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Supplemental Data

Prefrontal Acetylcholine Release Controls Cue Detection on Multiple Timescales

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Supplemental Results

In Vitro Calibration of Choline-Sensitive Microelectrodes

ChOase-coated microelectrodes (n = 26) used in these experiments exhibited a background current of 93.09 ± 15.10 pA and a sensitivity of 7.42 ± 0.66 pA/μM for choline. ChOase-coated recording sites yielded a highly linear response in current to cumulative additions of choline up to 80 μM (Figure S1B; Table S1). Addition of ascorbic acid (AA) produced negligible changes in currents measured from ChOase-coated and non-ChOase-coated recording sites, while the addition of DA resulted in similar increases in current obtained from ChOase-coated recording sites and sentinels. Self-referencing eliminated the effects of DA. The properties of the electrodes used for *in vivo* recordings are summarized in Table S1 and a representative calibration curve is shown in Figure S1B.

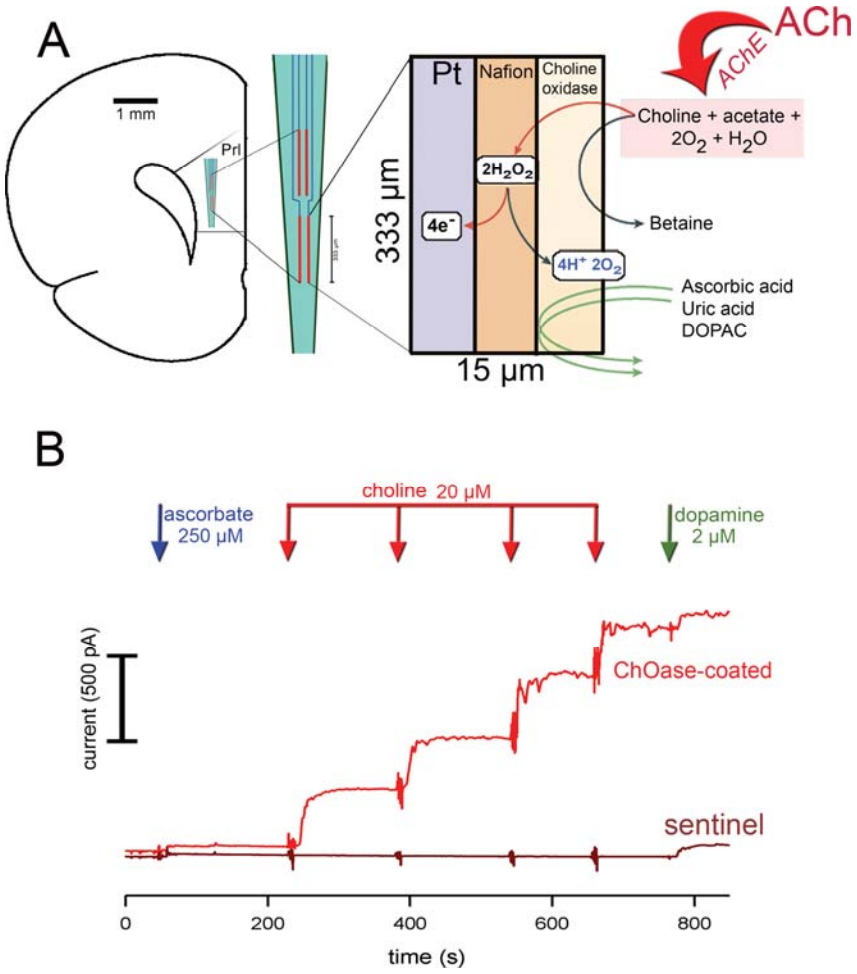

Figure S1. Illustration of the Measurement Scheme of ACh Release Using Choline-Sensitive Microelectrodes and *In Vitro* Calibration

(A) Illustration of a ceramic-based microelectrode equipped with four Platinum (Pt) recording sites and the position and approximate relative dimensions of the electrode when placed into the prelimbic (Prl) cortex. On the right, the main principles of the measurement scheme are illustrated. Choline is generated by hydrolysis of newly released acetylcholine (ACh) by acetylcholinesterase (AChE). The lower pair of recording sites was coated with choline oxidase (ChOase) while the upper pair was not coated with ChOase and used for self-referencing. Nafion serves to repel anions from the surface of the platinum electrode. Hydrogen peroxide (H_2O_2) is generated as a result of choline oxidation and measured using fixed potential amperometry. (B) *In vitro* microelectrode calibration depicting recordings (5 Hz sampling rate) of raw currents from a choline oxidase (ChOase)-coated (red) and a non-ChOase-coated recording site (dark red) in response to the addition of 250 µM ascorbic acid (AA), four cumulative additions of 20 µM choline, and 2 µM dopamine (DA; see Table S1 for calibration data).

Electrode Properties *In Vivo* Following Completion of Experiments

At the end of the experiments, 6-8 days following implantation, the sensitivity of chronically implanted enzyme-selective microelectrodes was determined by assessing signal amplitudes following infusion of choline into the recording region. As described in Supplemental Methods, data recorded during prior sessions and while animals performed the task were excluded from final analysis if implanted choline-sensitive microelectrodes failed to meet a set of response characteristics. Infusions of 5, 10 and 20 nmoles of choline produced dose-dependent increases in signal amplitudes, from 3.51 ± 0.15 to 11.15 ± 0.52 μM (R^2 = 0.968 ± 0.003; $F_{(2,13)}$ = 15.05, p = 0.001; Figure S2). Furthermore, potassium-evoked choline signals were attenuated by co-infusions of the AChE inhibitor neostigmine (Figure S2; $t_{(2)}$ = 4.45, p = 0.047), indicating that choline signals continued to reflect choline resulting from hydrolysis of ACh.

Supplemental Table

Table S1 *In vitro* calibration measures for choline-sensitive microelectrodes used for *in vivo* recordings

Analysis Mode	LOD (nM)	R^2	Choline:AA	Choline:DA
Single	153.39 ± 14.14	0.990 ± 0.002	650.48 ± 244.96	0.277 ± 0.055
Self-referencing	129.18 ± 16.59	0.991 ± 0.002	795.81 ± 268.61	>1000 ± 0.0

Data [Mean ± S.E.M]; are based on the calibration of 26 microelectrodes (LOD, limit of detection; R^2, linearity of the response of the microelectrode to increasing concentrations of choline; AA, ascorbic acid; DA, dopamine).

