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Differential Acetylcholine Release in the Prefrontal Cortex and Hippocampus During Pavlovian Trace and Delay Conditioning

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Abstract

Pavlovian trace conditioning critically depends on the medial prefrontal cortex (mPFC) and hippocampus (HPC), whereas delay conditioning does not depend on these brain structures. Given that the cholinergic basal forebrain system modulates activity in both the mPFC and HPC, it was reasoned that the level of acetylcholine (ACh) release in these regions would show distinct profiles during testing in trace and delay conditioning paradigms. To test this assumption, microdialysis probes were implanted unilaterally into the mPFC and HPC of rats that were pre-trained in appetitive trace and delay conditioning paradigms using different conditional stimuli in the two tasks. On the day of microdialysis testing, dialysate samples were collected during a quiet baseline interval before trials were initiated, and again during performance in separate blocks of trace and delay conditioning trials in each animal. ACh levels were quantified using high performance liquid chromatography and electrochemical detection techniques. Consistent with our hypothesis, results showed that ACh release in the mPFC was greater during trace conditioning than during delay conditioning. The level of ACh released during trace conditioning in the HPC was also greater than the levels observed during delay conditioning. While ACh efflux in both the mPFC and HPC selectively increased during trace conditioning, ACh levels in the mPFC during trace conditioning testing showed the greatest increases observed. These results demonstrate a dissociation in cholinergic activation of the mPFC and HPC during performance in trace but not delay appetitive conditioning, where this cholinergic activity may contribute to attentional mechanisms, adaptive response timing, or memory consolidation necessary for successful trace conditioning.

Keywords

microdialysis; attention; cholinergic system; nucleus basalis magnocellularis; medial septal area

Introduction

Pavlovian delay conditioning involves the contiguous pairing of a conditional stimulus (CS) and unconditional stimulus (US), such that the CS precedes and partly overlaps the US. In contrast, during Pavlovian trace conditioning, the CS and US are separated in time so that a stimulus-free trace interval (TI) occurs between CS offset and US onset. Delay conditioning

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critically depends on the cerebellum and brainstem (Sacchetti, Scelfo, & Strata, 2005, 2009; Supple & Leaton, 1990; Timmann et al., 2010), with the additional involvement of the amygdala in the case of fear conditioning (for review, see Fanselow & LeDoux, 1999). In contrast, trace conditioning critically depends on hippocampal (e.g., Esclassan, Coutureau, Di Scala, & Marchand, 2009) and medial prefrontal (e.g., McLaughlin, Skaggs, Churchwell, & Powell, 2002) systems, in addition to cerebellar and brainstem circuitry (Christian & Thompson, 2003; Clark, 2011; Clark, Manns, & Squire 2001, 2002; Thompson & Kim, 1996; Thompson & Steinmetz, 2009; Woodruff-Pak & Disterhoft, 2008). Although the hippocampus (HPC) and medial prefrontal cortex (mPFC) are critical for trace conditioning, neither region is necessary for the acquisition, retention, or performance in delay conditioning (for review, see Christian & Thompson, 2003; Clark, 2011).

In addition to these dissociations in the neuroanatomical substrates for trace and delay conditioning, these two types of associative learning depend on distinct cognitive processes. Evidence from both human and non-human animals suggests that delay conditioning depends on non-declarative or implicit memory, and is independent from awareness, while trace conditioning depends on declarative or explicit memory, and is critically dependent upon awareness of stimulus contingencies (see Clark, 2011; Clark et al., 2001, 2002; Manns, Clark, & Squire, 2002; Woodruff-Pak & Disterhoft, 2008). Additionally, trace conditioning depends on mechanisms of attention (see Han et al., 2003), where cortical cholinergic transmission to the mPFC plays a critical role in supporting attention (Kozak, Bruno, & Sarter, 2006; Passetti, Dalley, O'Connell, Everitt, & Robbins, 2000; Sarter, Gehring, & Kozak, 2006; Sarter, Hasselmo, Bruno, & Givens, 2005). Trace conditioning therefore depends on the interplay between hippocampal, medial prefrontal, and cholinergic systems, where the nature of this interplay evolves across time from the moment of initial learning to post-conditioning consolidation.

The HPC is necessary for initial acquisition and early consolidation of trace conditioning. For example, lesions of the HPC cause impairments in the acquisition of trace eye blink conditioning (Green, & Woodruff-Pak, 2000; Ivkovich & Stanton, 2001; Weiss, Bouwmeester, Power, & Disterhoft, 1999) and trace conditioned fear paradigms (Bangasser, Waxler, Santollo, & Shors, 2006; Fendt, Fanselow, & Koch, 2005; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; Yoon & Otto, 2007). Although the HPC is critical for trace conditioning acquisition, hippocampal involvement diminishes with the progressive consolidation of memory for the trace conditioning experience. For example, trace eye blink conditioning is disrupted by HPC lesions made 1 day after conditioning, but lesions have no effect when made 1 month after conditioning (Kim, Clark, & Thompson, 1995). Similarly, trace fear conditioning is impaired when the HPC is damaged early in the consolidation process, while performance is spared when HPC damage occurs after a sufficient consolidation period (Quinn, Ma, Tinsley, Koch, & Fanselow, 2008). As memory for trace conditioning is consolidated, the hippocampal system becomes progressively less involved and the mPFC emerges as the key structure supporting trace conditioning memory storage and retrieval (Clark, 2011; Quinn et al., 2008).

The majority of research on hippocampal involvement in trace conditioning has focused on aversive conditioning (e.g., eye blink or fear conditioning), there is some evidence for HPC involvement in appetitive trace conditioning (e.g., conditioned approach to food or sucrose, or conditioned licking or jaw movement). For example, rabbits show a learning-related increase in neurophysiological activity in the HPC that is related to the acquisition of a conditioned jaw movement during training in an appetitive trace conditioning paradigm where a tone CS was paired with water presentation following a 450 ms TI (Seager, Borgnis, & Berry, 1997). Additionally, systemic infusion of the cholinergic antagonist scopolamine reduced the magnitude of these conditioning-related hippocampal responses and caused

acquisition impairments in this form of appetitive trace conditioning (Seager, Asaka, & Berry, 1999). In contrast to these findings, others have reported that hippocampal lesions spare conditioned lick response acquisition in an appetitive trace conditioning paradigm where a 2s TI separated a tone CS from a highly palatable liquid US (Thibaudeau, Doré, & Goulet, 2009; Thibaudeau, Potvin, Allen, Doré, & Goulet, 2007; see also Kyd, Pearce, Haselgrove, Amin, & Aggleton, 2007).

The discrepancies between conditions where HPC lesions disrupt or fail to disrupt appetitive trace conditioning may be due to a number of specific factors relating to the nature of the CS and US involved, the behavior being measured, as well as the relative duration of the TI. Indeed, the critical role that the TI plays in determining whether trace conditioning impairments are observed has been demonstrated in tests of aversive conditioning. For example, dorsal hippocampus lesions impair trace fear conditioning performance when the CS and US are separated by a trace interval of 20 s, but spare performance when the trace interval is only 1 s or 3s in duration (Chowdury, Quinn, & Fanselow, 2005). Therefore, it appears that the HPC can play an important role in appetitive as well as aversive trace conditioning, provided that the TI employed is sufficiently demanding given the specific parameters of the conditioning task (see also Berger, Berry, & Thompson, 1986).

