The hippocampus and cerebellum are critically involved in trace eyeblink classical conditioning (EBCC). The mechanisms underlying the hippocampal-cerebellar interaction during this task are not well understood, although hippocampal theta (3-7 Hz) oscillations are known to reflect a favorable state for EBCC. Two groups of rabbits received trace EBCC in which a brain-computer interface administered trials in either the explicit presence or absence of naturally occurring hippocampal theta. A high percentage of robust theta led to a striking enhancement of learning accompanied by rhythmic theta-band (6-7 Hz) oscillations in the interpositus nucleus (IPN) and cerebellar cortex that were time-locked both to hippocampal rhythms and sensory stimuli during training. Rhythmic activity was absent in the cerebellum of the non-theta group. These data strongly suggest a beneficial impact of theta-based coordination of hippocampus and cerebellum and, importantly, demonstrate that hippocampal theta oscillations can be used to regulate the functional properties of the cerebellum.
CEREBELLAR THETA OSCILLATIONS ARE SYNCHRONIZED DURING HIPPOCAMPAL THETA-CONTINGENT TRACE EYEBLINK CONDITIONING

A Thesis

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Cerebellar Theta Oscillations are Synchronized during Hippocampal Theta-contingent Trace Eyeblink Conditioning

Overview

Eyeblink classical conditioning (EBCC) evolved from the pioneering ideas of learning theorists such as Pavlov and Estes and has had a long history as a model paradigm for assessing the functions of systems involved in learning and memory, simplified by a highly controlled environment. In the early 1960’s, Gormezano and colleagues adapted this preparation to rabbits (for review, see Gormezano, 1972). Due largely to R.F. Thompson’s systems mapping approach, dozens of empirical studies have demonstrated that acquisition and retention of all forms of EBCC rely on a highly localized brainstem-cerebellar circuit (for review, see Christian & Thompson, 2003; Steinmetz & Woodruff-Pak, 2000). Consequently, this paradigm is perhaps the most well-understood and extensively used neurobiological model for mammalian associative learning. This places EBCC in an ideal position for use as a model system to study the roles of less understood phenomena such as neurobiological oscillations. As with any model, the usefulness of EBCC is defined by the extent to which it can incorporate essential structures. For trace EBCC both hippocampus and cerebellum are required (see below). To date, questions concerning the possible timing, form, and location of hippocampal-modulated cerebellar responses during trace EBCC have not been adequately addressed by lesion or drug studies because the hippocampal inputs to cerebellum were absent or outside normal physiological limits (e.g. Berry & Thompson, 1979; Solomon, Vander Schaaf, Thompson & Weisz, 1986; Salvatierra & Berry, 1989; Kaneko & Thompson, 1997; Takehara, Kawahara & Kirino, 2003). The following sections review the existing literature on the critical roles of the cerebellum and hippocampus in EBCC, as well as emerging data that have begun to bridge these literatures into a cohesive network through addressing the extent to which interactions between critical structures may be coordinated by theta-band oscillations.

Cerebellum in EBCC

McCormick, Lavond, Clark, Kettner, and Thompson (1981) put forth the first evidence for the role of the cerebellum in EBCC by demonstrating that a unilateral cerebellar lesion, which included both the cortex and deep nuclei, abolished conditioned responses (CRs) during
short-delay EBCC. It has since been shown, for both delay and trace preparations, that lesions restricted to the anterior interpositus nucleus, completely and permanently prevent acquisition of CRs in naïve animals and abolish CRs in well-trained animals without affecting the reflexive, unconditioned response (UR) performance (McCormick, Clark, Lavond, & Thompson, 1982; Lincoln, McCormick, & Thompson, 1982; McCormick & Thompson, 1984a; Yeo, Hardiman, & Glickstein, 1985b). Reversible inactivation of IPN with low doses of muscimol during the first five sessions of acquisition resulted in complete prevention of learning with no savings at the beginning of post-inactivation training (Krupa & Thompson, 1997). In contrast, reversible inactivation of the magnocellular red nucleus (which receives CS-US output from the IPN) during acquisition had no effect on the UR but eliminated expression of the CR. Animals exhibited asymptotic learned performance of the CR immediately after reactivation (Krupa, Thompson, & Thompson, 1993). Furthermore, reversible inactivation of the cranial motor nuclei, which receive CS-US output from the red nucleus, completely prevented expression of both CRs and URs, and the animals, again, showed savings of the CS-US association, with asymptotic performance from the beginning of post-inactivation training (Krupa, Weng, & Thompson, 1996). These manipulations provide further support for a localized site of plasticity in the IPN by demonstrating that both the red nucleus and the motor nuclei are efferent to the essential site of the memory trace. Electrophysiological recordings in IPN reveal neural populations that respond to the conditioned and unconditioned stimuli as well as prior to CRs in a predictive manner (McCormick & Thompson, 1984b; Berthier & Moore, 1986; 1990). Additionally, it has been shown that microstimulation of brainstem areas afferent to cerebellar deep nuclei or fibers projecting directly to the cerebellum serves as an effective conditioned stimulus (CS) or unconditioned stimulus (US), depending on stimulus location and CRs elicited by stimulation are completely abolished by lesions of these upstream cerebellar regions (Mauk, Steinmetz, & Thompson, 1986; Steinmetz, Rosen, Chapman, Lavond, & Thompson, 1986; Steinmetz, Lavond, & Thompson, 1989; but also, see Chapman, Steinmetz, & Thompson, 1988 for detail on qualification mentioned above). Based on this line of research, the IPN has been widely-accepted as the site of CS-US convergence, critical for acquisition and for expression of CRs.

As evidence for the essential role of IPN accrued, some indication of possible critical involvement of cerebellar cortical areas, such as anterior lobe and Larsell’s hemispheric lobule IV (HVI), developed in parallel. As both HVI and anterior lobe send direct projections to the
IPN, these areas are considered likely substrates for EBCC-related plasticity. Similar to the electrophysiological responses of the IPN, cerebellar cortex displays learning related activity with responses modeling the amplitude time-course of the CR (McCormick & Thompson, 1984b). However, lesions of cortical areas have revealed inconsistent findings. Results of lesions to cortex range from complete abolition of EBCC responses (Yeo, Hardiman & Glickstein, 1985a) to a transient decrease in CR performance with recovery to pre-lesion levels of learning-related responding upon reacquisition/additional training (Woodruff-Pak, Lavond, & Thompson, 1985; Lavond, Steinmetz, Yokaitis, & Thompson, 1987; Perrett and Mauk, 1995). While these disparate findings support at least, a modulatory role of the cortex in EBCC, they shed light on the added topographical complexity of the cortex (a potential challenge from a methodological standpoint) and/or the ostensible intricacy of cortical involvement in EBCC. Furthermore, Thompson refutes the interpretation that cerebellar cortex is essential for the task by claiming that complete cerebellar cortical lesions cannot be created without damaging the IPN (see Christian & Thompson, 2003). As a further attempt to define the role of the cerebellar cortex in EBCC, lesions of the cerebellar cortex after pre-training revealed that while old learning wasn’t disrupted, the temporal properties of new learning were impaired (Garcia, Steele & Mauk, 1999). To date, subregional specifics reveal that damage to HVI significantly impairs the rate of learning and frequency of CR expression in learned animals and unit activity in this area show inhibitory and excitatory patterns of Purkinje cell activity that appear to be related to CS or US presentation and CR execution (e.g., Berthier and Moore 1986). Damage to the ansiform and paramedian lobules of anterior cortex is sufficient to alter the timing and magnitude of CRs (Perrett and Mauk, 1995), while individual Purkinje cell response profiles of anterior cortex during a short- versus long-ISI discrimination training task showed encoding on either one or both of the ISI conditions, with an overall pattern of Purkinje cell excitation followed by inhibition during the CS-US interval (Green & Steinmetz, 2005). Collectively, these results provide support for the modulatory role of the cerebellar cortex during EBCC in contributing information related to the timing, frequency, and magnitude of CRs, which facilitate normal acquisition and expression of CRs.
Hippocampus in EBCC