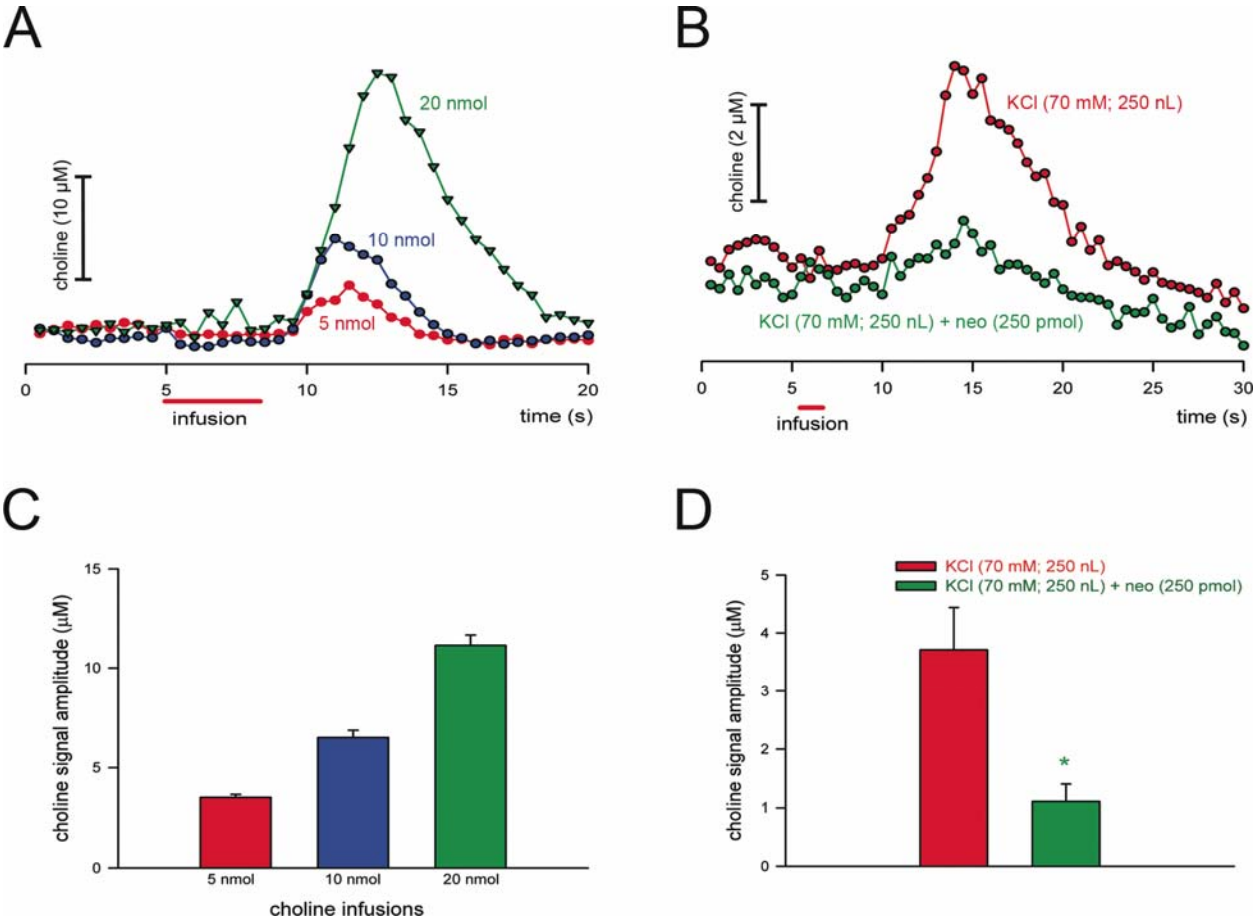

Figure S2. Properties of Implanted Electrodes Following the Completion of Recordings in Performing Animals.
(A) Self-referenced choline signals generated *in vivo* after infusion of choline (5, 10 and 20 nmoles; infusion rate = 250 nL/s) via a guide cannula attached to the microelectrode. Infusions were conducted 6-8 days following implantation into the mPFC (after completion of recordings in performing animals). Choline signal amplitudes increased in response to increasing doses of choline (C; n = 16). (B, D) The increases in cholinergic activity produced by infusing KCl (70 mM; 250 nL; red trace in B) were attenuated by co-infusion of the acetylcholinesterase inhibitor neostigmine (20 pmol; green trace in B; data in D are based on n = 3). These data indicate that following 6-8 days of implantation into the cortex, the electrodes continued to detect choline that resulted from the hydrolysis of newly released ACh.

Absence of Cue-evoked Cholinergic Transients in Motor Cortex

The limited topographic organization of the cortical cholinergic input system does not readily suggest that different subpopulations of cholinergic neurons are involved in different functions. Results from studies using microdialysis to

measure changes in ACh release in multiple areas did not suggest that changes in cholinergic activity differ drastically between cortical regions (Himmelheber et al., 1998; Laplante et al., 2005; Rasmusson and Szerb, 1976), supporting the traditional view of a cortex-wide, unitary gating function of ACh release (Sarter and Bruno, 1997). Alternatively, cholinergic transients in the mPFC could be assumed to be a result of local, prefrontal regulation of ACh release and/or the selective activation of prefrontally projecting basal forebrain cholinergic neurons, via afferent networks involving perhaps the nucleus accumbens (Neigh et al., 2004; Sarter et al., 2005a; Zmarowski et al., 2007). Therefore, cue-evoked cholinergic activity was recorded in a cortical control region, the motor cortex (forepaw region). This non-associational region was selected because it cannot be excluded that cholinergic inputs to other associational regions, particularly the posterior parietal cortex, are directly influenced by and complement the cognitive functions of cholinergic inputs to the mPFC (Broussard et al., 2006; Nelson et al., 2005). Prior experiments suggested a role of cholinergic inputs to the motor cortex in the learning of skilled reaching (Conner et al., 2005; Conner et al., 2003). As illustrated in Figure S3, neither detected nor missed cues, nor any other task-related event, evoked reliable and robust cholinergic transients in the motor cortex. Relatively small transient changes (<1 μM) were occasionally observed, for example while animals reared up to the food ports (Figure S3C). To compare cue-evoked cholinergic transients recorded in the motor cortex with those observed in the mPFC, the highest choline levels found during a 6-s post-cue period (detected) presentation were used to substantiate that “peak

amplitudes" in motor cortex were significantly smaller then the amplitudes evoked by detected cues in the mPFC ($t_{(9)}$ = 3.81, p = 0.004).

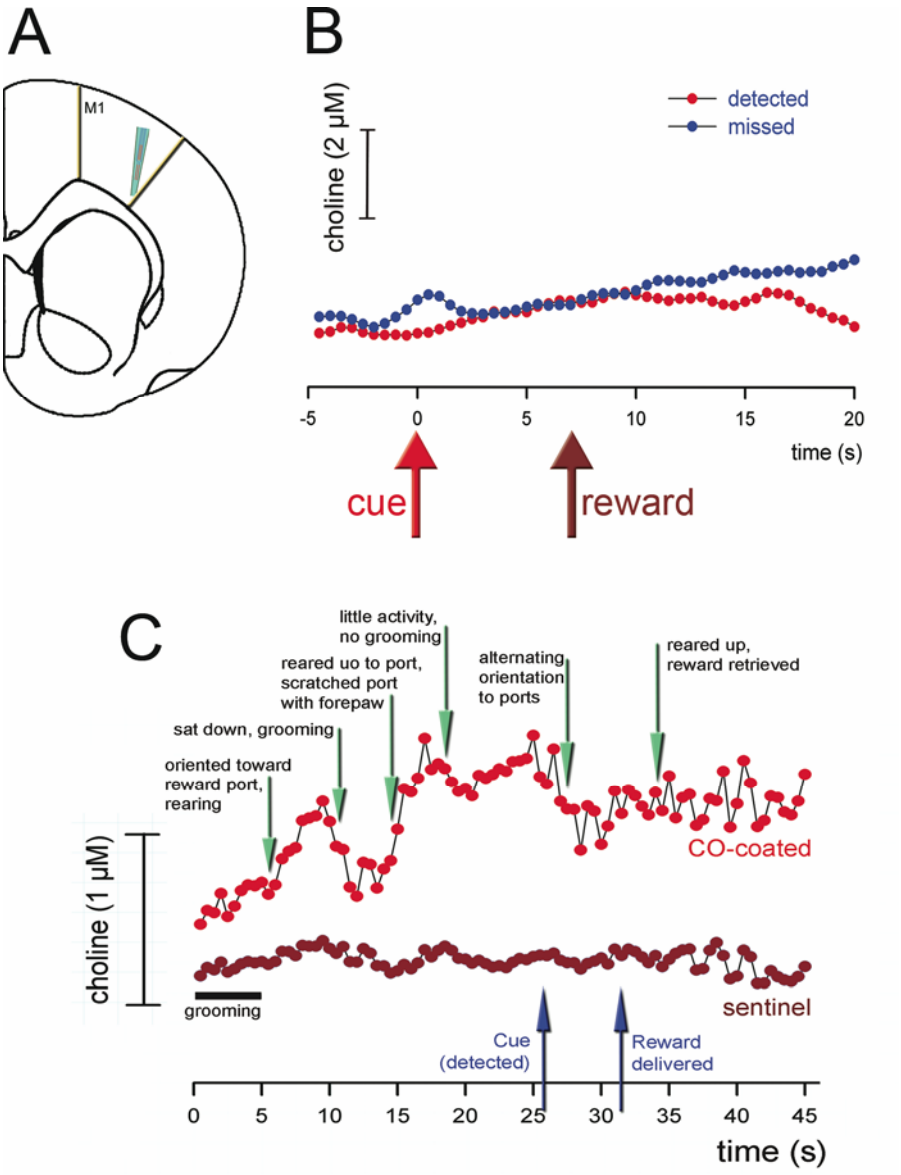

Figure S3. Absence of Cue-Evoked Cholinergic Transients in Motor Cortex
(A) Schematic illustration of the placement of a microelectrode in the forepaw region of the motor cortex and approximate dimensions of the part of electrode that features the four recording sites relative to the thickness of the cortex. (B) Representative self-referenced traces during trials involving cue detection (red) and a missed cue (blue). Reliable changes in cholinergic activity in motor cortex were not observed (based on recordings in x animals). (C) Raw trace (ChOase-coated channel: red; sentinel: dark red) exemplifying the occasionally occurring, small changes in motor cortex cholinergic activity, typically associated with forepaw activity but not evoked by cue or reward delivery (see arrows).