In addition to the HPC, the mPFC has been implicated in the acquisition (Kronforst-Collins & Disterhoft, 1998; Runyan, Moore, Dash, 2004; Takehara-Nishiuchi, Kawahara, & Kirino, 2005; Weible, McEchron, & Disterhoft, 2000) consolidation (Takehara-Nishiuchi, Nakao, Kawahara, Matsuiki, & Kirino, 2006) and memory retrieval (Oswald, Maddox, & Powell, 2008) in trace, but not delay, conditioning. A wealth of data support consolidation models that suggest that memories, including memory for trace conditioning, are initially stored in HPC and later are consolidated in the mPFC (see Clark, 2011; Dash, Hebert, & Runyan, 2004). While the mPFC becomes critical for trace conditioning only after a consolidation period, evidence suggests the mPFC is active throughout the acquisition and post-acquisition periods. For example, mPFC lesions made as early as one day to as much as one month following initial acquisition impairs trace conditioning performance, suggesting that the mPFC plays an important role well before memory consolidation is complete (Simon, Knuckley, Churchwell, & Powell, 2005). Similarly, infusion of the gamma-amino butyric acid type A (GABA_A) receptor agonist muscimol into mPFC disrupts trace fear conditioning whether the infusion is made early or late in training (Blum, Herbert, & Dash, 2006; see also Gilmartin & Helmstetter, 2010). Finally, electrophysiological evidence shows a progressive increase in mPFC activity corresponding to the acquisition of an eye blink CR in a trace conditioning paradigm, where mPFC neurons exhibited progressively more selective and sustained activity during the TI (Takehara-Nishiuchi & McNaughton, 2008). Together, these findings demonstrate a continued involvement of the mPFC in trace conditioning, where mPFC activity becomes increasingly critical as animals move from the acquisition to consolidation stage.

The HPC and mPFC are strongly influenced by cholinergic modulation by the medial septum (MS) and the nucleus basalis magnocellularis (NBM), respectively (see McKinney, Coyle, & Hedreen, 1983). Consequently, it is not surprising that manipulations of the cholinergic system have a significant effect on trace conditioning without impacting delay conditioning. For example, we have previously shown that combined MS and NBM lesions, which deplete both hippocampal and cortical ACh, impair acquisition in appetitive trace, but not delay conditioning (Butt et al., 2007). Pharmacological studies similarly reveal the critical involvement of the cholinergic system in trace conditioning. For example, systemic injections of the cholinergic antagonist scopolamine impair acquisition of trace conditioning but do not disrupt delay conditioning (Hunt & Richardson, 2007; Kaneko & Thompson, 1997; Seager et al., 1999). In contrast to the effects of cholinergic antagonists, systemic

administration of cholinergic agonists can facilitate trace conditioning in intact animals and can also ameliorate trace conditioning impairments otherwise observed in aged animals (Disterhoft et al., 1999; Disterhoft & Oh, 2003).

In addition to lesions or drug treatments that affect the entire basal forebrain, manipulations of either these pto-hippocampal orbaso-cortical projection system of the basal forebrain alone can disrupt trace conditioning while sparing delay conditioning. For example, immunotoxiclesions of the cholinergic neurons of the medial septum projections to the HPC (Fontán-Lozano, Troncoso, Múnera, Carrión, & Delgado-Garcia,2005) as well as micro-infusion of scopolamine directly into the HPC (Pang et al., 2010) disrupt trace eye blink conditioning. Similarly, Raybuck and Gould (2010) report that infusion of cholinergic antagonistsdihydro-beta-erythroidine (DHβE) or methyllycaconitine (MLA) into the dorsal HPC disrupt acquisition of trace fear conditioning while sparing delay fear conditioning. Dorsal hippocampal infusions of the cholinergic agonist nicotine, on the other hand, enhance acquisition in trace fear conditioning. Raybuck and Gould (2010) show that post-acquisition trace conditioning performanceis not affected by either cholinergic antagonist or agonist infusions into dorsal HPC. In contrast to these cholinergic effects on trace conditioning, the infusion of cholinergic drugs into the dorsal HPC had no effect on delay conditioning. Collectively, these findings suggest a critical role for cholinergic activity in dorsal HPC, especially during acquisition of trace fear conditioning.

Cholinergic involvement in trace fear conditioning is demonstrated not only in the HPC but also in the mPFC. For example, nicotine infusion into the mPFC enhances the acquisition of trace fear conditioning, while the infusion of the cholinergic antagonists DHβE and MLA impairs post-acquisition performance (Raybuck & Gould, 2010). Additionally, nicotine infusion into the mPFC had no impact on delay conditioning. Based on these findings, the authors conclude that cholinergic activation of the mPFC plays a critical role in trace but not delay conditioning, perhaps by contributing to the long-term storage of memory necessary for recall or performance in trace conditioning paradigms more than it contributes to early acquisition (Raybuck & Gould, 2010; see also Gilmartin & Helmstetter, 2010; Quinn et al., 2008; Runyan et al., 2004).

Although the cholinergic system has been clearly implicated in trace conditioning, the nature of endogenous ACh release in the HPC and mPFC during this type of conditioning has not yet been shown. The goal of the current experiment was therefore to explore potential differences in the pattern of ACh release in these brain are as during post-acquisition performance in trace and delay conditioning. First, rats were trained concomitantly in both trace and delay appetitive conditioning paradigms, where an auditory or visual CS signaled the delivery of a sucrose pellet (US) into a recessed food-cup. ACh levels in the HPC and mPFC were measured during a quiet baseline period and during subsequent performance in both conditioning tasks. Based on evidence of mPFC involvement in trace but not delay conditioning (e.g., Raybuck & Gould, 2010; Simon et al., 2005; Takehara-Nishiuchi et al., 2006) and on findings of increased ACh release in mPFC during attention-dependent task performance (e.g., Himmelheber, Sarter, & Bruno, 2001; Sarter et al., 2006; Sarter et al., 2005) it was predicted that ACh efflux would be greater in the mPFC during performance in trace conditioning as compared to delay conditioning. Additionally, based on the finding that hippocampal lesions made after initial acquisition have minimal effects on post-acquisition trace conditioning performance (Kim et al., 1995; Quinn et al., 2008), it was hypothesized that ACh efflux in the HPC during performance in the trace conditioning task would be no greater than in the delay conditioning task.

Methods

Guidelines for animal use

Subjects were cared for according to the requirements set by the Society for Neuroscience, the American Psychological Association, the California State University, San Bernardino Animal Care and Use Committee.

Animals

Subjects were 13 male Long-Evans rats (Harlan, Indianapolis, IN) weighing 300–350 g. upon arrival. Animals were housed in an environment maintained at 21–23 °C with *ad libitum* water on a 12-hour reverse light cycle (lights off 06:00 h). Beginning one week prior to training, animals were reduced to and maintained at 85% of their normal body weight for the duration of the experiment.

Apparatus

Training and testing was conducted in individual computer-controlled, sound-attenuating operant chambers (Coulbourn Instruments, Allentown, PA) equipped with a speaker for presenting white noise (80 dB) and equipped with a light located over the food magazine. US presentations consisted of the delivery of a single sucrose pellet (45 mg; MedAssociates, Lancaster, NH) into a food magazine (MedAssociates, Lancaster, NH) located at floor level. Snout entries into the food magazine were recorded using photo-beam response detectors (MedAssociates, Lancaster, NH) located at the entrance to the recessed food-cup. A 1 W white light located at the top of the chamber provided ambient illumination. The presentation of the white noise CS, light CS, and the delivery of the sucrose pellet US was controlled via computer interface (WINLINC, Coulbourn Instruments, Allentown, PA). The operant chambers had aluminum walls and ceiling, and Plexiglas door, with a 5-channel, motorized microdialysis liquid swivel (Instech Laboratories, Plymouth Meeting, PA) positioned at the top of the chamber. A small infusion pump (Bioanalytical Systems Inc, West Lafayette, IN), located outside the sound-attenuating chamber housing each unit, was connected via micro-tubing to the liquid swivel.

Behavioral procedures

Prior to training, animals were habituated to the testing environment, where they were allowed access to 10 sucrose pellets placed in the food-cup magazine. Food-deprived animals were connected to the tether and placed in the testing chamber for 1 h, during which they consumed the available sucrose pellets. The following day, animals began a 15-day pre-training phase where they underwent daily testing in a combined trace and delay appetitive conditioning protocol. Each daily session began with a 45 min stimulus-free baseline period to allow acclimatization to the test chamber. Next, animals were trained across two successive blocks of 30 trials of a given trial type (i.e., trace or the delay conditioning trials). The sequence of testing in the two types of trials determined pseudo-randomly such that each sequence of trial types (i.e., trace-then-delay, or delay-then-trace) was experienced on the same number of days.