For simple associative learning tasks, such as delay EBCC, the hippocampus is not essential, though it has been observed to play a facilitative role, as neural firing in the hippocampus does show a topography that correlates with the rabbit’s behavioral responses. As behavioral conditioning develops, the hippocampal response moves earlier in time, always preceding CR onset in latency (typically 25 to 35 ms) and paralleling the behavioral eyeblink response (Schmaltz & Theios, 1972; Berger, Alger & Thompson, 1976). As task complexity increases, as in trace conditioning, in which there is a temporal gap between the CS and the US (Solomon et al., 1986; Moyer, Deyo & Disterhoft, 1990), and discrimination reversal (where CS+ and CS- then becomes CS- and CS+, respectively; Gould & Steinmetz, 1994) the hippocampus becomes essential for normal acquisition. Lesions or pharmacological inactivation of the hippocampus and/or retrosplenial/cingulate cortex inhibit normal conditioned responding during trace EBCC, though short-latency, small-amplitude CRs are seen (e.g. Lesion: Solomon et al., 1986; Scopolomine: Kaneko & Thompson, 1997). Also, when hippocampal patterns are disrupted, but the efferent pathways are left intact, EBCC is severely delayed (Berry & Thompson, 1979; Solomon, Solomon, Vander Schaaf, & Perry, 1983; Salvatierra & Berry, 1989). This is most likely due to maladaptive information leaving the hippocampus and disrupting EBCC. Berger, Berry & Thompson (1986) proposed that the projections reached the cerebellum via the subiculum and retrosplenial cortex.

It has been determined that critical involvement of the hippocampus in trace EBCC is temporally restricted to the early phases of learning and retention, as evidenced by abolition of CRs in animals that received hippocampal lesions immediately after training versus lesions produced 1 month after training, which have no effect on CR performance (Kim, Clark, & Thompson, 1995; Takehara, Kawahara & Kirino, 2003). This is consistent with the literature on medial temporal lobe declarative memory deficits in humans and monkeys, which are characterized by severe anterograde amnesia and time-limited retrograde amnesia (Zola-Morgan & Squire, 1990). Electrophysiological recordings during trace EBCC, have shown that hippocampal neurons increase firing rates in response to conditioning stimuli (McEchron & Disterhoft, 1997; Griffin, Asaka, Darling, & Berry, 2004) and, perhaps critically, during the trace interval (Solomon et al., 1986; Griffin et al., 2004). Similarly to the above characterization of unit activity during delay EBCC, trace conditioning leads to unit responses that follow the
amplitude-time course of the CR. In effort to distinguish task-specific response profiles of the hippocampus, Green and Arenos (2007) recorded conditioned unit responses during delay and trace EBCC while holding the ISI constant (580-ms CS-US interval). Hippocampal pyramidal cell inhibition during non-CR trials was observed in trace, but not delay, conditioning in early sessions, while later sessions showed inhibition in both delay and trace preparations. Single unit activation to the CS during behavioral CR trials was observed very early in delay preparations, while in trace conditioning, activation to the CS tended to emerge later in training. Activation during the trace interval on behavioral CR trials, was not observed. This finding may be put in perspective by findings of Griffin and colleagues (2004), in which animals that received training trials in the explicit presence of theta showed activation during the trace interval by training day 2, while animals receiving trials in the explicit absence of theta did not. If theta is uncontrolled, as in the case of Green & Arenos, trace responding could be more heterogeneous and consequently less significant. In sum, the literature on hippocampal involvement during EBCC supports a critical role for the hippocampus during the early phases of acquisition and retention of trace EBCC and a facilitative role in delay conditioning.

**Theta in EBCC**

Neurobiological oscillations are known to act as timing signals in the brain, biasing input selection, facilitating synaptic plasticity, and coordinating activity within and across different regions (Singer, 1999; Welsh, Lang, Sugihara & Llinas, 1995; Buzsaki, 2002; Lisman, 2005; Hasselmo, 2005; Canolty, Edwards, Dalal, Soltani, Nagarajan, Kirsch, Berger, Barbaro, & Knight, 2006; Llinas, 2009). Hippocampal cholinergic theta oscillations, which are low frequency, sinusoidal waves ranging from 3-7 Hz, serve as an index of hippocampal functional state and are within the bandwidth of oscillations that have been proposed to synchronize large areas or across long distances (Green & Arduini, 1964; Buzsaki, 2002; Canolty et al., 2006). Cholinergic theta in the hippocampus relies on the integrity of inputs from medial septal nucleus and the lateral limb of the diagonal band of Broca, as lesions or pharmacological disruptions with anti-cholinergic drugs have been shown to significantly reduce the amount of theta or severely disrupt the regularity and amplitude of these waves (Berry & Thompson, 1979; Solomon & Gottfried, 1981; Stewart & Fox, 1990; Asaka, Griffin, & Berry, 2002; for review see Bland & Oddie, 2001). In 1978, Berry and Thompson discovered that pre-training hippocampal theta was correlated with a faster learning rate. This finding stressed the value of using extracellular local
field potentials (LFPs) as an index of neural processes, specifically hippocampal state, conducive to synaptic modification for learning. Recently, these results have been replicated in rabbits (Nokia, Penttonen, Korhonen, & Wikgren, 2008) and extended to human preparations (e.g. Caplan, Madsen, Raghavachari, & Kahana, 2001; Caplan, Madsen, Schulze-Bonhage, Aschenbrenner-Scheibe, Newman, & Kahana, 2003). Berry and Thompson (1979) then demonstrated that lesions to the medial septal nucleus not only reduced the amount of hippocampal theta significantly, but also disrupted the growth of hippocampal unit responses and slowed acquisition rate during EBCC. It has been well documented that treatments disrupting hippocampal theta are disruptive to the acquisition of EBCC (e.g. Solomon & Gottfried, 1981; Solomon et al., 1983; Salvatierra & Berry, 1989; Solomon, Groccia-Ellison, Flynn, Mirak, Edwards, Dunehew, & Stanton, 1993; Kaneko & Thompson, 1997). There have been relatively fewer studies demonstrating the benefit of theta to learning and memory by artificially eliciting or enhancing theta to accelerate behavioral learning (Landfield & Lynch, 1977; Wetzel, Ott, & Matthies, 1977; 1977; Deupree, Coppock, & Willer, 1982; Berry & Swain, 1989). One drawback to lesion and drug studies is that they produce unnatural brain states, due to their permanent modification of the systems involved in theta and their inability to specifically coordinate theta with individual conditioning trials. In a conditioning session, rabbit hippocampal theta typically occurs in epochs varying from 2 cycles to several seconds in duration, interrupted by periods of non-theta (either large irregular activity or sharp waves). This natural ebb and flow may be an important aspect of theta in cognitive processes (Buzsaki, 2002). Long-lasting or permanent treatments such as drugs or lesions prevent this natural fluctuation and may induce LFPs that look like theta but interact differently with the endogenous neural substrates. For example, electrical stimulation of medial septum (producing theta field potentials in hippocampus) has recently been shown to produce aberrant, “non-physiological” activity patterns in theta-related cells in the hippocampus (Scarlett, Dypvik, & Bland, 2004). One solution to this important problem of maximizing theta would be to let natural variation occur but restrict trials to make sure they coincide with endogenous hippocampal theta, as pursued in studies from our lab.