Cue Detection Following Removal of Cholinergic Input to Motor Cortex

To determine whether cholinergic inputs to the motor cortex are necessary for cue detection, we determined the behavioral effects of bilateral infusions of 192-SAP into the motor cortex. As illustrated in Figure S4A,B, bilateral infusions of the immmunotoxin resulted in a complete removal of cholinergic inputs to the primary motor cortex. Moreover, cholinergic input loss extended ventro-laterally into the sensory cortex, but not medially into cingulated regions. Despite these relatively large lesions, the proportion of cues that were detected was not affected ($F_{(3,16)}$ = 0.55, p = 0.67; Figure S4C). Furthermore, 192-SAP-induced cholinergic deafferentation of the motor cortex did not affect the exploratory and locomotor activity throughout the session as indicated by the number of port approaches ($F_{(3,16)}$ = 0.51, p = 0.68; Fig. S4D). These findings contrast with the effects of removal of cholinergic inputs to the mPFC. As these lesions also abolished session-related, tonic changes in cholinergic activity, these findings further substantiate the conclusion that cue-evoked cholinergic activity in the mPFC is necessary for cue detection.

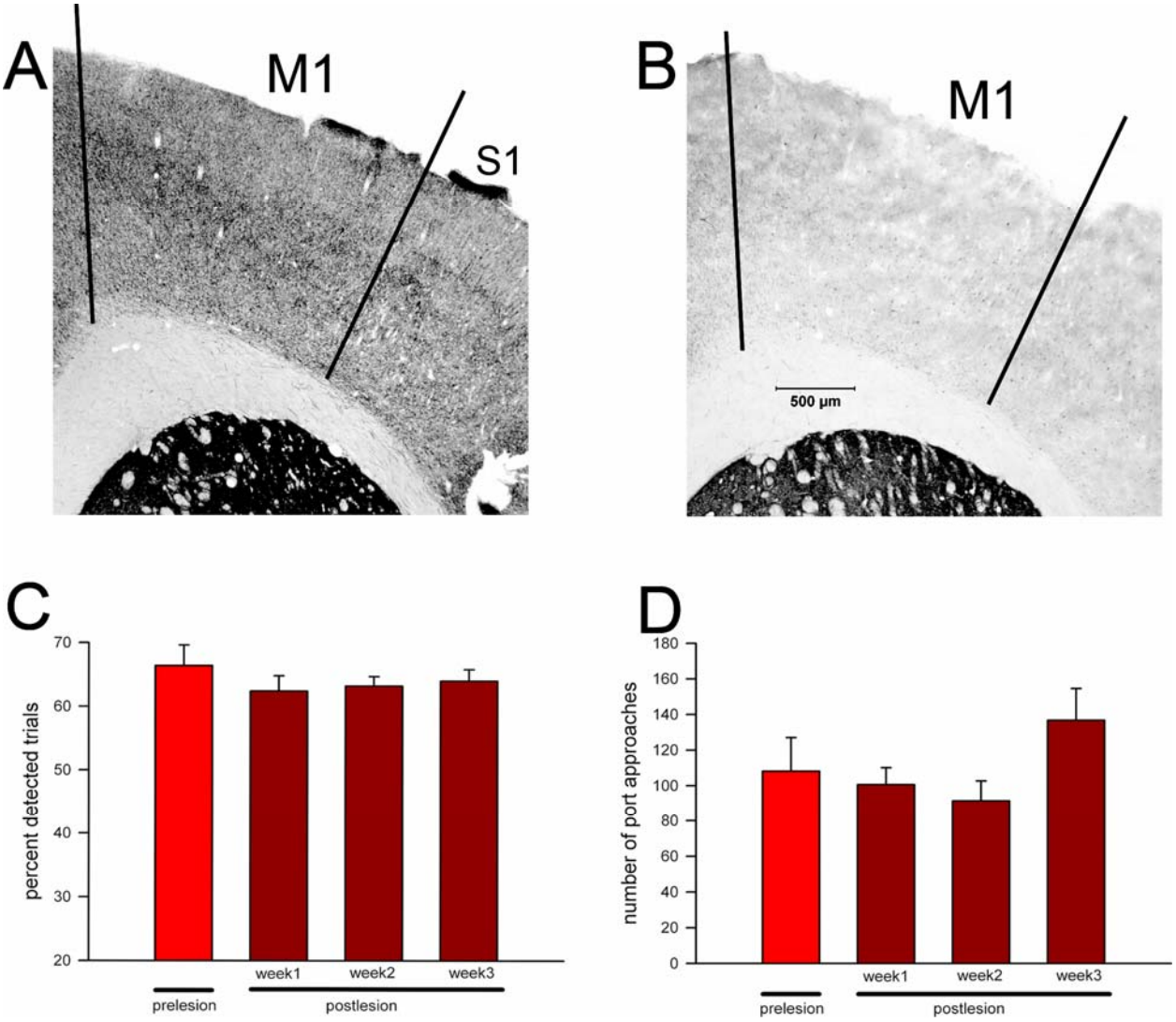

Figure S4. Cue Detection Following Removal of Cholinergic Input to Motor Cortex
(A,B) Coronal sections stained for the visualization of AChE-positive fibers in the primary motor cortex (M1) and adjacent sensory cortex (S1) of a sham-operated (A) and deafferented (B) animal (500 µm scale inserted). As illustrated in (B), the lesion resulted in the almost complete removal of cholinergic inputs this region. (C) The proportion of detected cues was not affected by this lesion. (D) Likewise, the lesion did not affect the total number of port approaches, a measure of performance-associated locomotor and exploratory activity (data based on n = 5 animals per condition).

Cue-evoked Cholinergic Transients in Trials Not Involving Reward Delivery

Cholinergic activity was recorded in trials not involving reward delivery in order to determine whether reward delivery and delivery-triggered port approach and

reward retrieval evokes cholinergic activity. After animals were trained to criterion in the final version of cued appetitive response task, subsequent training sessions involved three randomly placed “catch”-trials during which the cue was not followed by reward delivery. Such pre-exposure to catch trials was designed to minimize unspecific arousal effects of non-reward. Subsequent recording sessions likewise involved three such trials/session (n = 5, totaling 15 trials total; as 6 of these trials involved missed cues, data from 6 trials involving cue detection were randomly selected for analysis). Cue-evoked cholinergic transients were observed in detected but not in missed “catch” trials, and the amplitudes of detected cue-evoked cholinergic transients did not differ from those observed in regular trials (Fig. S5; detected: 1.86 ± 0.3 μM; missed: 0.40 ± 0.08 μM). These results, along with the absence of cue-evoked cholinergic transients during trials involving missed cues and during early acquisition trials (below) indicates that reward delivery, delivery-triggered port approach and reward retrieval do not evoke increases in cholinergic activity in the mPFC.