The US was signaled by either a 10 s white noise CS or a 10 s light discriminative CS, depending on whether animals were undergoing training in the trace or delay conditioning paradigm. Animals were randomly assigned to training conditions where the white noise CS signaled the US in the trace conditioning paradigm and the light CS signaled the US in the delay conditioning paradigm, or vice-versa. The assignments of each CS type to given conditioning paradigm were counter balanced to control for potential differences in conditioning to the two stimulus types in the two conditioning tasks.

In the trace conditioning paradigm, the designated CS (i.e., white noise or light) was followed after a 10 s trace interval (TI) by a sucrose pellet US, with an average inter-trial interval (ITI) of 40 s (range 20 – 60 s). In the delay conditioning paradigm, the designated CS was followed immediately by a sucrose pellet US, with an average ITI of 50 s (range 40 – 70 s). In order to ensure habituation to the microdialysis tether prior to microdialysis testing, animals were tethered during each testing session.

Surgery

Surgery was conducted upon completion of the pre-training phase of trace and delay conditioning. Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Sigma Aldrich, St. Louis, MO) and surgically implanted with two microdialysis probe guides fitted with sterile, stainless steel temporary stylets (MAB 4 series, SciPro, Inc., Sanborn, NY) with the tips of the probe guides positioned immediately above the right HPC target (AP: -5.8, ML: +5.0, DV: -2.5 from dura; see Pych, Chang, Colon-Rivera & Gold, 2005) and immediately above the right PFC target (AP: +2.7, ML: +0.8, DV: -2.5 from dura; Paxinos & Watson, 2004). Upon completion of surgery, animals received injections of analgesic (Ketaprofin, 2 mg/kg, s.c.; Western Medical Inc, Anaheim, CA) to control pain, and antibiotic (Baytril, 2.5 mg/kg s.c.; Western Medical Inc, Anaheim, CA) to prevent infection. Animals were finally returned to their home cages and allowed 36 hrs for recovery prior to initiating microdialysis testing.

Microdialysis

Thirty-six hours after recovery from surgery, animals underwent one day of dual-probe microdialysis sampling during behavioral testing in the trace and delay conditioning tasks. Prior to being placed in the testing chamber, rats were tethered and their stainless steel guide stylets were removed to allow insertion of a 2 mm microdialysis probe (Model 2.14.2 PES, SciPro Inc., Sanborn, NJ; shaft length 14 mm, active probe membrane length 2 mm, outer diameter 0.6 mm) into the implanted mPFC probe guide, and insertion of a 3 mm microdialysis probe (Model 2.14.3 PES, SciPro Inc., Sanborn, NJ; shaft length 14 mm, active probe membrane length 3 mm, outer diameter 0.6 mm) into the implanted HPC probe guide. Both probes were continuously perfused (0.5 µl/min) with artificial cerebrospinal fluid (aCSF) containing 50 nM of the acetylcholinesterase inhibitor neostigmine bromide (148 mMNaCl, 4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 50 nMneostigmine bromide; pH 6.8). The necessity of using neostigmine bromide was demonstrated in pilot experiments, where ACh efflux levels were found to be below the threshold for reliable detection using existing HPLC and electrochemical detection methods. This finding was consistent with data reported by Chang, Savage, and Gold (2006), who also demonstrated the need to include acetylcholinesterase inhibitor in the aCSF in order to consistently detect basal ACh levels. Unpublished observations from our lab show that neostigmine bromide had no statistically significant effects on behavior in either the delay or trace conditioning tasks.

Unlike pre-training procedures, rats underwent a 3 hr equilibration period in the testing chamber before beginning behavioral testing and microdialysis sampling in order to stabilize neurotransmitter release. This equilibration period was followed by another 1 hr baseline period, where microdialysis samples were collected for comparison to ACh samples taken during behavioral testing. At the end of the 1 hr baseline period, the delay and trace conditioning procedure was initiated. To reduce the likelihood of neurotransmitter carryover from one 30 min conditioning protocol to the next (i.e., between trace and delay conditioning trial blocks), a 1 hr stimulus-free washout period was introduced between the two blocks of conditioning trials. Dialysate samples were collected every 30 min throughout the baseline and testing phases, and during the washout phase. Samples were immediately frozen and stored for later quantification of ACh levels.

Acetylcholine assay and quantification procedures

Quantification of ACh levels was accomplished using high-performance liquid chromatography with electrochemical detection (Coulechem II; ESA Inc., Chelmsford, MA). A pre-column choline oxidase enzymatic reactor (model MF 6149; Bioanalytical Systems Inc., Lafayette, IN) was used to oxidize choline in the dialysate sample prior to separation of choline and ACh by a microbore column (model 6150; Bioanalytical Systems Inc., Lafayette, IN) using a sodium phosphate mobile phase. Post-column hydrolysis of ACh was achieved using an enzymatic reactor containing covalently-bound acetylcholinesterase and choline oxidase (model 6151; Bioanalytical Systems Inc., Lafayette, IN); ACh was hydrolyzed to acetate and choline, with choline subsequently oxidized to hydrogen peroxide (H₂O₂) and betaine. Electrochemical detection of the resulting H₂O₂ was achieved using a peroxidase-wired glassy carbon electrode (model MF-2095; Bioanalytical Systems Inc., Lafayette, IN) and analytical cell (model 5041; ESA Inc., Chelmsford, MA) set at a potential of -200 mV with a gain of 500 pA.

The absolute level of ACh in each sample was quantified by integrating the area under the ACh peak on the HPLC chromatogram and comparing it to a standard curve (based on the area for a range of known standard values of ACh). ACh release was then expressed as a percentage of baseline release as determined by dividing testing-induced peak area by the mean baseline peak area for the two 30-min baseline samples collected prior to each conditioning protocol.

Statistical Analyses

The duration of time spent with the snout in the recessed food-cup magazine was measured during the 10 s before each CS presentation (pre-CS baseline response period), during each CS presentation (10 s), and in the trace conditioning task, during the TI (10 s). Each of these 10 s intervals was comprised of five 2-s bins of data for duration of snout entry into the magazine. This raw data, summed across each of the 30 conditioning trials in either the trace or delay conditioning paradigms, was first used to calculate discrimination ratios designed to distinguish those animals that acquired a reliable CR in each task from those that failed to develop a CR in either or both tasks as described below.

Acquisition criterion performance

Animals were first divided into “learning” and “non-learning groups depending on whether they met an *a priori* performance criteria; animals with a conditioning ratio ≥ 0.60 in both the trace and delay tasks were placed in the learning group, while those with ratios < 0.60 in either trace or delay tasks were placed in the non-learning group. Rather than assessing cumulative acquisition performance, criterion discrimination ratios were based on performance from the last three days of the experiment (i.e., pre-training days 14 and 15, and the microdialysis test day). The discrimination ratio for the delay conditioning task was calculated as follows: [mean duration of CS responses $\div$ (mean duration of CS responses + mean duration of pre-stimulus responding)]. The discrimination ratio for the trace conditioning task was calculated in a similar manner, but rather than assessing relative responding during the CS, this measure assessed relative responding during the TI as follows: [mean duration of TI responses $\div$ (mean duration of TI responses + mean duration of pre-stimulus responding)].

These discrimination ratio criteria represent an increase of 50% or more in CS or TI conditioned responding relative to pre-CS baseline responding in each task in the delay and trace conditioning tasks, respectively. Animals with proportionately greater responding during the CS in delay conditioning and during the TI in trace conditioning were included in the learning group, regardless of how precisely the CR was timed with respect to the timing

of US delivery. Thus, rather than being placed in the non-learning group as a result of having relatively poor CR timing, animals that failed to meet criteria were classified as non-learners because they failed to show differential responding in the delay and trace conditioning tasks in general. Behavioral data for those animals retained in the learning group were subjected to a more detailed analysis of the response profile in each conditioning task (see Measures of adaptive responding below).