In trying to discern the significance of naturally occurring theta within conditioning sessions, Seager, Johnson, Chabot, Asaka, and Berry (2002) developed a brain-computer interface (BCI) to limit EBCC training to two naturally occurring extremes of hippocampal theta
such that each of two groups can be trained in either the explicit presence (T+) or absence (T-) of on-going theta. Trial presentations during theta lead to: 1) significant increases in learning rate (Seager et al., 2002; Griffin et al., 2004; Asaka, Mauldin, Griffin, Seager, Shurell, & Berry 2005), 2) a corresponding increase in hippocampal unit responses across days (Griffin et al., 2004), 3) a substantial reduction in age-related memory impairment (Asaka et al., 2005) and 4) an enhancement in neural activity of the prefrontal cortex (PFC) exclusive to theta-triggered animals (Darling, 2005). Thus, the working hypothesis is that this technique allows for the utilization of theta to coordinate trials with the timing of neural networks within the hippocampus and related structures that are important for acquiring the association between conditioning stimuli. This notion is very similar to parts of the model proposed by Hasselmo, Bodelon & Wyble, (2002), in which cue-related signals arriving in hippocampus (especially CA3) from association cortex, that occur in precise temporal relationship to peaks and troughs of theta, are thereby selected for strengthening or weakening (plasticity) or readout from memory (stability), respectively. This general idea of the role of theta is becoming more widely accepted as new data and models are generated (Buzsaki, 2002; Buzsaki & Dragun, 2004; Lisman & Otmakhova, 2001; Williams & Givens, 2003; Jensen, 2001).

**Hippocampal-Cerebellar Interactions during EBCC**

Few studies have directly assessed interactions between the hippocampus and areas of the cerebellum known to be important for normal acquisition of trace EBCC. Of these, questions of directionality of influence have not been fully assayed. It is known that lesions of the cerebellum disrupt learning-related responses of the hippocampus, though the underlying mechanisms of this outcome have not been determined (Clark, McCormick, Lavond & Thompson, 1984; Sears & Steinmetz, 1990; Ryou, Cho, & Kim, 1998). Effects of hippocampus on learning-related input in cerebellum have been less directly demonstrated through studies that manipulate hippocampal functioning to disrupt learning (Berry & Thompson, 1979; Solomon et al., 1983; Salvatierra & Berry, 1989; Kaneko & Thompson, 1997; Takehara et al., 2003). These studies imply that crucial hippocampal influences on cerebellum exist, though no studies have recorded from cerebellum during direct hippocampal manipulation. Recently, Kalmbach, Ohyama, Kreider, Riusech, and Mauk (2009) reported on prefrontal-cerebellar interactions during trace EBCC. These authors demonstrated prelimbic/infralimbic modulation of cerebellar responses. To the
extent that PFC receives learning-related input from hippocampus about CS-US associations and that hippocampal oscillatory activity entrains PFC response patterns, their study provides tacit support for hippocampal influence on cerebellar physiology and a viable route for exerting such influence.

At present, interactions between the hippocampus and circuitry of the cerebellum have yet to be extended to include the effects of theta-contingent training; nor do they include information on cerebellar LFPs. Thus, the current study investigated the nature of involvement of hippocampal-theta in cerebellar responses during trace EBCC. The resulting data demonstrate that hippocampal theta state can be used to synchronize hippocampal (CA1) and cerebellar LFPs into a rhythmic theta oscillation that accompanies a striking cognitive benefit over non-theta conditioning. During hippocampal theta, relationships between cerebellar regions are also theta-modulated, with cerebellar rhythmicity absent under non-theta hippocampal states. These findings provide a significant role for hippocampal theta in coordinating a widely-distributed memory system for trace EBCC and demonstrate the use of hippocampal state to modify cerebellar processing of conditioning stimuli.

METHODS

Subjects. Subjects were 11 New Zealand White rabbits (*Oryctolagus cuniculus*) supplied by Myrtle’s Rabbitry (Thompson Station, TN). All rabbits were maintained on a 12-hour light-dark cycle, with training conducted during the light phase. The rabbits were allowed free access to food and water in their home cages. All procedures involving animals were approved by the Miami University Institutional Animal Care and Use Committee.

Surgery. After each rabbit was pre-anesthetized with a ketamine (50 mg/kg i.m.) and xylazine (10 mg/kg i.m.) cocktail or Thiopental Sodium (Pentothal; 5% solution; approximately 0.3 ml or to effect, i.v.), a small nylon suture loop was placed in the nictitating membrane. Each animal was then secured in a stereotaxic frame and maintained on 2% isoflurane anesthesia for the duration of surgery. The dorsal surface of the skull was exposed to reveal landmarks of bregma and lambda, which were used to properly orient the head (lambda 1.5 mm ventral to bregma). All electrodes were constructed from stainless steel insect pins (size 000) insulated with
Epoxylite (The Epoxilite corp., Buffalo, NY) except for 50-70 μm at the tip. The impedance (200-300 kΩ) was verified using BAK-IMP-1 impedance tester. Coordinates for placement of bilateral hippocampal electrodes were: 4.5 mm posterior to bregma, 5.5 mm lateral to the midline suture and approximately 3.0 mm ventral to dura (Girgis & Shih-Chang, 1981; Fig. 8A for electrode locations). The dorsal-ventral placement was assisted by simultaneously monitoring the familiar electrophysiological properties as the electrode passed through cortex, corpus callosum and the target area of the stratum oriens or pyramidal layer of CA1 layer of the dorsal hippocampus. Cerebellar electrodes in IPN and Larsell’s lobule HVI were implanted ipsilaterally to the trained eye. Electrodes were lowered using a combination of stereotaxic coordinates and electrophysiological monitoring. Based on the previous recording studies of McCormick and Thompson (1984b) and Green and Steinmetz (2005), IPN coordinates were: (0.0-1.5 mm anterior, 2.0-6.0 mm lateral, 12.5-14.5 mm ventral to lambda) and anterior cerebellar cortex coordinates were: (3.0 mm anterior, 5.0 mm lateral, 10.0-15.0 mm ventral to lambda) (Fig. 8B and C for electrode locations). The deep cerebellar nuclei can be distinguished from the cerebellar cortex by the lack of cellular activity characteristic of the dense cell body layers of the cerebellar cortex. Target placement was reached when a signal-to-noise ratio of at least 10:1 was achieved for at least 2 observable units. All recordings were monopolar with a skull screw as reference. After implantation, the chronic electrode wires were soldered to a D-Sub connector (9-position male D-subminiature connector, Radioshack) that was then attached to the skull and anchor screws with dental acrylic. The incision was sutured and a pre-formed base with two 6-32 x 1/2-inch nylon machine screws was secured to the skull with dental acrylic (in order to be connected to a head stabilizer during conditioning).

**Apparatus.** During adaptation and training sessions, each animal was restrained in a standard Plexiglas box (Mitchell & Gormezano, 1970) inside a Faraday cage designed to attenuate extraneous sounds and reduce electrical interference. It should be noted that, unlike primate restraint chairs, a natural posture is afforded the rabbit by the type of restraint presented here. Animals wore headgear (attached to the 9-pin D-sub connector that was permanently mounted during surgery) throughout the conditioning sessions. The headgear included an immediate amplification with field effect transistors (FET) to avoid artifact, followed by input to custom-made biological amplifiers (Miami University Instrumentation Laboratory, Oxford, OH).
During training, the NM loop was attached to a small, lightweight ‘measurement arm,’ which was attached to a potentiometer. When the animal blinked, the arm rotated the potentiometer, creating a small voltage that was recorded and analyzed. The measurement arm has almost no mechanical resistance and thus did not interfere with the movement of the nictitating membrane.

Training Groups. Rabbits were randomly assigned to one of two groups, a theta-triggered (T+) group (n=6), which received trace EBCC trial presentation in the explicit presence of each animal’s naturally occurring hippocampal theta, or a nontheta-triggered (T-) group (n=5), which received trace EBCC in the explicit absence of the animal’s naturally occurring hippocampal theta. Procedures for theta-contingent trial presentation have been described previously (Seager et al., 2002). Briefly, neural activity from one hippocampal electrode was filtered (0.5–22.0 Hz) and then monitored in real time by a software program (LabVIEW, National Instruments Corporation, Austin, TX) designed to compute a spectral ratio of the proportion of power at theta (3.5–8.5 Hz) and nontheta (0.5–3.5 Hz and 8.5–22.0 Hz) for 640-ms scrolling time intervals, updated every 160 ms. For animals in the theta-triggered group T+, trials were given only when the spectral ratio exceeded 1.0 three times in a row (960 ms total pretrial duration). For animals in the T- group, trials were given only when the spectral ratio fell below 0.3 three times in a row. These criteria for T+ and T- trials were empirically determined to maximize (or minimize) the probability that theta would continue throughout the conditioning trial (Seager et al., 2002).