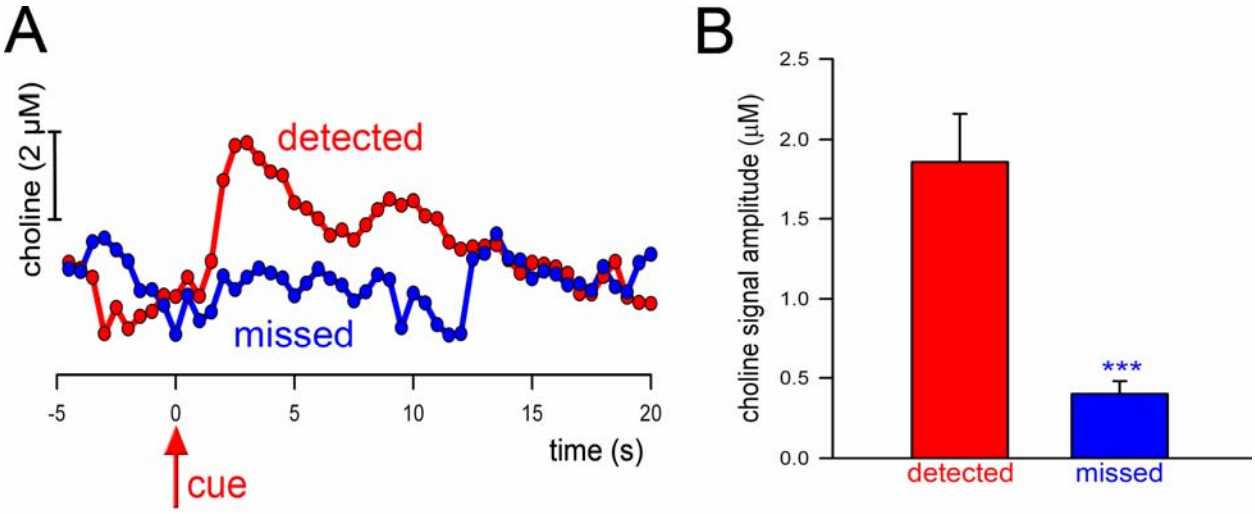

Figure S5. Cue-evoked Cholinergic Transients in Trials Not Involving Reward Delivery
(A) Representative self-referenced and boxcar-filtered (over two points) traces from trials involving cue detection (red trace) or a missed cue (blue trace) but which did not involve reward delivery and retrieval ("catch" trials). (B) The amplitude of detected cue-evoked cholinergic transients was significantly larger compared to missed trials (t_{10} = 4.69; ***, P < 0.001) and did not differ from the amplitudes observed during regular trials (M±SEM; n=5).

Cholinergic Activity During Early Training Sessions

Cholinergic activity in the mPFC was recorded during the initial three sessions of acquisition training of the final version of the task. Prior to the recording sessions, animals were trained to retrieve reward at the ports. During the first recording session, cues failed to trigger a behavioral response while, by day 3, cue-evoked termination of grooming behavior and orientation toward the ports began to emerge, yielding 19 ± 1.9 % trials meeting the criterion for cue detection. Figure S6 illustrates representative traces of cholinergic activity from trials on day 1 and day 3. During day 1, and while the cue did not evoke a behavioral response, port approach and reward retrieval was triggered by reward delivery. No systematic changes in cholinergic activity were observed, neither in response to the cue nor

to reward delivery and retrieval (Fig. S6). By day 3, detected cue-evoked cholinergic transients manifested, particularly during the second half of the session. A total of 10 trials involving detected cues were obtained from 4 animals for this analysis (3+3+2+2; signal amplitude: 1.92 ± 0.21 μM; t_{50}: 7.50 ± 2.59 s). It is interesting to note that these cholinergic transients decayed more slowly than those recorded in well-trained animals (see main results), suggesting that cue-evoked cholinergic transients become sharper with continued training. Importantly, these findings further support the conclusion that cholinergic transients are not evoked by reward and reward-evoked behavior, not even during the early stage of training. These findings contrast with the dynamic shift of dopaminergic transients recorded in the nucleus accumbens during associative learning (Day et al., 2007; see Discussion in main text).

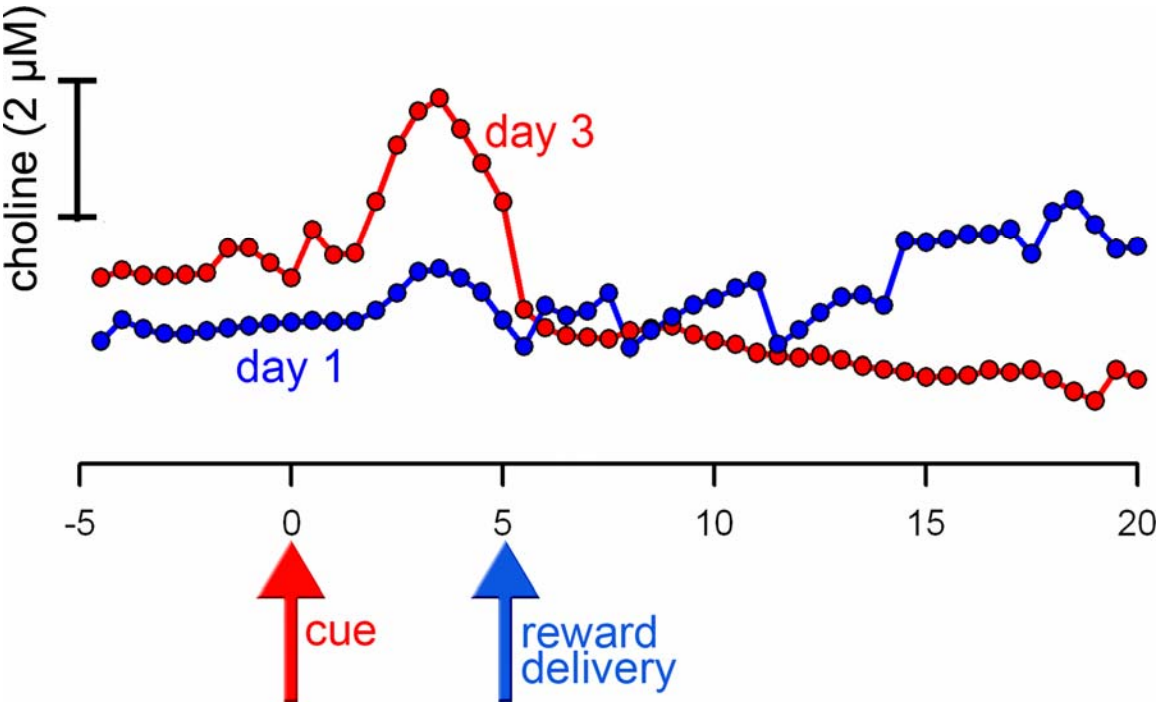

Figure S6. Cholinergic Activity During Early Training Sessions
Representative self-referenced traces (recorded at 2 Hz) from an animal during the first session (day 1; blue trace) and third (day 3; red trace) session of training of the cued appetitive response task. Prior to this stage, animals were trained to retrieve reward upon reward delivery (see Methods). During day 1, the cue failed to evoke a behavioral response and transient increases in cholinergic activity were not observed, neither in response to cue presentation nor reward delivery and retrieval. As the cue began to trigger termination of grooming behavior and orientation toward the reward ports during the second half of day 3, cue-evoked cholinergic transients began to manifest (based on n = 4). As detailed in the Supplemental Results, at this early stage, detected cue-evoked cholinergic transients were similar in amplitude when compared with those recorded in well-trained animals (see Supplemental Results for statistical findings). However, the decay rate of these early cholinergic transients was slower than that observed in well-trained animals, suggesting a "sharpening" of cholinergic transients in the course of cue acquisition.