Measures of adaptive conditioned responding

The pattern of responding in the appetitive trace and delay conditioning paradigms used here differs substantially, with animals showing less responding during the CS and early part of the TI in the trace conditioning task than during the latter part of the TI, with the greatest level of responding occurring immediately before US presentation (unpublished observations). In delay conditioning, responding was characterized by progressively greater levels of food-cup approach across the duration of the CS, reaching a maximum just before US presentation. In order to capture the precisely timed, adaptive nature of the CR in the trace and delay conditioning tasks, we calculated difference scores on data from each task as follows. Trace conditioning CR difference scores were derived by subtracting the cumulative duration of food-cup entry during the last 4 s of the TI across each of the 30 trace conditioning trials presented each test day (120 s total TI duration) from the mean duration of responding per 120 s of the pre-CS baseline interval. Greater TI difference scores reflect systematically greater levels of food-cup approach during the last 4 s of the TI relative to the average duration of baseline food-cup approach. Delay CR difference scores were determined similarly, with the cumulative duration of food-cup entry during the last 4 s of the CS across trials subtracted from the mean duration of responding during an equivalent pre-CS baseline interval. Here, greater CS difference scores reflect systematically greater levels of food-cup approach during the last 4 s of the CS relative to the average duration of baseline food-cup approach. TI difference scores from the trace conditioning task and CS difference scores from the delay conditioning task were analyzed using separate within-subjects one-way analyses of variance (ANOVA).

Effects of auditory vs. visual conditioned stimuli

Potential differences in responding to the auditory and visual conditioned stimuli used in a given task were assessed using 2-tailed Student's *t*-tests. Comparisons were made on the trace conditioning CR measure (i.e., TI difference scores) in animals trained using a visual CS ($n = 3$) and those trained with an auditory CS ($n = 4$). Additional comparisons were made on the delay conditioning CR measure (i.e., CS difference scores) for the complementary groups. Similar comparisons were made on the level of ACh release in the HPC or mPFC during trace and delay conditioning to test for potential cue-dependent differences between animals assigned to one stimulus type/task combination or the other.

Biochemistry analyses

Levels of ACh released during testing, expressed as a percent of baseline release were analyzed via two-way (Conditioning Task $\times$ Brain Region) within-subjects ANOVA. Specific planned comparisons of ACh levels (percent of baseline) in each task, and comparisons of absolute levels of testing-induced ACh release and baseline ACh release were conducted using paired *t*-tests.

Histology

Upon completion of behavioral testing, rats were killed by a lethal dose of sodium pentobarbital (80 mg/kg, i.p.; Sigma, St. Louis, MO) followed by cardiac perfusion with 0.9% saline for 5 min and 10% formalin for 30 min. Brains were extracted and placed a 25%

sucrose solution for 48 hrs prior to freezing and sectioning. Sections (60 μ m) were stained with thionin and examined to verify probe placements.

Results

Probe Placement Verification

For verification of placement of microdialysis probes, brains were sectioned and stained. All probe placements were determined to be within the boundaries of the mPFC and HPC, respectively. As illustrated in Figure 1, the mPFC probes were located within the prelimbic cortex and cingulate cortex, while hippocampal probes occupied the CA1, CA2, and CA3 sub-regions of the hippocampal formation.

Behavioral Results

Acquisition criterion performance—Seven animals demonstrated adequate learning as defined by a conditioning ratio ≥ 0.60 for both conditioning tasks, while six animals failed to meet criteria for inclusion in the learning group. Animals in the learning group ($n = 7$) had an average trace conditioning ratio of 0.69 and an average delay conditioning ratio of 0.68, where as animals in the non-learning group ($n = 6$) had an average trace conditioning ratio of 0.55 and an average delay conditioning ratio of 0.63. Subsequent analyses of data from those animals retained in the learning group were conducted as described below, while no further statistical analysis was performed on behavioral data from animals in the non-learning group.

Effects of auditory vs. visual conditioned stimuli—Student's t -tests were used to test for potential differences in the response to the visual and auditory CS stimuli used in the two conditioning tasks. These tests showed that there were no differences in the trace conditioning CR measure (i.e., difference score) in animals trained in that task using a visual CS ($n = 3$) and those trained with an auditory CS ($n = 4$). Cue-dependent differences in the delay conditioning CR measure, however, did occur in these two groups. Rats trained in the delay conditioning task using the auditory CS showed greater levels of conditioned responding than those trained with the visual CS ($t(5) = 3.24, p < .05$). This difference likely reflects the fact that visual cues predicting food trigger conditioned rearing, which would momentarily preclude food-cup approach, whereas auditory cues typically cause a conditioned head jerk, which would interfere less with food-cup approach (see Holland, 1980). Despite these cue-dependent differences in the level of conditioned responding in the delay conditioning task, analogous t -tests conducted on the neurochemistry results showed that there were no cue-dependent differences in the level of testing-induced ACh efflux in either the HPC or mPFC during either trace or delay conditioning.

CR response difference scores—Visual analysis of the pattern of behavioral responding demonstrates that rats developed adaptively timed conditioned responding in both behavioral tasks (see also Galvez, Weible, & Disterhoft, 2007; Moore, Desmond, & Berthier, 1989), consistent with our earlier observations (see Figure 2). By the final day of pre-training, animals in the learning group showed comparatively little responding during the CS or during the early part of the TI in the trace conditioning task. Instead, the duration of food-cup approach increased progressively across the CS interval and the TI, reaching a maximum just before US presentation. This pattern of responding reflects the adaptive timing of the CR to coincide with US delivery, while non-adaptive premature responding during the CS and early TI occurred at much lower levels. In delay conditioning, adaptive responding was characterized by progressively greater levels of food-cup approach across the duration of the CS, reaching a maximum just before US presentation. Adaptive

responding was quantified in terms of TI difference scores in the trace conditioning task, and CS difference scores in the delay conditioning task as described above.

CR difference scores in the trace conditioning task increased across pre-training and testing days; ANOVA yielded a significant day main effect, $F(15, 90) = 5.18, p < .001$. An increase in CR difference scores also occurred across days in the delay conditioning task; ANOVA yielded a significant day main effect, $F(15, 90) = 5.29, p < .001$. There were no significant mean differences in performance between the day of microdialysis and the final day of pre-training for either the trace ($t(6) = 1.96, p > .05$) or delay ($t(6) = 2.06, p > .05$) conditioning task. Collectively, these findings therefore confirm that animals in the learning group showed statistically reliable acquisition in both the trace and delay conditioning tasks, and that post-operative performance did not differ from pre-operative performance in either task (see Figure 3).

Neurochemistry Results

Comparisons of percent of baseline ACh release in mPFC and HPC during trace and delay conditioning (see Figure 4) revealed a significant main effect of both task and brain region; ANOVA yielded a task effect of $F(1, 6) = 8.29, p < .05$ and a brain region effect of $F(1, 6) = 6.96, p < .05$. Collapsed across brain regions, ACh efflux was greater during trace conditioning ($m = 159\%$, $sd = 0.41$) than during delay conditioning ($m = 103\%$, $sd = 0.47$). Collapsed across task conditions, ACh efflux was greater in the mPFC ($m = 149\%$, $sd = 0.53$) than the HPC ($m = 113\%$, $sd = 0.35$). The interaction between brain region and task condition was not statistically significant; $F(1, 6) = 4.47, p > .05$.