Behavioral Training. Following 5-7 days of postsurgical recovery and two days of adaptation to the conditioning chamber (where the rabbit was restrained and no stimuli were presented for ~45mins), each animal underwent theta-contingent trace EBCC. This hippocampus-dependent form of EBCC consisted of a 100-ms tone CS (1 kHz, 85 dB) followed by a 500-ms stimulus-free period and a subsequent 100-ms corneal airpuff US (3 psi). A daily, paired session was approximately 90 minutes in duration with a minimum of 60 seconds between trials. The number of trials was dependent upon the criteria based spectral analysis computed by the brain-computer interface, typically 50. Animals were trained to a behavioral criterion of eight CRs in any nine consecutive trials and for a minimum of 4 days.
Data Acquisition and Analysis. All neural and behavioral data were gathered with a DataWave interface (DT304) using the 16-channel, 12-bit, 400-kHz aggregate analog to digital converter. Behavioral data were analyzed using traditional learning criteria in the literature. Specifically, the number of trials to the third CR, which is indicative of initial acquisition of the CS-US contingency (Prokasy, 1972; 1987; Gormezano, Kehoe, & Marshall, 1983; Thompson, Berry, Rinaldi, & Berger, 1979), percent CRs by day, and cumulative CRs, which give an accurate overview of learning trajectories, were scored by hand based on oscilloscope readouts. A CR was defined as nictitating membrane movement occurring between 80-499 ms after tone offset that reached 0.5 mm.

Electrophysiological activity from all electrodes was band-pass filtered at 1-200 Hz (Miami University Instrumentation Laboratory, Oxford, OH) and sampled at 500 Hz (DataWave Technologies, Broomfield, CO). LFP activity from each electrode tip was averaged across trials for individual subjects and across animals to produce daily group histograms (Fig. 2). Frequency analyses were computed on the whole trial period, which extended from CS-onset to 1 second after US-offset. Spectral analyses were computed on group average LFPs (Fig. 3), and percent power was computed individually by subject and then averaged (Fig. 4). In order to exclude the impact of temporal regularities induced by the inter-stimulus interval and the waveform of the non-rhythmic EPs, time series analyses of theta were performed on a 1-second sample of rhythmic activity after the end of the large amplitude LFP response evoked by the US. Time series analyses, shown in figure 5-7, were computed on averaged group data of the post-US period (beginning at 1550 ms into the trail recording period, and lasting for 1 second). Files of interest were shifted in 1-ms increments for calculation of correlation coefficients (see x-axis for time shift between files, denoted as lag time from a zero-ms shift in time-relation of data files). MatLab programming software (The Mathworks, Inc., Natick, MA) and Microsoft Excel (Microsoft Corp., Redmond, WA) were used for all offline analyses and creation of data plots.

Histology. At the conclusion of training, each rabbit was anesthetized and a brief stimulating pulse-train (alternating current) was passed through some of the IPN electrodes in order to evoke an EB and thus verify proper electrode placement. Additionally, small marking lesions were made at the tips of all recording electrodes, by passing a 200-μA, 10-s direct current through each recording electrode (Grass Stimulator Model SD-9, Grass Instruments, West
Warwick, RI). Animals were then euthanized with an overdose of sodium pentobarbital (Euthasol, 0.2205 mg/kg IV) and perfused transcardially with physiological saline (0.9%) and formalin (10%) solutions. Serial frozen sections (40 µm) were taken, mounted on gelatin-coated slides and stained with Prussian blue (Sigma, St. Louis, MO), which reacts with the iron ions displaced from the electrode tip by the lesion current. A safranin counterstain was also applied for background stain of somas (Sigma, St. Louis, MO). Slides were then examined with a compound microscope (Nikon, Japan) for verification of electrode locations. Only animals with electrodes properly placed in CA1, IPN, and HVI were included in the study. Hippocampal LFP recordings for animals with more ventral hippocampal placements in locations toward the hippocampal fissure (e.g. lacunosum moleculare) were inverted for field potential analyses (see Buzsaki, 2002 for detailed explanation).

RESULTS

Behavior

Behavioral results demonstrate that initiating acquisition trails based on CA1 theta-state enhances learning rate substantially. Groups differed significantly in percent CRs on days 2, 3, and 4 (D2: MT+=25.0%, MT-=3.3%, F(1,9)=6.02, p=0.037; D3: MT+=53.4%, MT-=3.1%, F(1,9)=15.43, p=0.003; D4: MT+=54.6%, MT-=9.6%, F(1,9)=12.03, p=0.007). Figure 1A shows the percentage of CRs across days 1-4, illustrating the significant difference in group performance. Although, group differences in percent CRs were not significant on day 1(MT+=12.6%, MT-=0%, F(1,9)=3.69, p=0.087), trails to the third CR (classically thought to demarcate the end of learning phase 1, see Prokasy, 1972, 1987) was significantly different between groups (MT+=41.8, MT-=174.2, F(1,9)= 7.54, p=0.023). Trial 41 occurred on the first day of acquisition for T+ animals, while trial 174 typically occurred on day 4 for T- animals. The averaged cumulative CR plot shows the learning trajectories of T+ and T- groups diverging within the first 50 trails of acquisition day 1 (Fig. 1B), which is consistent with our prior findings (Berry & Thompson, 1978; Seager et al., 2002; Griffin et al., 2004; Asaka et al., 2005; Darling, 2005) and those of others (Nokia et al., 2008). Trials to a criterion of 8 out of 9 consecutive CRs (which classically denotes asymptotic levels of responding, see Gormezano et al., 1983) was not significant between groups (MT+=195.7, MT-=365.2, F(1,9)=3.64, p=0.089).
Neural Recordings

Overview. Averaged monopolar recordings of LFPs in hippocampal field CA1, cerebellar interpositus nucleus (IPN), and Larsell’s hemispheric lobule VI (HVI) of cerebellar cortex of the T+ group displayed strong evoked potentials (EPs) followed by an immediate emergence of robust, time-locked theta oscillations in response to stimulus presentations as early as day 1 (Fig. 2). Specifically, a regular hippocampal theta rhythm was triggered by both CS and US in T+ while smaller, irregular theta following each EP was present in T-. In cerebellum, rhythmic theta in the averaged LFPs appeared early in the T+ group, with the first positive peak of rhythmic theta at ~300 ms after CS and US onsets, approximately out of phase with and appearing to attenuate the negative EP compared to the larger nontheta negative peak of the EP seen in T-. Conversely, in T-, each EP ended with a clear, single negative peak showing little or no theta of regular periodicity during the trace period following the CS or after the US (Fig. 2 and 3). Thus, the state of the hippocampus clearly differentiated the T+ and T- groups in terms of cerebellar evoked and rhythmic responses to both conditioning stimuli. All LFPs of the T+ group exhibited a consistent 6-7 Hz theta frequency during the trial period, which was synchronized across structures (Fig. 6 and 7), while the T- group displayed less rhythmicity and greater variability in frequency between structures as well as across days (Fig. 3-7). Two animals were excluded from the current study due to poor signal quality (see histological results for additional exclusions). Of those animals used in the current study, one animal from the T+ group had corrupt data files for all three recording locations on acquisition day 2 and, in the T- group, a data file for cerebellar HVI LFPs on day 1 was lost for an animal.