Session-Related Tonic Changes in Cholinergic Activity: Comparison with ACh Release Measured Using Microdialysis

Given the predominance of microdialysis in research on the role of neurotransmitters in behavioral and cognitive performance, we tested the hypothesis that session-related tonic changes in cholinergic activity, calculated

from amperometric recordings, correspond with measures of ACh release generated by microdialysis. In separate groups of task-performing animals, ACh release was measured in the PFC and motor cortex (Figure S7). Performance session-related amperometric data were re-calculated to match the 8-min microdialysis collection intervals, and both sets of data were transformed for dimension-free expression and to allow statistical comparisons between methods and cortical regions. The results from a mixed factor ANOVA over the effects of cortical region and method (between-subject factors) and block (within-subject measures) indicated that session-related increases in cholinergic activity did not differ between methods, regions and blocks. Furthermore, there were no interactions between method, region, and block (main effects and interactions involving the factor block: all $F_{(4,60)} < 2.9$, all $p > 0.1$; main effect of method: $F_{(1,15)} = 1.56$, $P = 0.23$; main effect of region $F_{(1,18)} = 0.116$, $P = 0.75$). Thus, session-related tonic changes in cholinergic activity were identical irrespective of the method used to measure these changes and irrespective of cortical region (Figure S7). These findings suggest that, in contrast to the cue-evoked cholinergic transients that were only found in the mPFC, session-related, slowly changing levels of cholinergic activity may occur cortex-wide, reflecting general performance-associated increases in the readiness for cortical input processing or cortical arousal (Pepeu and Giovannini, 2004). These results also specify the nature of ACh release measured by microdialysis as indicating session-related tonic changes in cholinergic activity.

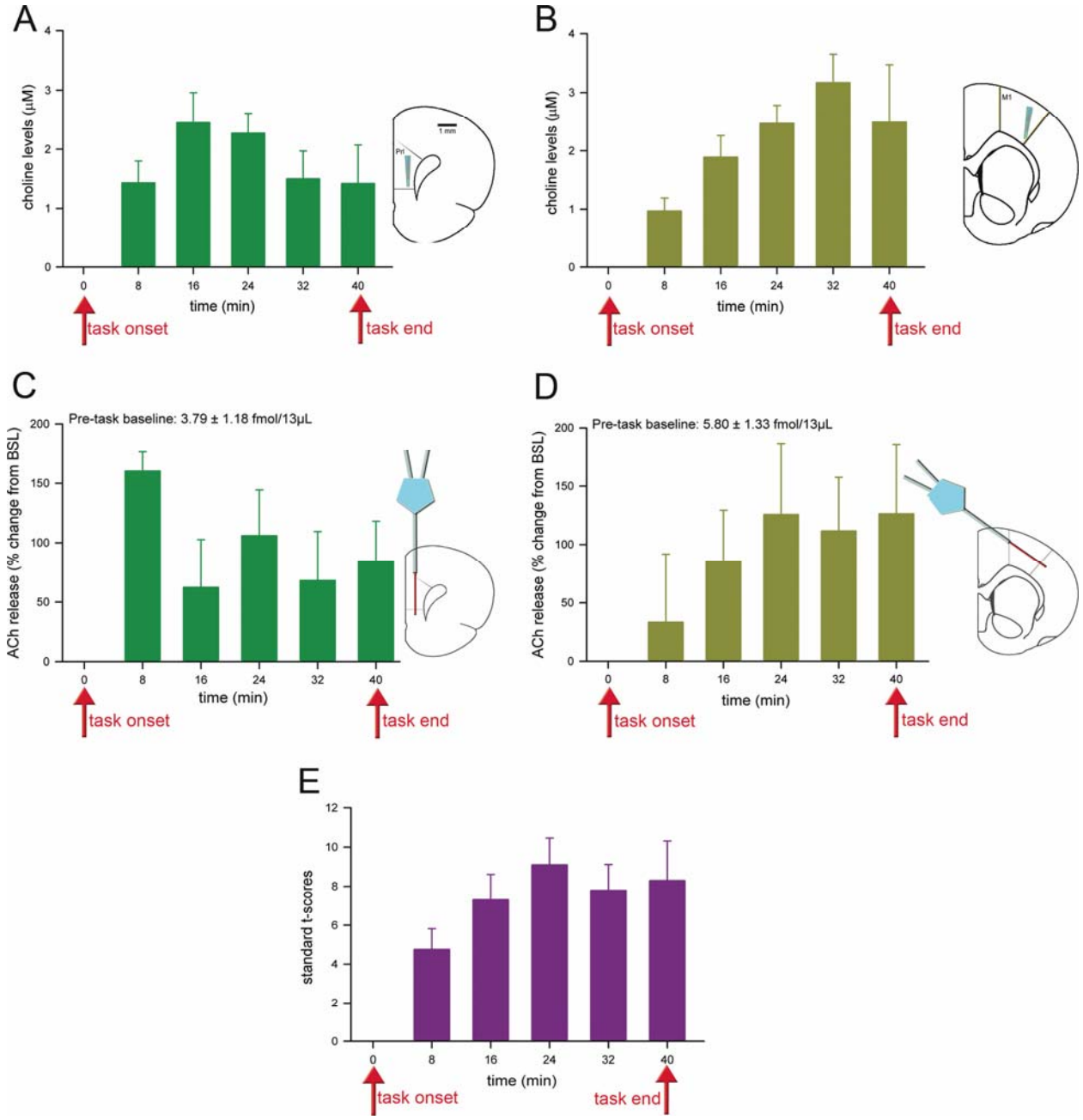

Figure S7. Session-Related changes in Cholinergic Activity Determined by Amperometry and Microdialysis

Performance session-related changes in ACh release determined by using amperometry (A,B) or microdialysis (C,D) in the PFC (A,C) or motor cortex (B,D) in animals performing the cued appetitive response task. In both cortical regions and as measured by both methods, performance was associated with increases in cholinergic activity. In order to compare the data generated by two different methods, amperometric measures were expressed against a 3-min pre-task baseline and averaged over 8 min blocks to match the dialysate collection intervals (data were taken from the experiment described in the main text; n = 4 for the microdialysis experiment). Furthermore, data from both methods were transformed to indicate dimension-free expression of performance-

associated changes in cholinergic activity (see Supplemental Methods). The analysis of these data indicated that session-related increases in cholinergic activity did neither differ between the two cortical regions nor between the two methods, and there were no interactions between methods, regions, and time blocks (see Supplemental Results). (E) Based on the absence of significant differences, this graph depicts transformed data averaged over the two regions and methods, and plotted by time block. Collectively, these results suggest that session-related (or tonic) increases in cholinergic activity may occur cortex-wide and can be measured with both methods (microdialysis and amperometry).

Supplemental Discussion

Which neuronal mechanisms may be responsible for the manifestation of session-related tonic cholinergic activity?

The neuronal mechanisms regulating performance session-related levels of tonic cholinergic activity cortex-wide likewise remain unclear. A significant component of this tonic activity may be controlled by the ascending noradrenergic system (Berridge and Waterhouse, 2003). Furthermore, Aston-Jones and colleagues demonstrated that the noradrenergic neurons in the locus coeruleus (LC) encode decisions concerning responses and trial outcomes in monkeys performing tasks involving demands on sustained attention (Aston-Jones and Cohen, 2005). Given the collateralized organization of the noradrenergic system, volume transmission of noradrenaline, and evidence indicating noradrenergic control of basal forebrain cholinergic neurons (Fort et al., 1995), cholinergic activity therefore may be profoundly influenced by noradrenergic afferents from the LC. The finding that cortical evoked potentials involving noradrenergic activation are attenuated by removal of basal forebrain cholinergic neurons (Berntson et al., 2003) or by blocking noradrenergic α1 receptors in the basal forebrain (Knox et al., 2004)

confirm the potential significance of such noradrenergic-cholinergic interactions.