Comparisons of the absolute level of testing-induced ACh efflux to baseline efflux for each brain region during each task revealed that during trace conditioning the mPFC had a significant increase in testing induced ACh release over baseline release; a two-tailed paired samples t -test yielded $t(6) = 3.05, p < .05$. During trace conditioning, the HPC did not show a significant increase in testing induced ACh release over baseline release when analyzed by two-tailed t -test. However, a one-tailed paired-samples t -test (where potential differences in testing-induced and basal ACh levels would reasonable be expected to show testing-induced increases rather than decreases) yielded a significant difference between testing and baseline levels of ACh; $t(6) = 2.21, p < .05$. In contrast, during delay conditioning testing induced ACh release in mPFC was not greater than baseline release; a two-tailed paired-samples t -test yielded $t(6) = 0.40, p = .35$. Also, during delay conditioning the HPC did not have a significant increase in testing induced ACh release over baseline release; a two-tailed paired-samples t -test yielded $t(6) = -0.44, p = .34$.

Comparisons of percent of baseline ACh release in mPFC and HPC during trace and delay conditioning in the non-learning group (see Figure 5) revealed no significant main effect of either task ($F(1, 5) = 0.20, p > .05$) or brain region ($F(1, 5) = 0.27, p > .05$) and no interaction ($F(1, 5) = 0.13, p > .05$).

Discussion

The goal of the current experiment was to examine the pattern of ACh release in the HPC and mPFC during performance in trace and delay conditioning paradigms. As hypothesized, the level of ACh release in the mPFC was greater during trace than during delay conditioning. Prefrontal ACh levels during trace conditioning were also greater than hippocampal levels in both tasks. In addition to the elevated levels of ACh observed in the mPFC, hippocampal ACh levels were also greater during trace conditioning than during delay conditioning. The observed differences in ACh release were derived from comparisons of the proportion of testing-induced ACh release relative to baseline ACh

release within each brain region. Further comparisons of the absolute levels of baseline and testing-induced ACh release in the mPFC and HPC during trace or delay conditioning corroborate our initial findings; absolute levels of testing-induced ACh release during trace conditioning were greater than baseline levels in both the mPFC and HPC, whereas the level of testing-induced and baseline ACh release did not differ in the delay conditioning task. Collectively, these results demonstrate differential cholinergic activation of the mPFC and HPC during performance in appetitive trace conditioning. We suggest that the increase in ACh release observed during trace conditioning in this experiment may contribute to attentional mechanisms, adaptive response timing, or memory consolidation.

The differential, task-dependent pattern of ACh efflux was observed only in animals that reached acquisition performance criteria during initial training. In those rats that failed to reach performance criteria, there was instead a generalized, task-independent increase in ACh release in both the HPC and mPFC. This pattern of ACh release in the rats that failed to learn the trace conditioning may reflect non-associative factors such as auditory or visual stimulation caused by CS presentations, gustatory stimulation in response to sucrose, or behavioral arousal associated with access to sucrose pellets in the testing environment (see Acquas, Wilson, & Fibiger, 1996; Inglis, Day, & Fibiger, 1994; Rasmussen, Smith, & Semba, 2007). The difference in the pattern of ACh release in the mPFC and HPC observed in those animals that met acquisition criteria and those that did not provides strong support for the argument that the differential pattern of ACh release observed in animals that successfully acquired the conditioning tasks reflects mnemonic processes specific to the trace conditioning paradigm. Collectively, results demonstrate that performance in trace conditioning is selectively associated with cholinergic activation of the HPC and to a greater extent the mPFC, while performance in delay conditioning does not cause an increase in ACh release in either brain region.

Based on previous findings that the HPC is not necessary for post-consolidation performance of trace conditioning (e.g., Kim et al., 1995; Quinn et al., 2008) and with the assumption that extended training would have allowed sufficient time for consolidation in the current experiment, we did not expect ACh levels in the HPC to increase during performance in the trace conditioning task. However, hippocampal ACh levels showed task-specific increases during trace conditioning performance, when ACh levels were significantly greater than basal levels of ACh release and were greater than ACh levels in the delay conditioning task. These results are consistent with several findings suggesting that the HPC plays an important role in the acquisition (Bangasser et al., 2006; Esclassan et al., 2009; Fendt et al., 2005; Misane et al., 2005; Yoon & Otto, 2007) and early consolidation of trace conditioning (Burman & Gewirtz, 2007; Kim et al., 1995; Quinn et al., 2008). Indeed, the elevated levels of ACh release in the HPC observed during trace conditioning in the current experiment may reflect continued acquisition or consolidation of memory for the trace conditioning experience. It is possible that the added difficulty of acquiring both the trace and delay conditioning tasks concurrently placed considerable demands on hippocampal memory formation and consolidation, such that cholinergic activity in the HPC persisted well into the training period.

In addition to its role in memory formation and consolidation in trace conditioning, the HPC has also been implicated in the inhibition of maladaptive behaviors (see Cho & Jeantet, 2009; Davidson & Jarrard, 2004; McNaughton & Wickens, 2003). In the current experiment, cholinergic modulation of the HPC may have contributed to the inhibition of non-adaptive responding during the CS and during the early part of the TI in the appetitive trace conditioning task. A role for hippocampal involvement in controlling adaptive responding in trace conditioning is further suggested by the finding that knock-out mice lacking the NMDA receptor subunit of the CA3 pyramidal cells in the HPC show significant

disruptions in the timing of the CR during trace eye blink acquisition (Kishimoto, Nakazawa, Tonegawa, Kirino, & Kano, 2006). Thus, while cholinergic activity in the HPC may serve primarily to consolidate memory for trace conditioning, elevated levels of hippocampal ACh may have also contributed to the inhibition of non-adaptive responding in the appetitive trace conditioning paradigm used here.

Task-dependent differences in ACh levels also occurred in the mPFC, where dramatic increases in ACh release were observed during trace conditioning but not delay conditioning performance. These findings are consistent with the argument that cholinergic modulation of the mPFC plays a critical role in mediating attention (e.g., Kozak et al., 2006; Passetti et al., 2000). We suggest that the attention-dependent nature of the trace conditioning paradigm (see Han et al., 2003) is reflected in the persistent elevation in ACh release observed in the mPFC in animals engaged in the trace conditioning task, while the attention-independent nature of the delay conditioning task is characterized by an absence of cholinergic activation in the mPFC. Attention to CS on set or off set may be critical for the adaptive timing of the conditioned approach to the food-cup observed in the appetitive trace conditioning task used here (see also Galvez et al., 2007). Indeed, the failure to attend to these stimulus parameters would result in poorly timed conditioned responses and may even result in the failure to acquire the CS-US association in trace conditioning.

The view that the mPFC participates in attending to the CS, and that it maintains attention during the TI in the trace conditioning paradigm is consistent with electrophysiological evidence showing that mPFC neurons in trained animals fire persistently throughout the TI in trace eye blink conditioning (Takehara-Nishiuchi & McNaughton, 2008). This prefrontal activity plays a critical role in the development of the trace eye blink CR by signaling the cerebellum via the lateral pontine nuclei (see Kalmbach, Ohyama, Kreider, Riusech, & Mauk, 2009; Kalmbach, Ohyama, & Mauk, 2010; Weible et al., 2000). In delay eye blink conditioning, associative learning takes place in the cerebellum in response to overlapping input from the mossy fibers activated by an auditory CS and from climbing fibers activated by the aversive US (Kalmbach et al., 2009). The challenge of trace conditioning, however, is that the CS input to cerebellum does not persist long enough to be effectively paired with the US signal. Recent evidence shows that mPFC inputs onto a distinct population of mossy fibers in the cerebellum are activated by the CS and remain active throughout the TI; this prefrontal input effectively substitutes for the direct CS input by overlapping with the US signal to the cerebellum to produce associative learning in a manner analogous to learning in delay conditioning (see Kalmbach et al., 2009, 2010). The finding of a persistent mPFC signal to cerebellum during the TI (e.g., Kalmbach et al., 2009, 2010; Takehara-Nishiuchi & McNaughton, 2008), along with the current finding of a task dependent increase in cholinergic transmission to the mPFC, suggests that the cortically-projecting cholinergic NBM may critically contribute to trace conditioning by driving the mPFC during the TI. Cholinergic activation of the mPFC might in turn increase prefrontal output to the lateral pontine nuclei, which ultimately drive cerebellar mossy fibers that remain active until the US input arrives in the cerebellum. While eye blink conditioning is clearly dependent upon the cerebellum (for review, see Christian & Thompson, 2003; Clark, 2011; Clark et al., 2001, 2002; Woodruff-Pak & Disterhoft, 2008), the role of cerebellar circuitry in conditioned appetitive approach is less well established (see Martin-Soelch, Linthicum, & Ernst, 2007). Cholinergic support of persistent mPFC activity during the TI, where this activity effectively serves to bridge the temporal gap between CS and US presentations, may nevertheless play an essential role in appetitive trace conditioning.