EP Peak Amplitudes. For evoked responses to conditioning stimuli, peaks were denoted P1, P2, P3, and P4, where P1 and P2 corresponded to the first and second phase, respectively, of the CS-related EP and P3 and P4 correspond to the first and second phase, respectively, of the US-related EP (see high-amplitude LFP peaks at each stimulus presentation in Fig. 2). Taken individually, the amplitudes of the initial phase of each evoked response to the CS and US (P1 and P3), were generally not significantly different between T+ and T- groups across days 1-4 in any structure. One-way analysis of between group differences on peak amplitude of EP peaks 1 and 3 (P1; P3), corresponding to the first phase of responses to CS and US, respectively, revealed a significant difference in amplitudes of P1 in IPN on day 3 (M_{T+}=429.90, M_{T-}=650.39,
F(1, 9) = 5.63, p = 0.042). No other significant differences for either peak on days 1-4 of acquisition for CA1, IPN or HVI were found (See Appendix: Analysis of EP peaks 1 and 3). However, throughout T+ training, the IPN showed larger responses to CS than US in terms of positive peak amplitudes. Within-group difference scores, comparing P1 and P3 response amplitudes in IPN, differed significantly between theta groups on days 1-3 (D1: M_{T+} = -73.81, M_{T-} = -110.33, F(1, 9) = 6.20, p = 0.034; D2: M_{T+} = -68.83, M_{T-} = -208.10, F(1, 8) = 17.44, p = 0.003; D3: M_{T+} = -90.97, M_{T-} = -146.79, F(1, 9) = 6.14, p = 0.035), but not on day 4 (M_{T+} = -75.27, M_{T-} = 129.92, F(1, 9) = 5.07, p = 0.051). Within-group difference scores, comparing P1 and P3 response amplitudes corresponding to the first phase of the evoked responses to CS and US, respectively, were not significant on days 1-4 for CA1 and HVI, (See Appendix: Analysis of difference scores comparing amplitudes of EP peak 1 and 3).

The second phase of each EP showed distinct differences in cerebellar responses as a function of hippocampal state. For CS responses in IPN, amplitudes differed significantly between theta groups. Specifically, one-way ANOVAs on P2 amplitude (negative-going component of EPs to the CS) in IPN revealed that this EP peak was significantly larger in amplitude in the T- condition on days 1-3 (D1: M_{T+} = -272.58, M_{T-} = -508.61, F(1, 9) = 13.36, p = 0.005; D2: M_{T+} = -216.33, M_{T-} = -455.01, F(1, 8) = 13.84, p = 0.006; D3: M_{T+} = -248.46, M_{T-} = -505.75, F(1, 9) = 19.45, p = 0.002), but not D4 (M_{T+} = -238.01 M_{T-} = -352.85, F(1, 9) = 2.28, p = 0.165). Similar results occurred in HVI for P2, in that the negative-going EP peak was significantly larger in amplitude in the T- condition on days 1-3 (D1: M_{T+} = -234.64, M_{T-} = -600.73, F(1, 8) = 18.23, p = 0.003; D2: M_{T+} = -262.20, M_{T-} = -479.64, F(1, 8) = 5.37, p = 0.049; D3: M_{T+} = -305.92, M_{T-} = -553.06, F(1, 9) = 9.84, p = 0.012), but not D4 (M_{T+} = -356.45 M_{T-} = -418.48, F(1, 9) = 0.16, p = 0.699). No group difference in P2 was observed in area CA1 of hippocampus. In fact, analysis of the amplitudes of CA1 P2 and P4, corresponding to the second phase of responses to CS and US, respectively, revealed no significant between-group differences across days 1-4 of acquisition (See Appendix: Analysis of CA1 EP peaks 2 and 4).

For EPs to the US, the amplitude of P4 in IPN was not significantly different on day 1 between groups (M_{T+} = -79.52, M_{T-} = -268.22, F(1, 9) = 2.49, p = 0.149), but was significantly larger in the T- group on days 2-4 (D2: M_{T+} = -128.07, M_{T-} = -357.01, F(1, 8) = 6.34, p = 0.036; D3: M_{T+} = -99.64, M_{T-} = -357.42, F(1, 9) = 17.83, p = 0.002; D4: M_{T+} = -165.74, M_{T-} = -318.38, F(1, 9) = 8.66, p = 0.016). The amplitude of P4 in HVI was significantly larger in the T- group on days 3 and 4.
Frequency. Power spectral analyses of whole-trial (extending from CS-onset to 1 sec after US-offset) average LFPs show that the large, slower waveforms of each EP produced frequency components with consistent power at 3 and 5 Hz (Fig. 3). These spectral peaks are single, non-rhythmic evoked responses (positive-negative-positive for cerebellar structures and the inverse for CA1). Specifically, the 5 Hz component reflects the first half of the EP, while the 3 Hz component reflects the second, opposite polarity part of the EP in responses to both CS and US. Group differences in percent power at 3 and 5 Hz in CA1, IPN and HVI were not significant (See Appendix: Frequency analysis). As power in these EP frequency components does not vary between theta groups or structures, and are not significantly different between groups for each frequency, our analysis concentrated on the faster, rhythmic LFPs that differentiated our treatment groups and corresponded to differences in learning rate.

Spectral analysis of whole-trial LFPs on days 1-4 show that, for rhythmic theta frequencies, power at approximately 6.5 Hz was apparent in all three structures of the T+ group, while the T- group displayed significantly lower power at 6.5 Hz (as determined by percent power analysis below, see Fig. 4). When peaks did occur in the T- group they were higher frequency (7-9 Hz) and consistently smaller than the 6.5 Hz peaks of the T+ group (Fig. 3). Specifically, T- CA1 showed an expected theta response to the conditioning stimuli but at higher frequency than T+, T- IPN displayed small and variable peaks, and T- HVI had no salient frequency peaks above 5 Hz. These trends continued across the first four days of acquisition. Analyses comparing percent power at 6-7 Hz during the first four days of acquisition revealed significant differences between groups in CA1 and IPN on days 1-3 (CA1: D1: M_{T+}=17.2\%, M_{T-}=1.1\%, F(1,9)=8.48, p=0.017; D2: M_{T+}=20.8\%, M_{T-}=3.3\%, F(1,9)=8.40, p=0.02; D3: M_{T+}=11.2\%, M_{T-}=2.4\%, F(1,9)=9.43, p=0.015; IPN: D1: M_{T+}=7.8\%, M_{T-}=0.1\%, F(1,9)=6.50, p=0.031; D2: M_{T+}=9.5\%, M_{T-}=1.0\%, F(1,8)=6.13, p=0.038; D3: M_{T+}=5.8\%, M_{T-}=0.6\%, F(1,9)=37.29, p=0.000) but not on D4 (CA1: D4: M_{T+}=7.7\%, M_{T-}=1.6\%, F(1,9)=4.52, p=0.062;
**IPN:** D4: $M_{T+}=5.0\%, M_{T-}=1.0\%$, $F(1,9)=3.84, p=0.082$; Asterisks above histogram bars in Fig. 4 represent statistical significance at the 0.05 level). In HVI, group differences were not significant (D1: $M_{T+}=9.2\%, M_{T-}=0.1\%$, $F(1,9)=5.23, p=0.051$; D2: $M_{T+}=10.0\%, M_{T-}=0.5\%$, $F(1,9)=3.85, p=0.085$; D3: $M_{T+}=5.7\%, M_{T-}=0.4\%$, $F(1,9)=5.17, p=0.053$; D4: $M_{T+}=5.1\%, M_{T-}=0.7\%$, $F(1,9)=2.80, p=0.129$). Together, these frequency analyses provide significant quantitative support for group differences in the visual appearance of rhythmicity in the LFPs (Fig. 2).