Supplemental Experimental Procedures

Behavioral Apparatus

Behavioral training and testing was carried out using a 45(w)x45(d)x60(h) cm plywood-constructed chamber, with a full-size front door and a 30x30 cm observation window, 3.75 cm (in diameter) ventilation ports on top and bottom and on both sides. Reward ports were 3.75 cm in diameter, located on the left and right side walls, and with the center of the openings placed 6 cm above the floor. Background illumination was provided by 6 Red T1 ¾ LED's (arranged in 10" circle around camera port). The cue light was presented via a water clear White T1 ¾ LED attached to the ceiling. The luminescence measured at the level of animal on the surface of the chamber floor, with the background light illuminated and in the absence and presence of the cue light, was 0.88 lux and 1.17 lux, respectively. A CCTV bullet camera was installed into the ceiling. Video tracking of the animals' behavior was accomplished using a 14" 3 channel monitor, a Panasonic WJ 810 time/date generator, and a Panasonic DMR-ES10 DVD recorder. For amperometric recordings in performing animals, the chamber was shielded with copper wire screen (wire diameter: 0.458 mm; aperture: 1.131 mm) to avoid interference from static energy. Furthermore, the chamber was equipped with a removable top to center a 3.12 mm (diameter) low impedance commutator (Ailrflyte Electronics, Bayonne, NJ), swivel (Quanteon LLC, Nicholasville, KY) and counter weight to allow animals to move freely inside the

chamber during recording sessions.

Classification of Trials: Reliability

The trials from recording sessions were rated, in terms of detected versus missed cues, by two students blind to the recording data. The inter-observer agreement for the rating of detected and missed cues was assessed by calculating Cohen's Kappa (Cohen, 1960; Watkins and Pacheco, 2001). Kappa was 0.62, and the equivalent Pearson's Chi square (with Yate's correction) was 47.26 (P = 0.00001). Thus, inter-observer ratings were significantly different from "no agreement" and the kappa score indicates "good agreement" between the two observers

Preparation and Calibration of Choline-Sensitive Microelectrodes

Ceramic-based, multi-site microelectrodes featuring four 15x333 μm Platinum-recording sites arranged in side-by-side pairs (Quanteon; see Figure S1A) were prepared for enzyme coatings as described earlier (Burmeister et al., 2003; Parikh et al., 2004; Parikh and Sarter, 2006). Briefly, the microelectrodes were coated with Nafion™ prior to enzyme application to prevent potentially interfering compounds other than hydrogen peroxide from accessing the Platinum (Pt) surface of the individual recording sites. Choline oxidase (ChOase) was cross-linked with the BSA-glutaraldehyde mixture and immobilized onto the bottom pair of recording sites (Fig S1A). The remaining two recording sites were coated only with the BSA-glutaraldehyde solution and served to record background activity

(see below for data processing). Enzyme-coated microelectrodes were air-dried for 48-72 hrs prior to calibration. Microelectrodes were modified by soldering one end of four enamel-coated magnet wires (30 ga) to the terminals on the electrode panel and the other end to gold-pin connectors. Reference electrodes were constructed by soldering Ag/AgCl reference electrodes prepared from 0.008" silver wire (A-M Systems, Carlsberg, WA) to gold-pin connectors. The gold-pins were inserted into a miniature 9-pin plastic strip connector that was glued to the microelectrode using epoxy (for details on the design and construction of enzyme-coated microelectrodes for recordings in freely-moving animals, see Rutherford et al., 2007). ChOase-coated microelectrodes were calibrated using a FAST-16 electrochemical recording system (Quanteon) for choline sensitivity and selectivity prior to implantation in animals.

Microelectrodes were dipped in 0.05M PBS for 30 min prior to calibration. Calibrations were performed using fixed potential amperometry by applying a constant voltage of 0.7 V versus Ag/AgCl reference electrode in a beaker containing a stirred solution of 0.05M PBS maintained at 37ºC. Amperometric currents were digitized at a frequency of 5 Hz. After baseline currents stabilized, aliquots of stock solutions of ascorbic acid (AA; 20 mM), choline (20 mM), and dopamine (DA; 2 mM) were added to the calibration beaker such that the final concentrations were 250 μM AA, 20, 40, 60 and 80 μM choline and 2 μM DA (Figure S1B). Normalized currents recorded from non-ChOase-coated sites were subtracted from normalized currents recorded via ChOase-coated sites ("self-referencing") to yield currents that selectively reflect choline concentrations

(Burmeister and Gerhardt, 2001; Parikh et al., 2004; Parikh and Sarter, 2006). The slope (sensitivity), limit of detection (LOD), linearity (R^2) for choline and the selectivity ratio for AA and DA were calculated for each individual recording site and recordings from ChOase-coated sites were self-referenced. To be implanted into the cortex, electrodes were required to meet the following characteristics: 1) sensitivity for detecting choline: >3 pA/μM, with a background current of <200 pA; 2) limit of detection (LOD): <300 nM choline; 3) selectivity for choline:AA: >80:1; 4) detection of increasing choline concentrations (20-80 μM): R^2: >0.98.

Collectively, the selectivity of the electrode responses was a combined result of; 1) the presence of ChOase on the recording sites; 2) the calibration with AA and DA; 3) the application of a voltage that selectively oxidizes hydrogen peroxide on Pt-recording sites; 4) a Nafion-coating to block electroactive interferents from reaching the Pt sites; and 5) self-referencing against recording sites monitoring interferents reaching the Pt recording sites despite Nafion-coating.

After calibration, an infusion guide cannula (26 g; Plastics One Inc., Roanoke, VA) was attached to the microelectrode assembly so that the tip of the cannula was positioned between the two pairs of platinum recording sites, ~200 μm away from the ceramic-based microelectrode. The assembly was then implanted into the cortex.

Surgery and *In Vivo* Recording of Cholinergic Activity

Surgeries were performed under aseptic conditions. Anesthesia was induced

with 4-5% isoflurane using an anesthesia machine (Anesco/Surgivet, Waukesha, WI). Anesthetized rats with shaved heads were placed in stereotaxic frame (David Kopf, Model # 962, Tujunga, CA) and their body temperature was maintained at 37ºC using Deltaphase isothermal pad (Braintree Scientific, Braintree, MA). Anesthesia was maintained with 2% isoflurane along with oxygen at a flow rate of 1 mL/min throughout the surgical procedure. The scalp was cleansed with betadine scrub and incised along the midline for 10mm. Three stainless steel screws were threaded into the cranium to anchor microelectrode assembly with dental cement. The microelectrode assembly was slowly lowered into the prelimbic region of the right mPFC (AP: +3.2 mm; ML: -0.7 mm, measured from Bregma; DV: -3.5 mm, measured from dura; for justification for selecting the right prelimbic cortex see (Apparsundaram et al., 2005; Martinez and Sarter, 2004) or the forelimb area of the right motor cortex (Conner et al., 2005; Conner et al., 2003) (AP: +1.5 mm; ML: -3.75 mm; DV: -1.7 mm) using a microdrive (MO-10; Narishige, International, East Meadow, NY). A stylet (32 ga) was placed into the infusion cannula to prevent obstruction. Topical antibiotic (bacitracin, polymixin and neomycin) was applied to the wound immediately after surgery. All animals received an antibiotic (amikacin; 100 mg/kg; s.c.), an analgesic (buprenorphine, 0.05 mg/kg; s.c.), and saline (1.0 mL; i.p.) daily for two days post-operatively while remaining in their home cages with food and water *ad libitum*. Thereafter, the food restriction schedule was again implemented and the animals were habituated to the recording environment located in a shielded chamber. Animals were connected to a miniature potentiostat headstage (mk-II

RAT HAT; Quanteon) via a shielded cable connected to a low impedance commutator (Airflyte). The shielded cable attached to the commutator passed through a swivel (Quanteon) to allow the animal to freely move inside the chamber. The tethered animals were re-trained until they returned to criterion performance, typically after 3-5 days of additional training sessions postsurgery. During recording sessions, the outputs of the commutator were connected to the FAST-16 system. Amperometric recordings were collected every 500 ms by applying a fixed potential of 0.7 V to the microelectrode using the FAST-16 recording system. Data were digitized using FAST-16 software. Amperometric recordings were time-locked and synchronized by marking task events using TTL pulses. Time stamps generated by the FAST-16 system were logged by one experimenter while a second experimenter controlled the events governing the behavioral session. The video recorder was equipped with the time-date generator; the timing of the events generated by the time date generator were synchronized with the time stamps logged on the FAST-16 system during data analyses. In order to maintain uniformity throughout all recording sessions, the ITI of 90 ± 30 s was implemented by randomly inserting ITIs lasting 60, 75, 90, 105 or 120 s.