Finally, it is possible that the observed increase in ACh levels in the mPFC contributes to the precisely timed and highly adaptive conditioned responding seen in the appetitive trace conditioning task. The ability to inhibit premature, non-adaptive conditioned responding

during the CS interval and early TI may depend attentional processes, which themselves are clearly linked to cholinergic modulation of the mPFC. Consistent with this viewpoint, previous research shows that post-acquisition lesions of the cholinergic inputs to the mPFC impair performance in an operant visual attention paradigm; cholinergic denervation of the mPFC caused an increase in response perseveration anticipatory responding, especially under conditions of high attentional demand (Dalley et al., 2004).

In contrast to the task-dependent increases in ACh release in the mPFC and HPC observed during trace conditioning, ACh levels did not show an increase in either of these regions during delay conditioning. These findings are in agreement with previous experiments suggesting that delay conditioning does not depend on cholinergic activation in either the mPFC or the HPC (Raybuck & Gould, 2010; see also Hunt & Richardson, 2007; Kaneko & Thompson, 1997). The lack of hippocampal involvement in delay conditioning is clearly illustrated by evidence showing that even complete lesions of the HPC do not significantly affect delay conditioning (e.g., Wanisch, Tang, Mederer, & Wotjak, 2005). The contrast in the pattern of ACh release in mPFC and HPC during trace and delay conditioning in the current experiment provides further evidence that post-acquisition performance in these two classes of associative learning relies upon different neural mechanisms and supports the argument that these two types of learning represent truly different forms of memory (see Clark et al., 2001, 2002; see also Hasselmo, 2006).

In conclusion, results from the present study suggest that cholinergic activation of the mPFC and HPC is important for post-acquisition performance in trace but not delay conditioning. The release of ACh into the mPFC may critically contribute to attentional processes necessary in trace conditioning, such as attending to CS onset or offset. Cholinergic activation of both the mPFC and HPC might also be related to the suppression of premature, non-adaptive responding during the CS interval in appetitive trace conditioning paradigms. Finally, cholinergic activation of the HPC could continue to play a role in memory consolidation even after initial acquisition of the CR in the trace conditioning task.

Highlights

Pavlovian trace conditioning critically depends on the medial prefrontal cortex (mPFC) and hippocampus (HPC), whereas delay conditioning does not depend on these brain structures. Microdialysis of acetylcholine (ACh) released in these areas showed distinct profiles during testing in trace and delay conditioning paradigms; ACh release in both the mPFC and HPC was greater during trace conditioning than during delay conditioning. While ACh efflux in both the mPFC and HPC selectively increased during trace conditioning, ACh levels in the mPFC during trace conditioning testing showed the greatest increases observed. The observed increases in cholinergic activity in the mPFC and HPC during trace conditioning are interpreted in terms of attentional mechanisms, adaptive response timing, and memory consolidation necessary. This report is, to the best of our knowledge, the first to directly demonstrate a dissociation in the level of cholinergic activation of the mPFC and HPC during performance in trace and delay appetitive conditioning.

References

- Acquas E, Wilson C, Fibiger HC. Conditioned and unconditioned stimuli increase frontal cortical and hippocampal acetylcholine release: effects of novelty, habituation, and fear. *The Journal of Neuroscience*. 1996; 16:3089–3096. [PubMed: 8622138]
- Bangasser DA, Waxler DE, Santollo J, Shors TJ. Trace conditioning and the HPC: the importance of contiguity. *The Journal of Neuroscience*. 2006; 26(34):8702–8706. [PubMed: 16928858]

- Berger, TW.; Berry, SD.; Thompson, RF. Role of the hippocampus in classical conditioning of aversive and appetitive behaviors. In: Isaacson, RL.; Pribram, KH., editors. *The Hippocampus*. Vol. vol. 4. New York: Plenum; 1986. p. 203-239.
- Blum S, Hebert AE, Dash PK. A role for the prefrontal cortex in recall of recent and remote memories. *Neuroreport*. 2006; 17(3):341–344. [PubMed: 16462609]
- Burman MA, Gewirtz JC. Timing of fear expression in trace and delay conditioning measured by fear-potentiated startle in rats. *Learning and Memory*. 2004; 11:205–212. [PubMed: 15054136]
- Butt AE, Amodeo D, Flesher MM, Holt R, Marsa K, Lladones R, Haynes J, Olney R, Dash N, Kinney-Hurd B. 192 IgG-Saporin lesions of the cholinergic basal forebrain disrupt selective attention and trace conditioning but spare delay and long-delay conditioning. [Abstract]. *Society for Neuroscience Abstracts*. 2007; 424.9
- Chang Q, Savage LM, Gold PE. Microdialysis measures of functional increases in ACh release in the hippocampus with and without inclusion of acetylcholinesterase inhibitors in the perfusate. *Journal of Neurochemistry*. 2006; 97:697–706. [PubMed: 16579834]
- Cho YH, Jeantet Y. Differential involvement of prefrontal cortex, striatum, and hippocampus in DRL performance in mice. *Neurobiology of Learning and Memory*. 2010; 93(1):85–91. [PubMed: 19703576]
- Chowdhury N, Quinn JJ, Fanselow MS. Dorsal hippocampus involvement in trace fear conditioning with long, but not short, trace intervals in mice. *Behavioral Neuroscience*. 2005; 119(5):1396–1402. [PubMed: 16300446]
- Christian KM, Thompson RF. Neural substrates of eyeblink conditioning: acquisition and retention. *Learning & Memory*. 2003; 10:427–455. [PubMed: 14657256]
- Clark RE. Eyeblink conditioning and systems consolidation: An ironic yet powerful pairing. *Neurobiology of Learning and Memory*. 2011
- Clark RE, Manns JR, Squire LR. Trace and delay conditioning: Contrasting phenomena of declarative and nondeclarative memory. *Psychological Science*. 2001; 12:304–308. [PubMed: 11476097]
- Clark RE, Manns JR, Squire LR. Classical conditioning, awareness, and brain systems. *Trends in Cognitive Science*. 2002; 6:524–531.
- Dalley JW, Theobald DE, Bouger P, et al. Cortical cholinergic function and deficits in visual attentional performance in rats following 192 IgG-saporin-induced lesions of the medial prefrontal cortex. *Cerebral Cortex*. 2004; 14(8):922–932. [PubMed: 15084496]
- Dash PK, Hebert AE, Runyan JD. A unified theory for systems and cellular memory consolidation. *Brain Research Reviews*. 2004; 45:30–37. [PubMed: 15063098]
- Davidson TL, Jarrard LE. The hippocampus and inhibitory learning: a 'Gray' area? *Neuroscience and Biobehavioral Review*. 2004; 28(3):261–271.
- Disterhoft JF, Kronforst-Collins M, Oh MM, Power JM, Preston AR, Weiss C. Cholinergic facilitation of trace eyeblink conditioning in aging rabbits. *Life Sciences*. 1999; 64:541–548. [PubMed: 10069521]
- Disterhoft JF, Oh MM. Modulation of cholinergic transmission enhances excitability of hippocampal pyramidal neurons and ameliorates learning impairments in aging animals. *Neurobiology of Learning and Memory*. 2003; 80:223–233. [PubMed: 14521865]
- Esclassan F, Coutureau E, Di Scala G, Marchand AR. Differential contribution of dorsal and ventral hippocampus to trace and delay fear conditioning. *Hippocampus*. 2009; 19(1):33–44. [PubMed: 18683846]
- Fanselow MS, LeDoux JE. Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. *Neuron*. 1999; 23:229–232. [PubMed: 10399930]
- Fendt M, Fanselow MS, Koch M. Lesions of the dorsal HPC block trace fear conditioned potentiation of startle. *Behavioral Neuroscience*. 2005; 119(3):834–838. [PubMed: 15998205]
- Fontán-Lozano A, Troncoso J, Múnera A, Carrión AM, Delgado-García JM. Cholinergic septo-hippocampal innervation is required for trace eyeblink classical conditioning. *Learning & Memory*. 2005; 12:557–563. [PubMed: 16287719]
- Galvez R, Weible AP, Disterhoft JF. Cortical barrel lesions impair whisker-CS trace eyeblink conditioning. *Learning & Memory*. 2007; 14(1):94–100. [PubMed: 17272654]