*Rhythmicity.* Time-series analyses (auto- and crosscorrelations) of oscillations were limited to the post-US period (a 1-s period beginning at 1500 ms) so that the estimate of phase and rhythmicity would not be distorted by the large, nonrhythmic EPs that appeared to be stimulus driven in all structures in both groups (as shown in above power analysis, Fig. 3). Autocorrelograms of averaged LFPs showed clear rhythmicity in CA1 and both cerebellar structures of the T+ group. The T- group displayed less robust rhythmicity and a faster frequency in CA1, and importantly, no rhythmicity in either cerebellar structure (Fig. 5). Crosscorrelograms of CA1 and cerebellar LFPs in figure 6 revealed rhythmic coordination of CA1 with IPN and lobule HVI at theta frequency only in the T+ condition. A negative correlation at lag time zero indicates that CA1 and cerebellar waveforms are essentially 180° out of phase (although some hippocampal laminae, e.g. adjacent to the hippocampal fissure, would likely be out of phase with CA1 theta (Buzsaki, 2002) and therefore in phase with cerebellum). The dominant frequency of phase synchronization is approximately 6-7 Hz between CA1 and both cerebellar structures in the T+ condition. Conversely, in the T- condition, crosscorrelograms reveal a weak periodicity, mostly due to the rhythmicity in CA1 that does not co-occur in cerebellum. Crosscorrelograms of IPN and HVI are precisely in phase for both conditions, however, T+ triggering leads to strong, rhythmic coordination across regions of the cerebellum at 6-7 Hz (Fig. 7). The T- condition displayed a clearly aperiodic relationship, indicating a possible dependence of cerebellar coordination on hippocampal state or a common generator of theta rhythms. Results of time-series analyses document the nearly identical periodicity seen across structures in the T+ LFPs and the variability of oscillations in the T- LFPs.
Histology

Serial section diagrams shown in figure 8, depict placements of recording electrodes in CA1 (A), IPN (B), and lobule HVI (C). Placements were determined using a combination of stereotaxic atlas measurements, visual observation, scaled mapping, and stimulation (for some of IPN placements). Two animals were excluded from the present study due to improper electrode placements in either IPN or cerebellar lobule HVI (not shown in fig. 8). Additionally, one animal was excluded due to hippocampal tissue damage. Eight of the 11 animals used in the current study received AC pulse-train stimulation of IPN electrodes to verify placement of recording. Of these animals, clear left eye or eyeblink movements were elicited in five animals (T+ n = 2, T- n = 3), while three animals gave no readily observable eye-related response (T+ n = 2, T- n = 1). Six animals (T+ n = 4, T- n = 2) had electrode placements below CA1 pyramidal cell layer, thus were subject to hippocampal signal inversion.

DISCUSSION

The above findings demonstrate a significant impact of hippocampal theta-triggered training in regulating important functional properties of a widely distributed system for trace EBCC that includes essential cerebellar circuits. The major findings are that the presence of pre-trial hippocampal theta leads to a substantial increase in acquisition rate accompanied by 1) amplitude modulation of cerebellar evoked responses to conditioning stimuli, 2) cerebellar theta oscillations that are time-locked to the sensory stimuli in awake, behaving animals, and 3) synchronization of hippocampus and cerebellar IPN and HVI LFPs at 6-7 Hz theta frequency.

Prior work had shown that, for trace EBCC, the hippocampus was part of the necessary circuit but its role was not clear (Solomon et al., 1986; Moyer et al., 1990; McEchron & Disterhoft, 1999; Takehara et al., 2003; Green & Arenos, 2007). Most interpretations center around hippocampal involvement in sustaining CS-related activity through the trace period until US onset (e.g. Solomon et al., 1986; Clark, Manns, & Squire, 2002; Griffin et al., 2004; Darling, 2005; Kalmbach et al., 2009). Our results show a strong, coordinated rhythmicity at theta during this period in the T+ group but not the T- group in both hippocampus and cerebellum. This data suggest that robust oscillations might help facilitate neural plasticity, known to be associated with theta frequencies in hippocampus (e.g. Larson & Lynch, 1986; 1989; Larson, Wong &
Lynch, 1986; Pavlides, Greenstein, Grudman, & Winson, 1988; Huerta & Lisman, 1993; Hasselmo et al., 2002; Buzsaki, 2002; McCartney, Johnson, Weil & Givens, 2004), and cerebellum (Maex & De Schutter, 1998; Medina & Mauk, 2000; D’Angelo, Nieus, Maffei, Armano, Rossi, Taglietti, Fontana, & Naldi, 2001; Dugue, Brunel, Hakim, Schwartz, Chat, Levesque, Courtemanche, Lena, & Dieudonne, 2009). The coordination of hippocampal and cerebellar theta in the T+ group of our current study may phase-lock their excitability at a periodicity that favors long-distance communication (Buzsaki, 2002; Canolty et al., 2006).

In our EP analyses, the smaller negative EP in response to the CS in the T+ condition may, in part, be attributed to the rapid emergence of robust theta oscillations (theta reset), with a positive peak during the negative phase of the EP (Fig. 3). Our current result demonstrates a significant relationship of hippocampal theta state with the CS response in the cerebellum (both IPN and HVI) and may imply that the behavioral benefit of theta-triggering involves a reduction in the amplitude of the negative cerebellar EP and a continuation of theta rhythmicity into the trace. Further, the relative CS versus US positive peak amplitudes for IPN differed as a function of hippocampal theta states, showing an effect across the trace interval. This could be critically important in trace conditioning in which the CS terminates hundreds of milliseconds before the US arrives. Williams and Givens (2003) discovered theta reset during a continuous conditional discrimination task in rats. Subsequently, Darling (2005) observed an immediate theta reset of hippocampal LFPs of T+ animals (but not as well-timed in T-) early in training. Interpretations of these findings, which are consistent with our present results, include suggestions that theta reset might provide optimal conditions for long-term potentiation and in the case of theta-contingent training, more consistent arrival time of the US-related EP in relation to theta phase and perhaps a resultant larger US-evoked EP response (corresponding to optimal behavioral learning) in the T+ condition.

It is well-established that involvement of the hippocampus during trace EBCC, and consequently the facilitative influences of hippocampal theta, occurs in the early phases of learning (Kim, Clark, & Thompson, 1995; Takehara, Kawahara & Kirino, 2003; Griffin et al., 2004; Darling et al., 2005). Our results showing an early distinction in learning rate and corresponding LFP activity between T+ and T- conditions are consistent with the above findings. The present study also provides more precise specification of the timeline for hippocampal theta involvement by demonstrating an attenuation of group differences in percent power at 6-7 Hz.
and EP peak amplitudes by acquisition day 4 when the T-animals begin to exhibit more CRs. This change over days had not previously been demonstrated for LFPs, but is consistent with Disterhoft’s demonstration of attenuated hippocampal unit responding with overtraining (McEchron & Disterhoft, 1999).

The circuitry of the cerebellum has been shown to support resonant frequencies within the theta bandwidth (3-7 Hz) (Medina & Mauk, 2000; D’Angelo et al., 2001; Dugue et al., 2009). Moreover, there is evidence that such oscillations synchronize activity within cerebellar hemispheres as well as between cerebellar and cortical regions (Hartmann & Bower, 1998; O’Connor, Berg & Kleinfeld, 2002). Those studies on cerebellar oscillations at theta frequency suggest that our methods may be engaging resonant frequencies of cerebellar circuits that favor plasticity and optimize timing. Our finding extends this by showing stimulus time-locked cerebellar oscillations that are coordinated with hippocampal rhythmicity at consistent 6-7 Hz periodicity, and accompanied by a substantial cognitive enhancement.