Microelectrode Sensitivity *In Vivo*

After completion of recording sessions, 5, 10 and 20 nmoles of choline were infused through the guide cannula to determine the sensitivity of the microelectrode to choline (Fig S2A,C). Choline signal amplitudes and R^2 were

determined. In order for the recording data to be included into the final set of data, electrodes were required to generate a signal amplitude ≥1 μM following a 5 nmol infusion and, across the effects of all three concentrations, a linear response to increasing choline concentrations ($R^2 > 0.90$).

Additionally, and in order to confirm that the responses of the implanted microelectrode reflects choline resulting from the hydrolysis of endogenously generated ACh (Parikh et al., 2004; Parikh and Sarter, 2006), the effect of neostigmine, an acetylcholinesterase (AChE) inhibitor, on potassium-evoked choline signals was determined in three animals. KCl (70 mM; 250 nL) was infused through the guide cannula in the presence and absence of neostigmine (co-infused at 250 pmoles; Figure S2B,D).

Choline Signal Analysis and Group Sizes

Data for final analyses was extracted from 26 animals divided into five main groups (this excludes recordings from deafferented animals; see below). Group I (n = 6) and II (n = 5): recordings from mPFC and motor cortex, respectively, in animals performing the cued appetitive response task involving the long cue-reward interval (6 ± 2 s). Group III and IV (n = 3, each): recordings from mPFC and motor cortex, respectively, from trained but non-performing animals (task not turned on to determine potential effects of training context). Group V (n = 5): recordings from medial PFC from animals trained in a version of this task using a shorter cue-reward interval (2 ± 1s; see Figure S1A). Group VI (n = 4): recordings from medial PFC from animals during task acquisition.

Self-referencing. The recordings obtained from ChOase-coated channels were self-referenced against responses from non-ChOase-coated channels to yield currents that selectively indicate changes in choline concentration. The self-referencing procedure involves normalization of currents recorded via all 4 sites based on the currents produced by DA during electrode calibration *in vitro*. The normalized currents recorded on non-ChOase-coated sites were then subtracted from normalized current recorded via ChOase-coated channels. Self-referenced currents were then converted to equivalent values of choline concentration in accordance with the linear calibration function (Burmeister and Gerhardt, 2001; Parikh et al., 2004; Parikh and Sarter, 2006).

Event-evoked cholinergic activity. From each session, amperometric data from 10 trials (5 with and 5 without cue detection) were analyzed. In order to select trials that spanned the entire session, all trials were grouped into five 8-min blocks. From the total of five trials that occurred on average during each 8-min block, data from one trial involving cue detection and one involving a missed cue were selected for analysis.

Self-referenced amperometric recordings were boxcar-averaged over 1-3 data points to minimize noise levels (determined by averaging the difference between two successive data points across the 10 s pre-cue baseline) to ≤200 nM. Seven parameters were derived for the analysis of event-evoked choline signals: 1) cue-evoked choline signal peak amplitudes (μM) were calculated based on the highest choline levels observed during the interval between cue presentation and reward delivery (6 ± 2 s or 2 ± 1 s) relative to the average

choline levels during a two-second period prior to cue presentation; 2) signal decay rate for cue-elicited choline signals (t_{50}; time required for the signal to decline by 50% of peak amplitude); 3) to determine whether the cue-evoked cholinergic transients reflected presynaptic ACh release, PFC choline signals following cholinergic deafferentation were recorded over a total of 16 s per trial (8 s pre-cue and 8 s post-cue) and expressed against the average choline signal level during a 2-s pre-trial period. The resulting data were averaged, maintaining the 2 Hz resolution, over all trials that formed the basis for the final analysis (above); comparisons between recordings from sessions with long versus short cue-reward intervals were conducted likewise; 4) the time elapsed from cue presentation to cue-evoked choline signal peak amplitude; 5) to test the possibility that increases in cholinergic activity, in addition to or as opposed to, peak amplitudes, index a behavioral or cognitive operation, the time point at which the cue-evoked increases in cholinergic activity increased by 25% relative to the pre-cue 2-s average; 6) reward delivery-evoked signal amplitudes (μM) were calculated on the basis of highest signal levels observed during a 5-s period after reward delivery, relative to the average of signal levels during a 2-s period prior to this event; 7) to determine whether the direction of the slope of cholinergic activity prior to the cue predicts cue detection, the slope of choline signal levels over a 20 s period prior to cue presentation was determined based on boxcar-filtered (over 20 points) data and subsequent linear regression.

Session-related changes in cholinergic activity. With the beginning of the first trial, we observed slow or “tonic” changes in choline signal levels (on the

scale of minutes). Cue-evoked transient increases in cholinergic activity were superimposed over these session-related changes. Session-related changes in cholinergic activity were determined by boxcar-averaging self-referenced choline levels over 20 points. Choline levels were determined for each min and expressed as change from pre-session baseline levels (average of data points over the last 3 min prior to the first trial). To determine whether session-related increases in choline levels correlate with performance measures averaged over blocks of trials, and with microdialysis data, changes in choline signal levels, expressed against 3-min pre-session baselines, were averaged over 8-min blocks. Additionally, to further confirm that task performance is necessary for eliciting tonic increases in cholinergic activity, and to determine whether changes in choline levels vary over blocks of trials, 3-min pre-session baselines were divided into two baselines of 90 s each (BL1 and BL2); averages across BL2 and block of trials were expressed as a change from BL1 for each session.

To reveal potential relationships between session-related and cue-evoked cholinergic activity, correlations between the peak amplitudes of cue-evoked signals and a 2-s pre-cue period, both expressed against the average of a 3-min pre-session baseline, were analyzed (methods used compare session-related tonic changes in cholinergic activity with microdialysis release data, including the methods used for the microdialysis experiments, are described in Supplemental Materials).

Boxcar-Filtering. Boxcar averaging (or filtering) represents a method for reduction of noise. Moving averages are calculated over a defined number of

data (also called weighted averaging). For example, an averaging interval of 5 would mean that the value x would be replaced by the average over t,u,v,w,x, and that the subsequent value y would be replaced by the average over u,v,w,x,y, and so forth (e.g., Cook & Miller, 1992).

Cholinergic Transients During Acquisition

Rats (n=4) were habituated in the behavioral apparatus and trained to retrieve food pellets at a randomly selected port as described above. This initial shaping step lasted for up to 14 sessions. Thereafter, choline-sensitive microelectrodes were implanted in the mPFC. Rats were allowed to recover for 48 hrs and then trained for two additional sessions to retrieve reward at baited ports (25 reward deliveries/session). Amperometric recordings were conducted during three sessions (postsurgery days 5-7) of training of the final version of the task (above). Each session consisted of 25 trials (1-s cue followed by reward delivery 6 ± 2s later; ITI: 90 ± 30s). As trials involving detected cues emerged during the second half of the 3rd session, the analysis of detected cue-evoked signals was based on a total of ten trials classified as such (3+3+2+2).