- Gilmartin MR, Helmstetter FJ. Trace and contextual fear conditioning require neural activity and NMDA receptor-dependent transmission in the medial prefrontal cortex. *Learning & Memory*. 2010; 17(6):289–296. [PubMed: 20504949]
- Green JT, Woodruff-Pak DS. Eyeblick classical conditioning: hippocampal formation is for neutral stimulus associations as cerebellum is for association-response. *Psychological Bulletin*. 2000; 126(1):138–158. [PubMed: 10668353]
- Han CJ, O'Tuathaigh CM, van Trigt L, Quinn JJ, Fanselow MS, Mongeau R, Koch C, Anderson DJ. Trace but not delay fear conditioning requires attention and the anterior cingulate cortex. *PNAS*. 2003; 100:13087–13092. [PubMed: 14555761]
- Hasselmo ME. The role of acetylcholine in learning and memory. *Current Opinions in Neurobiology*. 2006; 16(6):710–715.
- Himmelheber AM, Sarter M, Bruno JP. The effects of manipulations of attentional demand on cortical acetylcholine release. *Brain Res Cogn Brain Res*. 2001; 12(3):353–370. [PubMed: 11689296]
- Holland PC. Influence of visual conditioned stimulus characteristics on the form of Pavlovian appetitive conditioned responding in rats. *Journal of Experimental Psychology. Animal Behavior Processes*. 1980; 6(1):81–97. [PubMed: 7373228]
- Hunt PS, Richardson R. Pharmacological dissociation of trace and long delay fear conditioning in young rats. *Neurobiology of Learning and Memory*. 2007; 87:86–92. [PubMed: 16904919]
- Inglis FM, Day JC, Fibiger HC. Enhanced acetylcholine release in hippocampus and cortex during the anticipation and consumption of a palatable meal. *Neuroscience*. 1994; 62:1049–1056. [PubMed: 7845585]
- Ivkovich D, Stanton ME. Effects of early hippocampal lesions on trace, delay, and long-delay eyblink conditioning in developing rats. *Neurobiology of Learning and Memory*. 2001; 76(3):426–446. [PubMed: 11726246]
- Kalmbach BE, Ohyama T, Kreider JC, Riusech F, Mauk MD. Interactions between prefrontal cortex and cerebellum revealed by trace eyelid conditioning. *Learning & Memory*. 2009; 16(1):86–95. [PubMed: 19144967]
- Kalmbach BE, Ohyama T, Mauk MD. Temporal patterns of inputs to cerebellum necessary and sufficient for trace eyelid conditioning. *Journal of Neurophysiology*. 2010; 104(2):627–640. [PubMed: 20484534]
- Kaneko T, Thompson RF. Disruption of trace conditioning of the nictitating membrane response in rabbits by central cholinergic blockade. *Psychopharmacology (Berlin)*. 1997; 131(2):161–166. [PubMed: 9201804]
- Kim JJ, Clark RE, Thompson RF. Hippocampectomy impairs the memory of recently, but not remotely, acquired trace eyblink conditioned responses. *Behavioral Neuroscience*. 1995; 109(2):195–203. [PubMed: 7619310]
- Kishimoto Y, Nakazawa K, Tonegawa S, Kirino Y, Kano M. Hippocampal CA3 NMDA receptors are crucial for adaptive timing of trace eyblink conditioned response. *The Journal of Neuroscience*. 2006; 26:1562–1570. [PubMed: 16452679]
- Kozak R, Bruno JP, Sarter M. Augmented prefrontal acetylcholine release during challenged attentional performance. *Cerebral Cortex*. 2006; 16:9–17. [PubMed: 15788700]
- Kronforst-Collins MA, Disterhoft JF. Lesions of the caudal area of rabbit medial PFC impair trace eyblink conditioning. *Neurobiology of Learning and Memory*. 1998; 69:147–162. [PubMed: 9619994]
- Kyd RJ, Pearce JM, Haselgrove M, Amin E, Aggleton JP. The effects of hippocampal system lesions on a novel temporal discrimination task for rats. *Behavioural Brain Research*. 2007; 187:159–171. [PubMed: 17950928]
- Manns JR, Clark RE, Squire LR. Standard delay eyblink classical conditioning is independent of awareness. *Journal of Experimental Psychology: Animal Behavior Processes*. 2002; 28:32–37. [PubMed: 11868232]
- Martin-Soelch C, Linthicum J, Ernst M. Appetitive conditioning: neural bases and implications for psychopathology. *Neuroscience and Biobehavioral Reviews*. 2007; 31(3):426–440. [PubMed: 17210179]