These observations have important implications for the locus and mechanisms of essential participation of the hippocampus in trace conditioning, such as the modulation of neural pathways by which CS and US information may be transmitted to the cerebellum. Prominent models of EBCC suggest that the US activates the inferior olive (IO), which provides strong synaptic input to cortical Purkinje cells via climbing fibers, with collaterals to IPN (Gould, Sears & Steinmetz, 1993; Maex & De Schutter, 1998; Medina & Mauk, 2000; Allen, Myers & Gluck, 2001). These models also implicate pontine nuclei (PN), activated by the CS, as providing mossy fiber input to granule and Golgi cells, also with collateral input to IPN, and ultimately to the parallel fiber system, which synapses onto Purkinje cells. While the IO/climbing fiber system can generate oscillatory potentials in the theta range (Llinas and Yarom, 1981a,b; Bal & McCormick, 1997; for review see Llinas, 2009), our results suggest the need for studies on the latter (CS) pathway for two major reasons. First, if the role of hippocampus in trace EBCC is hypothesized to be a continuation of the excitatory CS response into and through the trace interval, our results would predict major differences in amplitude and/or duration of PN responses depending on hippocampal theta state. There is significant evidence of theta periodicity in unit activity of cerebellar granule and Golgi cells in this pathway (D’Angelo et al., 2001; Dugue et al., 2009), but their dependence on hippocampal theta is unknown. One recent proposal by Kalmbach and others (2009) for trace EBCC is that mossy fiber input, controlled by
forebrain structures such as hippocampus and prefrontal cortex, serves to sustain CS responses through the trace interval to overlap with the US so that cerebellar circuits can form CS-US associations. Our results suggest that cerebellar theta (which occurred here only in the T+ condition) may create rhythmic patterns of mossy/parallel fiber excitability through the trace interval that allow time-locked US responses from the IO to arrive under optimal conditions for plasticity. Furthermore, to the extent that hippocampal theta entrains forebrain areas during learning- and memory-related tasks (e.g. Hyman et al., 2005; Siapas et al., 2005), the mechanisms put forth by Kalmbach et al. (2009) for post-CS forebrain activation of PN mossy fibers would likely be modulated by hippocampal theta. In fact, our lab has demonstrated theta-contingent differences in prefrontal unit response during the CS and trace intervals (Darling, 2005).

Secondly, the PN have long been implicated in the initiation and pacing of hippocampal theta (Anchel & Lindsley, 1972; Vertes & Kocsis, 1997) so that, if there is a common generator for the oscillations observed in both hippocampus and cerebellum, patterns of activity in PN should interact with hippocampal theta states controlled by our BCI and address questions about the direction of influence between the structures. It should be noted that the precise phase locking over the distance between hippocampus and cerebellum (indicated by the peak at zero lag in the crosscorrelations) would be surprising if one region were directly driving the other, but would be consistent with a common pacemaker for both structures. Evidence for causal linkages between hippocampal and cerebellar functions exist, primarily disruption of hippocampal, learning-related neural responses after cerebellar lesions (Clark, McCormick, Lavond & Thompson, 1984; Sears & Steinmetz, 1990), but our findings support the idea of strong influences from forebrain to cerebellum during trace EBCC.

Generalizing across species, studies have shown theta oscillations to be implicated in human cognitive processing, with beneficial effects in acquisition, retrieval, verbal working memory tasks and spatial navigation (Kahana, Sekuler, Caplan, Kirschen, & Madsen, 1999b; Caplan et al., 2001; Raghavachari, Kahana, Rizzuto, Caplan, Kirschen, Bourgeois, Madsen, & Lisman, 2001; Caplan et al., 2003). If tasks could be acquired and performed when there are periods of maximal theta, cognitive processes might be enhanced. This raises the possibility that our methods could be used to ameliorate human cognitive deficits, such as age-related memory impairment, which we have already demonstrated using EBCC in animals (Asaka et al., 2005).
This technology provides a useful means of observing and manipulating clearly different functional brain states, without lesions or drugs, that could be adapted in the future to other species, brain structures, or oscillatory frequencies.
REFERENCES


Chapman, P.F., Steinmetz, J.E., & Thompson, R.F. (1988). Classical conditioning does not occur when direct stimulation of the red nucleus or cerebellar nuclei is the unconditioned stimulus. *Brain research, 442*(1), 97-104.


Fig. 1. Behavioral learning rates for T+ (black trace; n=6) and T- (gray trace; n=5) animals during acquisition. Error bars represent +/- 1 SEM. (A) Average percent conditioned responses (CRs) on days 1-4 of acquisition (top) shows that theta-contingent trial presentation (T+) leads to a significantly faster (asterisks denote p = 0.05) learning rate compared to T- triggering. (B) Average cumulative CRs across training trials 1-50 (bottom) shows precise learning trajectories for each theta group. Standard error bars indicate divergence between T+ and T- groups within the first 50 trials of acquisition day 1. A daily training session consisted of approximately 50 trials.
Fig. 2 Average LFPs in CA1 (left), IPN (center), and lobule HVI (right) on days 1-4 of acquisition for T+ (black trace) and T- (gray trace) groups. T+ triggering leads to an immediate emergence of robust and time-locked theta oscillations during the trace and post-US periods in both hippocampus and cerebellum. Large and small tick marks on the x-axis denote stimulus onset and offset, respectively, for the tone (CS) beginning at 500 ms and airpuff (US) beginning at 1100 ms (for T+, n=6 on days 1, 3, and 4; n=5 on day 2, for T-, n=5 for all days except, n=4 on D1 for HVI due to a corrupted data file).
Fig. 3. Power Spectra from 2-20 Hz of average LFPs in CA1 (left), IPN (center), and HVI (right) during whole trial period (from CS-onset to 1 sec after US-offset) on days 1-4 show power peaks at approximately 6.5 Hz in the T+ condition (black trace) that persist over days. In the T- condition (gray trace), power at ~7 Hz is present in CA1 (persisting across days), while power in cerebellum is much lower at the 6-7 Hz frequencies and is more variable across days (most commonly ~8 Hz). Area within gray rectangle highlights 6-7 Hz bandwidth, the frequency range of interest (for T+, n=6 on days 1, 3, and 4; n=5 on day 2; for T-, n=5 for all days except, n=4 on D1 for HVI).
Fig. 4. Percent power at 6-7 Hz in average LFPs during whole trial period (from CS-onset to 1 sec after US-offset) in CA1, IPN, and lobule HVI on days 1-4 of acquisition. Line graphs display significantly greater power in this frequency band in CA1 and IPN of the T+ group compared to T- on days 1-3. Error bars represent +/- 1 SEM. Asterisks represent significance at the $p = 0.05$ level.
Fig. 5. Autocorrelograms of average LFPs during the post-US period (a 1-second period beginning at 1550 ms) in CA1 (left), IPN (center), and HVI (right) on days 1-4 of acquisition. Lag time represented on the x-axis reflects the temporal shift in data files being correlated at each point in the correlation function. In CA1 and cerebellum of the T+ group (black; \( n = 6 \)), correlations are dominated by rhythmic theta frequency (6.5 Hz). In the T- group (gray; \( n = 5 \)) some periodicity is elicited by the US in CA1 (as expected) but is not observed in the cerebellum.
Fig. 6. Crosscorrelograms of average LFPs during the post-US period (a 1-second period beginning at 1550 ms) between CA1 and IPN (A) and CA1 and HVI (B) on days 1-4 of acquisition. Lag time represented on the x-axis reflects the temporal shift in data files being correlated at each point in the crosscorrelation function. T+ triggering leads to a systematic covariation of theta in hippocampus and cerebellum. Phase synchronization repeats at approximately 6-7 Hz between hippocampus and both cerebellar structures in the T+ condition (left). The absence of clear theta rhythmicity in the crosscorrelation of CA1 with either cerebellar structure in the T- group (right) may be due to different frequencies in the two structures as revealed by power spectra (Fig. 4).
**Fig. 7.** Crosscorrelograms of average LFPs during the post-US period (a 1-second period beginning at 1550 ms) between IPN and HVI on days 1-4 of acquisition. Lag time represented on the x-axis reflects the temporal shift in data files being correlated at each point in the crosscorrelation function. Crosscorrelations between IPN and HVI reveal that cerebellar areas are in phase in both T conditions, but that only the T+ group is modulated at 6-7 Hz theta periodicity.
Fig. 8. Serial sections diagrams depicting electrode tip placements (open circles) in CA1, IPN, and HVI. (A) hippocampus region CA1 (4-5 mm posterior to the skull landmark bregma), (B) IPN (0.5-1.5 mm anterior to skull landmark lambda), and (C) lobule HVI of lateral anterior cerebellar cortex (2-3.5 mm anterior to skull landmark Lambda). Abbreviations: crus II (crII), dentate nucleus (DE), dorsal paraflocculus (DPFL), fastigial nucleus (FA), fibers (f), Larsell’s hemispheric lobule 6 (HVI), Interpositus Nucleus (IPN), paramedian lobe (PM), ventral paraflocculus (VPFL).
Analysis of EP peaks 1 and 3. One-way analysis of between group differences on peak amplitude of EP peaks 1 and 3 (P1; P3), corresponding to the first phase of responses to CS and US, respectively, revealed a significant difference in amplitudes of P1 in IPN on day 3 (MT+=429.90, MT-=650.39, F(1,9)=5.63, p=0.042). No other significant differences for either peak on days 1-4 of acquisition for CA1, IPN or HVI were found (P1: CA1: D1: MT+=-1185.26, MT-=547.59, F(1,9)=4.53, p=0.062; D2: MT+=-1304.65, MT-=629.90, F(1,8)=5.20, p=0.052; D3: MT+=-896.01, MT-=660.10, F(1,9)=1.63, p=0.234; D4: MT+=-825.48, MT-=537.78, F(1,9)=2.80, p=0.129; IPN: D1: MT+=502.30, MT-=582.01, F(1,9)=0.45, p=0.518; D2: MT+=501.93, MT-=667.81, F(1,8)=2.32, p=0.166; D4: MT+=445.81, MT-=667.81, F(1,8)=2.32, p=0.166; P3: CA1: D1: MT+=-1507.84, MT-=868.60, F(1,9)=3.22, p=0.106; D2: MT+=-1701.20, MT-=983.08, F(1,8)=5.11, p=0.054; D3: MT+=-1287.78, MT-=722.66, F(1,9)=2.79, p=0.129; D4: MT+=-1348.82, MT-=829.92, F(1,9)=2.17, p=0.175; IPN: D1: MT+=576.11, MT-=471.68, F(1,9)=0.78, p=0.402; D2: MT+=570.76, MT-=983.08, F(1,8)=5.11, p=0.054; D3: MT+=521.09, MT-=461.61, F(1,9)=0.81, p=0.391; HVI: D1: MT+=518.95, MT+=817.28, F(1,8)=0.11, p=0.747; D2: MT+=128.57, MT+=271.32, F(1,8)=1.32, p=0.587; D3: MT+=557.74, MT+=820.24, F(1,9)=1.01, p=0.341; D4: MT+=580.34, MT+=819.38, F(1,9)=0.73, p=0.416).