Microdialysis Methods

*S*eparate groups of rats (n = 4 per group) were trained to criterion and then underwent surgery for microdialysis guide cannula implantation. Guide cannula (MAB 4.15.IC; SciPro, Sanborn, NY) were implanted either into the right mPFC (n = 4; AP: +3.0 mm; ML: -0.6 mm, DV: -0.6 mm) or motor cortex (n = 4; AP: +1.6

mm; ML: -1.6 mm (50º side angle), DV: -0.6 mm). In order to prevent clogging of the cannula shafts, stainless steel stylets were inserted into the microdialysis guide cannula. After surgery, rats were returned to their home cages and allowed to recover for 7 days with free access to food and water. Following the post-operative recovery period, the food deprivation schedule was resumed and animals were returned to behavioral training until they regained criterion performance. At this stage, stylets were removed and polyethylene tubing was attached during additional training sessions in order to habituate the animals to the final microdialysis testing conditions. Once the animals regained stable performance, dialysates were collected during two sessions per animal. Results from previous experiments indicated that cortical ACh release remains highly dependent on neuronal membrane depolarization for three test sessions and repeated probe insertions (Moore et al., 1999; Moore et al., 1995). Each dialysis session started by the removal of the stylets and an insertion of concentric probe with a 3.0 mm membrane tip (Model: MAB4; membrane o.d. 0.24 mm; SciPro Inc. Sanborn, NY). Prior to insertion, probe efficiency was determined *in vitro*, by placing the probe into a solution containing 1.0 pmol of ACh and taking one 10-min collection. Probes were perfused at a rate of 2.0 µL/min with artificial cerebrospinal fluid (aCSF), pH 6.9 ± 0.1, containing the following (in mM): 126.5 NaCl, 27.5 $NaHCO_3$, 2.4 KCl, 0.5 NA_2SO_4, 0.5 KH_2PO_4, 1.2 $CaCl_2$, 0.8 $MgCl_2$ and 5.0 glucose. Note that the perfusion medium did not contain an acetylcholinesterase inhibitor. Probes with recovery rates of >8% were used. Rats were placed into the operant chambers for 212 min prior to task onset;

probes were perfused for 180 min to allow ACh efflux to stabilize. Collections of samples began 32 min prior to the task onset. Dialysates were collected every 8 min. The last three collections prior to task onset were used to calculate basal ACh efflux. Following the onset of the task, the timing of dialysates collections was adjusted to correct for the dead volume of the probe and outlet tubing. Five samples were collected while the animal performed the task. Three additional dialysates were collected following the completion of the task and while animals remained in the chamber. Thereafter, microdialysis probes were removed and stylists inserted, and the rats were returned to their cages.

Dialysate samples were frozen at –80º C until analyzed for ACh levels by high performance liquid chromatography (HPLC) with electrochemical detection (ESA, Chelmsford, MA) as described earlier (Kozak et al., 2006). The detection limit of this system averaged 2.0 fmol/13 µL injection. ACh levels are expressed as percentage ± SEM of baseline. Baseline was defined as the average of four dialysis samples collected immediately before the start of behavioral testing. Dialysis data were transformed as described above to allow statistical comparisons with session-related changes in choline signal levels measured amperometrically.

Comparison between Minute-Based Tonic Changes and Microdialysis data

To determine whether such session-related changes in choline signal levels corresponded with changes in ACh release as measured by microdialysis (below) and performance measures, microdialysis release data and

amperometric data first needed to be standardized to generate dimension-free data allowing statistical comparisons. For this purpose, the mean (M) and standard deviation (SD) of amperometric data from a 3-min pre-session period and the pre-session microdialysis baseline data were calculated. Session-related amperometric data and release data (x) were then transformed into t-scores ($t = (x - M)/SD$) to express session-related changes in cholinergic activity, generated by both methods, in terms of the number of SDs based on the baseline distribution. For example, a t-score of 2.3 would indicate that the value was 2.3 SDs above the mean of the baseline data. The average of transformed choline signal values over 8-min blocks was calculated and compared with transformed ACh release data obtained from 8-min microdialysis collection intervals.

Effects of Bilateral Removal of Cholinergic Inputs to Motor Cortex

To determine whether cholinergic innervation of the motor cortex is necessary for the performance of the cued appetitive response task, the effects of bilateral removal of cholinergic inputs into the motor cortex on performance were tested in a separate group of animals ($n = 5$). Cholinergic deafferentation of the motor cortex was produced by infusing 192-SAP (100 ng/0.5 µL) bilaterally at two sites per hemisphere (AP: +1.5 and 0.0 mm; ML: ± 3.75 mm; DV: -1.7 mm; see also Conner et al., 2005). Similar to the experiment on the effects of cholinergic deafferentation of the mPFC on performance, cholinergic inputs were removed after animals reached criterion performance (see above for additional details concerning surgical methods). Following two days of post-surgery recovery, with

food and water available *ad libitum*, animals were returned to the deprivation regimen and daily test sessions. Animals' performance was assessed daily for an additional three weeks. Sessions were videotaped once a week for behavioral analysis.

Histology and AChE Histochemistry

After completion of the experiments, animals were given an overdose of sodium pentobarbital and transcardially perfused with 100 mL of ice-cold heparinised saline followed by 300 mL of 4% paraformaldehyde in 0.1M PBS (pH 7.4). The brains were removed and post-fixed overnight at 4ºC and stored in 30% sucrose in 0.1M PBS for 72 hrs. Coronal sections (50 µm) were cut using a freezing microtome (Leica CM 2000R, Leica Microsystems Inc., Chantilly, VA) and stored in cryoprotectant solution (30% glucose, 60% ethylene glycol and 0.04% sodium azide in 0.05M PBS, pH 7.4) at -20ºC until further processing. Serial sections from mPFC (between 3.7 and 2.7 mm anterior to bregma) and motor cortex (1.0-1.5 mm anterior to bregma) were either Nissl-stained or processed for the histochemical visualization of AChE-positive fibers. Microelectrode/dialysis probe placements were verified based on Nissl-stained sections. Loss of cholinergic inputs to the cortex were documented and quantified (below) on the basis of sections stained for visualization of AChE-positive fibers using a modification of Tago's method (Tago et al., 1986) that utilized tetraisopropylphosphoramide to inhibit nonspecific butylcholinesterases (Burk et al., 2002). AChE-positive fiber density in the medial PFC was quantified using a grid counting technique

described earlier (Burk and Sarter, 2001; McGaughy et al., 1996), employing a Leica DM 4000B digital microscope (Nuhsbaum, McHenry, IL). AChE-positive fibers were counted in layers III/IV and based on an area of 0.16 mm^2 at 400X magnification and expressed as average count from three sections.

Statistical Analyses

Statistical analyses were performed using SPSS/PC+ (V13.0; SPSS, Chicago, IL). Repeated measure mixed factor ANOVAs were used to analyze the effects of group (intact and unilateral lesion, two levels; pre-lesion (bilateral) and post-lesion; four levels), task (standard and shorter cue-reward interval; two levels) and trial blocks (five levels) on behavioral performance. *Post-hoc* multiple comparisons for analysis of significant main effects were performed using Least Significance Difference (LSD) test or independent t-tests. One way ANOVAs or planned multiple two-tailed unpaired t-tests were employed to test group differences with respect to the proportion of detected cues, reward retrieval latencies, and port approach frequencies. The effect of trial blocks on the proportion of cue that were detected was examined using one-way ANOVA.

Repeated measures ANOVA with group (intact versus unilateral lesion; two levels), task version (standard and shorter cue-reward interval; two levels) and recording region (mPFC and motor cortex; two levels) as between-subject variables were used to analyze choline signal changes across pre- and post-cue periods. Group- (intact versus lesion, performing versus non-performing, both two levels) and region- (two levels) based comparisons on session-related

changes in cholinergic activity were analyzed using repeated measure mixed factor ANOVAs. Changes in session-related choline signal levels over blocks of trials were analyzed using one-way ANOVAs. For the comparison between session-related choline signal levels and microdialysis ACh release data, the effects of method (amperometry and dialysis), collection interval and region were analyzed using repeated measure ANOVAs and standardized data (described above). Multiple comparisons were carried out using LSD or multiple *t*-tests. To determine whether the slopes (positive or negative) of the tonic changes in choline levels predict subsequent cue detection, a 2x2 contingency table was analyzed using the χ^2 test. Correlations between the accordance of cue-evoked behavioral changes and increases in choline levels, between session-related tonic levels and the amplitude of cue-evoked cholinergic transients, and between tonic levels and performance measures or port approach frequency were analyzed using Pearsons' r and testing correlation coefficients for statistical significance. The effects of 192-SAP on the density of AChE-positive fibers were determined using two-tailed unpaired Student's t-test (alpha = 0.05 for all statistical tests). Exact p-values were reported (Greenwald et al., 1996).

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