related to the temporal characteristics and familiarity of the stimuli (McEchron et al. 2003; Weible et al. 2003; Griffin et al. 2004; Hasselmo and Stern 2006; Bang and Brown 2009). Interestingly, some of these reports have demonstrated this persistent activity is reliant upon the cholinergic system, which is a main component in driving the 3-7Hz 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 gradually habituate over six days of paired conditioning or within 30 trials in the pseudoconditioned group suggesting its importance in detecting and adapting behaviorally to relevant environmental stimuli (Weible et al. 2003). In the present study, late trace excitation was significantly different between T+ and T- paired animals until 8 conditioned responses out of 9 consecutive trials (8/9 CRs), which was on day 6.7 ± 0.59 SEM when collapsed across theta groups. This is essentially a replication of the habituated late trace response in paired animals since neither the T+ paired nor T- paired group’s late trace excitatory responses were significantly greater than baseline at this point of conditioning (Figs. 8,9). 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 if the tone is behaviorally significant and the firing of hippocampal neurons is highly periodic at theta frequencies. With only two days of unpaired conditioning, we
do not know whether the T+ unpaired tone response would eventually habituate and at what rate
compared to other groups. It is clear, however, that the presence of hippocampal theta is
accompanied by a prolonged excitatory mPFC response to a tone.

The lateral pontine nuclei (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 co-terminating 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 upon the pre-trial 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, dorso-medial thalamic nuclei, lateral pontine nuclei, cerebellum, and subregions of the hippocampus and mPFC. By assessing the electrophysiological responses of neurons throughout the brain while 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.
Theta-triggered hippocampal and medial prefrontal responses

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Figure Legends

**Figure 1.** Histological verification of electrode placement in the mPFC (ACC) and hippocampus. Top images show the most rostral (left) and the more typical (right) recording location in caudal portions of the mPFC. Bottom image shows bilateral hippocampal electrode locations in the stratum oriens layer of CA1. Slices with electrolytic lesions were stained with Prussian blue to help identify recording locations.

**Figure 2.** Behavioral analyses of paired animals demonstrated accelerated learning in T+ animals (n = 8) compared to T- (n = 6). Cumulative conditioned responses (CRs) (A) and percent CRs (B) for all animals over the first four days of conditioning. Number of days (C) and trials (D) for animals to reach the early learning criterion of the 5<sup>th</sup> CR revealed that T+ animals detected the CS-US contingency early than T- animals. Error bars represent SEM.

**Figure 3.** Averaged hippocampal LFPs during paired and unpaired conditioning. EPs were elicited by both the tone and airpuff in all groups. The stimulus-elicited phase coherence (6-8 Hz) is most robust in the T+ paired group. Compare to the 7 Hz sine wave above. Notice the small averaged pre-CS baseline in all groups due to a lack of phase coherence in the LFPs prior to stimulus onset. Thus, any theta rhythmicity in the T+ groups is a reset of ongoing theta; in T-, any theta in the averaged LFP is evoked by conditioning stimuli against a pretrial nontheta background. These waveforms did not generally differ over days. The ordinate is in normalized mV and the abscissa is in ms. Arrows denote tone and airpuff onset. (T+ paired n=5; T- paired n=5; T+ unpaired n= 3; T- unpaired n=3)

**Figure 4.** Differences in hippocampal LFP spectral components following the tone and airpuff relative to their pre-stimulus baseline. A decrease in 3-5 Hz frequencies was apparent in all groups and an increase in 6-8 Hz frequencies was greatest in T+ paired animals. Compare to the
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time domain in Figure 3. The frequency spectra did not generally differ over days. Error bars represent SEM. (T+ paired n=5; T- paired n=5; T+ unpaired n=3; T- unpaired n=3)

**Figure 5.** Hippocampal multiple unit histograms for paired animals (day of 5th conditioned response) and unpaired animals (day 1). The T+ paired group demonstrated greater excitation in response to the tone compared to all other groups. See figure 6 for quantitative analysis. Histograms are in 10ms bins with 150ms baseline and 900ms thereafter. Arrows denote tone and airpuff onset. (T+ paired n=8; T- paired n=8; T+ unpaired n=4; T- unpaired n=3)

**Figure 6.** Hippocampal multiple unit analysis. A) The late trace period (250 ms) excitation is significantly greater in the T+ paired group compared to T- paired animals and both unpaired groups over the first two days of stimulus presentation. B) T+/T- paired differences occurred at the earliest stages of learning (5th conditioned response - CR) and persisted through asymptotic responding (8/9 CR). Error bars represent SEM. C) Autocorrelation of unit histograms during the trace period on the day of 5th CR reveals periodicity ~6.25Hz (160ms) in T+ paired animals but no obvious periodicity in T- paired animals. Dashed line represents first peak at 160ms. (T+ paired n=8; T- paired n=8; T+ unpaired n=4; T- unpaired n=3)

**Figure 7.** Averaged mPFC LFPs during paired and unpaired conditioning. EPs were elicited by both the tone and airpuff in all groups with the largest averaged EPs from the airpuff in unpaired animals. Notice the small averaged pre-CS baseline in all groups due to a lack of phase coherence in the LFPs prior to stimulus onset. No rhythmically coherent responses to stimuli were present in any group other than a <2 Hz response in all groups. These waveforms did not generally differ over days. The ordinate is in normalized mV and the abscissa is in ms. Arrows denote tone and airpuff onset. (T+ paired n=5; T- paired n=5; T+ unpaired n=3; T- unpaired n=3)
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**Figure 8.** mPFC multiple unit histograms for paired animals (day of 5th conditioned response) and unpaired animals (day 1). The T+ paired group demonstrated a period of inhibition followed by excitation in response to the tone (indicated with the box) that was not present in any other group, and a slightly larger response to the airpuff. The T+ groups had an extended excitatory response following the tone compared to T- animals that actually showed suppression. See figure 9 for quantitative analysis. Histograms are in 10ms bins with 150ms baseline and 900ms thereafter. Arrows denote tone and airpuff onset. (T+ paired n=5; T- paired n=5; T+ unpaired n=4; T- unpaired n=3)

**Figure 9.** mPFC multiple unit analysis. A) A tone-evoked inhibitory/excitatory sequence was present in T+ paired animals that was significantly greater than T- paired animals and both unpaired groups after day 1 of stimulus presentation (left) as well as throughout paired conditioning (right). B) Late trace period (250ms) excitation was significantly greater in T+ animals over the first two days of stimulus presentation (left), with T+ paired animals demonstrating greater excitation than T- paired animals through the day of 5th conditioned response (CR) but not at asymptotic (8/9) CR performance (right). C) Although not highly significant ($p \leq 0.08$), T+ paired animals tended to have more airpuff-elicited excitation compared to all other groups over the first two days of stimulus presentation (left) and throughout paired conditioning (right). Error bars represent SEM. (T+ paired n=5; T- paired n=5; T+ unpaired n=4; T- unpaired n=3)