- McEchron MD, Bouwmeester H, Tseng W, Weiss C, Disterhoft JF. Hippocampectomy disrupts auditory trace fear conditioning and contextual fear conditioning in the rat. *Hippocampus*. 1998; 8:638–646. [PubMed: 9882021]
- McKinney M, Coyle JT, Hedreen JC. Topographic analysis of the innervation of the rat neocortex and HPC by the basal forebrain cholinergic system. *The Journal of Comparative Neurology*. 1983; 217:103–121. [PubMed: 6875049]
- McLaughlin J, Skaggs H, Churchwell J, Powell DA. Medial PFC and Pavlovian conditioning: Trace versus delay conditioning. *Behavioral Neuroscience*. 2002; 116:37–47. [PubMed: 11895181]
- McNaughton N, Wickens J, Hebb, pandemonium and catastrophic hypermnesia: the hippocampus as a suppressor of inappropriate associations. *Cortex*. 2003; 39(4–5):1139–1163. [PubMed: 14584571]
- Misane I, Tovote P, Meyer M, Spiess J, Ogren SO, Stiedl O. Time-dependent involvement of the dorsal HPC in trace fear conditioning in mice. *Hippocampus*. 2005; 8:418–426. [PubMed: 15669102]
- Moore JW, Desmond JE, Berthier NE. Adaptively timed conditioned responses and the cerebellum: a neural network approach. *Biological Cybernetics*. 1989; 62(1):17–28. [PubMed: 2590676]
- Oswald BB, Maddox SA, Powell DA. Prefrontal control of trace eyeblink conditioning in rabbits: role in retrieval of the CR? *Behavioral Neuroscience*. 2008; 122(4):841–848. [PubMed: 18729637]
- Pang M-H, Kim N-S, Kim I-H, Kim H, Kim H-T, Choi J-S. Cholinergic transmission in the dorsal hippocampus modulates trace but not delay fear conditioning. *Neurobiology of Learning and Memory*. 2010; 94(2):206–213. [PubMed: 20685338]
- Passetti F, Dalley JW, O'Connell MT, Everitt BJ, Robbins TW. Increased acetylcholine release in the rat medial prefrontal cortex during performance of a visual attentional task. *European Journal of Neuroscience*. 2000; 12:3051–3058. [PubMed: 10971646]
- Paxinos, G.; Watson, C. *The rat brain in stereotaxic coordinates*. San Diego: Academic; 2004.
- Pych JC, Chang Q, Colon-Rivera C, Gold PE. Acetylcholine release in hippocampus and striatum during testing on a rewarded spontaneous alternation task. *Neurobiology of Learning & Memory*. 2005; 84(2):93–101. [PubMed: 15950501]
- Quinn JJ, Ma QD, Tinsley MR, Koch C, Fanselow MS. Inverse temporal contributions of the dorsal hippocampus and medial prefrontal cortex to the expression of long-term fear memories. *Learning & Memory*. 2008; 15(5):368–372. [PubMed: 18441294]
- Rasmusson DD, Smith SA, Semba K. Inactivation of prefrontal cortex abolishes cortical acetylcholine release evoked by sensory or sensory pathway stimulation in the rat. *Neuroscience*. 2007; 149:232–241. [PubMed: 17850979]
- Raybuck JD, Gould TJ. The role of nicotinic acetylcholine receptors in the medial prefrontal cortex and hippocampus in trace fear conditioning. *Neurobiology of Learning & Memory*. 2010; 94(3):353–363. [PubMed: 20727979]
- Runyan JD, Moore AN, Dash PK. A role for prefrontal cortex in memory storage for trace fear conditioning. *Journal of Neuroscience*. 2004; 24:1288–1295. [PubMed: 14960599]
- Sacchetti B, Scelfo B, Strata P. The cerebellum: synaptic changes and fear conditioning. *Neuroscientist*. 2005; 11:217–227. [PubMed: 15911871]
- Sacchetti B, Scelfo B, Strata P. Cerebellum and emotional behavior. *Neuroscience*. 2009; 162(3):756–762. [PubMed: 19409218]
- Sarter M, Gehring WJ, Kozak R. More attention must be paid: the neurobiology of attentional effort. *Brain Research Review*. 2006; 51:145–160.
- Sarter M, Hasselmo ME, Bruno JP, Givens B. Unraveling the attentional functions of cortical cholinergic inputs: interactions between signal-driven and cognitive modulation of signal detection. *Brain Research Reviews*. 2005; 48:98–111. [PubMed: 15708630]
- Seager MA, Asaka Y, Berry SD. Scopolamine disruption of behavioral and hippocampal responses in appetitive trace classical conditioning. *Behavioural Brain Research*. 1999; 100:143–151. [PubMed: 10212061]
- Seager MA, Borgnis RL, Berry SD. Delayed acquisition of behavioral and hippocampal responses during jaw movement conditioning in aging rabbits. *Neurobiology of Aging*. 1997; 18(6):631–639. [PubMed: 9461061]

- Simon B, Knuckley B, Churchwell J, Powell DA. Post-training lesions of the medial prefrontal cortex interfere with subsequent performance of trace eyeblink conditioning. *The Journal of Neuroscience*. 2005; 25:10740–10746. [PubMed: 16291947]
- Supple WF Jr, Leaton RN. Lesions of the cerebellar vermis and cerebellar hemispheres: effects on heart rate conditioning in rats. *Behavioral Neuroscience*. 1990; 104(6):934–947. [PubMed: 2285492]
- Takehara-Nishiuchi K, Kawahara S, Kirino Y. NMDA receptor-dependent processes in the medial PFC are important for acquisition and the early stage of consolidation during trace, but not delay eyeblink conditioning. *Learning and Memory*. 2005; 12:606–614. [PubMed: 16322362]
- Takehara-Nishiuchi K, McNaughton BL. Spontaneous changes of neocortical code for associative memory during consolidation. *Science*. 2008; 322:960–963. [PubMed: 18988855]
- Takehara-Nishiuchi K, Nakao K, Kawahara S, Matsuiki N, Kirino Y. Systems consolidation requires postlearning activation of NMDA receptors in the medial PFC in trace eyeblink conditioning. *The Journal of Neuroscience*. 2006; 26(19):5049–5058. [PubMed: 16687496]
- Thibaudeau G, Dore´ FY, Goulet S. Additional evidence for intact appetitive trace conditioning in hippocampal-lesioned rats. *Behavioral Neuroscience*. 2009; 123:707–712. [PubMed: 19485578]
- Thibaudeau G, Potvin O, Allen K, Dore´ FY, Goulet S. Dorsal, ventral, and complete excitotoxic lesions of the hippocampus in rats failed to impair appetitive trace conditioning. *Behavioural Brain Research*. 2007; 185:9–20. [PubMed: 17707920]
- Thompson RF, Kim JJ. Memory systems in the brain and localization of a memory. *Proceedings of the National Academy of Sciences*. 1996; 93(24):13438–13444.
- Thompson RF, Steinmetz JE. The role of the cerebellum in classical conditioning of discrete behavioral responses. *Neuroscience*. 2009; 162:732–755. [PubMed: 19409234]
- Timmann D, Drepper J, Frings M, Maschke M, Richter S, Gerwig M, Kolb FP. The human cerebellum contributes to motor, emotional and cognitive associative learning. A review. *Cortex*. 2010; 46(7): 845–857.
- Wanisch K, Tang J, Mederer A, Wotjak CT. Trace fear conditioning depends on NMDA receptor activation and protein synthesis within the dorsal HPC of mice. *Behavioral Brain Research*. 2005; 157:63–69.
- Weible AP, McEchron MD, Disterhoft JF. Cortical involvement in acquisition and extinction of trace eyeblink conditioning. *Behavioral Neuroscience*. 2000; 114:1058–1067. [PubMed: 11142638]
- Weiss C, Bouwmeester H, Power JM, Disterhoft JF. Hippocampal lesions prevent trace eyeblink conditioning in the freely moving rat. *Behavioural Brain Research*. 1999; 99:123–132. [PubMed: 10512579]
- Woodruff-Pak DS, Disterhoft JF. Where is the trace in trace conditioning? *Trends in Neuroscience*. 2008; 31(2):105–112.
- Yoon T, Otto T. Differential contributions of dorsal vs. ventral HPC to auditory trace fear conditioning. *Neurobiology of Learning and Memory*. 2007; 10:1016.

Figure 1. Illustration of microdialysis probe targets in the mPFC and HPC (Paxinos & Watson, 2004). Probes (grey area indicates probe membrane, black area indicates probe shaft) in them PFC occupied prelimbic and cingulate cortex, and probes in the HPC occupied the CA1, CA2, and CA3 sub regions of the hippocampal formation.

Figure 2.

Temporal profile of the CR of conditioned approach in the trace conditioning (A) and delay conditioning (B) paradigms on final pre-training day. Note the adaptive response strategy of inhibiting responding during the CS interval and during the early part of the TI in the trace conditioning task, while increasing food-cup approach during the last 4 s of the TI (shaded box in A) as US delivery nears. In the delay paradigm, the greatest levels of conditioned responding occurred during the latter part of the CS, reaching a peak during the last 4 s of the CS (shaded box in B) just before the sucrose pellet US was delivered.

Figure 3. Mean ($\pm$ SEM) CR difference scores in trace (A) and delay (B) conditioning across the acquisition training period.

Figure 4.

Mean ($\pm$ SEM) testing-induced ACh release expressed as a percent of baseline release in the PFC and HPC during the trace and delay conditioning paradigms. ACh levels in both the mPFC and HPC were significantly greater ($*p < .05$) during trace conditioning (left) than during delay conditioning (right). ACh levels in the mPFC obtained during the trace conditioning task were higher than all other testing-induced levels of ACh, regardless of conditioning paradigm.

Figure 5.

Mean ($\pm$ SEM) testing-induced ACh release (percent of baseline release) in the PFC and HPC during the trace and delay conditioning paradigms in animals that failed to reach acquisition criteria. ACh efflux did not differ ($p > .05$) as a function of brain region (i.e., mPFC or HPC) or conditioning paradigm, in contrast to results from those animals that did reach criteria.