Analysis of difference scores comparing amplitudes of EP peak 1 and 3. For CA1 and HVI, within-group difference scores, comparing P1 and P3 response amplitudes corresponding to the first phase of the evoked responses to CS and US, respectively, were not significant on days 1-4 (CA1: D1: MT+=322.58, MT+=321.01, F(1,9)=0.00, p=0.993; D2: MT+=396.55, MT+=353.18, F(1,8)=0.54, p=0.822; D3: MT+=391.76, MT+=62.55, F(1,9)=2.20, p=0.172; D4: MT+=523.34, MT+=291.15, F(1,9)=0.87, p=0.376; HVI: D1: MT+=218.02, MT+=290.53, F(1,8)=0.11, p=0.747; D2: MT+=128.57, MT+=271.32, F(1,8)=1.19, p=0.308; D3: MT+=63.01, MT+=35.05, F(1,9)=1.26, p=0.290; D4: MT+=35.05, MT+=134.46, F(1,9)=0.839, p=0.383).
**Analysis of CA1 EP peaks 2 and 4.** One-way analysis of between group differences in CA1 peak amplitude of peaks 2 and 4 (P2; P4), corresponding to the second phase of responses to CS and US, respectively, revealed no significant differences for either peak on days 1-4 of acquisition (P2: D1: $M_{T+}=414.97, M_{T-}=278.71, F(1,9)=0.08, p=0.783$; D2: $M_{T+}=840.37, M_{T-}=164.90, F(1,8)=2.83, p=0.131$; D3: $M_{T+}=722.37, M_{T-}=263.34, F(1,9)=2.14, p=0.177$; D4: $M_{T+}=782.38, M_{T-}=212.20, F(1,9)=3.04, p=0.115$; P4: D1: $M_{T+}=254.52, M_{T-}=112.78, F(1,9)=0.41, p=0.538$; D2: $M_{T+}=365.43, M_{T-}=174.67, F(1,8)=1.09, p=0.328$; D3: $M_{T+}=259.95, M_{T-}=87.54, F(1,9)=1.13, p=0.316$; D4: $M_{T+}=246.45, M_{T-}=175.95, F(1,9)=0.1, p=0.76$.

**Frequency analysis.** Group differences in percent power at 3 and 5 Hz during the whole trial period (500-2200ms) in CA1, IPN and HVI were not significant (3 Hz: CA1: D1: $M_{T+}=10.0\%, M_{T-}=10.5\%, F(1,9)=0.6, p=0.457$; D2: $M_{T+}=9.5\%, M_{T-}=6.4\%, F(1,8)=1.3, p=0.288$; D3: $M_{T+}=10.8\%, M_{T-}=9.8\%, F(1,8)=1.12, p=0.321$; D4: $M_{T+}=11.8\%, M_{T-}=6.9\%, F(1,9)=3.99, p=0.077$; IPN: D1: $M_{T+}=15.1\%, M_{T-}=17.9\%, F(1,9)=2.14, p=0.177$; D2: $M_{T+}=15.6\%, M_{T-}=17.4\%, F(1,8)=0.31, p=0.594$; D3: $M_{T+}=17.4\%, M_{T-}=16.5\%, F(1,8)=0.15, p=0.71$; D4: $M_{T+}=17.8\%, M_{T-}=13.5\%, F(1,9)=2.35, p=0.16$; HVI: D1: $M_{T+}=13.6\%, M_{T-}=20.6\%, F(1,8)=2.80, p=0.133$; D2: $M_{T+}=11.5\%, M_{T-}=15.8\%, F(1,8)=0.04, p=0.84$; D3: $M_{T+}=12.9\%, M_{T-}=19.0\%, F(1,8)=0.37, p=0.561$; D4: $M_{T+}=17.1\%, M_{T-}=15.4\%, F(1,9)=1.79, p=0.214$; 5 Hz: CA1: D1: $M_{T+}=4.5\%, M_{T-}=6.9\%, F(1,9)=0.0, p=0.993$; D2: $M_{T+}=6.4\%, M_{T-}=4.7\%, F(1,8)=4.0, p=0.81$; D3: $M_{T+}=6.0\%, M_{T-}=3.4\%, F(1,8)=1.84, p=0.212$; D4: $M_{T+}=6.0\%, M_{T-}=2.6\%, F(1,9)=2.93, p=0.121$; IPN: D1: $M_{T+}=3.2\%, M_{T-}=3.6\%, F(1,9)=3.95, p=0.078$; D2: $M_{T+}=6.6\%, M_{T-}=3.7\%, F(1,8)=0.29, p=0.608$; D3: $M_{T+}=5.7\%, M_{T-}=4.7\%, F(1,8)=0.00, p=0.958$; D4: $M_{T+}=4.0\%, M_{T-}=4.2\%, F(1,9)=0.67, p=0.436$; HVI: D1: $M_{T+}=2.8\%, M_{T-}=1.3\%, F(1,8)=2.01, p=0.195$; D2: $M_{T+}=4.6\%, M_{T-}=2.5\%, F(1,8)=0.00, p=0.956$; D3: $M_{T+}=4.4\%, M_{T-}=3.5\%, F(1,8)=0.01, p=0.938$; D4: $M_{T+}=2.9\%, M_{T-}=2.7\%, F(1,9)=0.40, p=0.545$.