

Association learning-dependent increases in acetylcholine release in the rat auditory cortex during auditory classical conditioning

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ABSTRACT

The cholinergic system has been implicated in sensory cortical plasticity, learning and memory. This experiment determined the relationship between the acquisition of a Pavlovian conditioned approach response (CR) to an auditory conditioned stimulus (CS) and the release of acetylcholine (ACh) in the primary auditory cortex in rats. Samples of ACh were collected via microdialysis during behavioral training in either an auditory classical conditioning task or in a non-associative control task. The conditioning group received daily pairings of a white noise CS with a sucrose pellet unconditioned stimulus (US), while the control group received an equal number of CS and US presentations, but with these stimuli being presented randomly. Training was conducted on three consecutive days, with microdialysis samples being collected on Days 1 and 3 in separate sub-groups. The level of ACh released in the auditory cortex during conditioning trials increased from the first to the third day of training in the conditioning group as rats acquired the CR, but did not change in the control group, which did not acquire a CR. These data provide direct evidence for the hypothesis that ACh release increases in the primary auditory cortex during natural memory formation, where cholinergic activation is known to contribute to the formation of specific associative representational plasticity in conjunction with specific memory formation.

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1. Introduction

Cholinergic activation of the neocortex is necessary in many aspects of cognition, learning and memory. Acetylcholine (ACh) is released in the neocortex by the cholinergic neurons of the nucleus basalis magnocellularis (NBM; Mesulam, Mufson, Wainer, & Levey, 1983), where NBM activity is involved in modulating attention (e.g., Chiba, Bucci, Holland, & Gallagher, 1995; McGaughy, Dalley, Morrison, Everitt, & Robbins, 2002; Sarter, Bruno, & Givens, 2003; Waite, Wardlow, & Power, 1999), contributing to cognitive flexibility (Butt et al., 2003; Cabrera, Chavez, Corley, Kitto, & Butt, 2006; De Bartolo et al., 2008), supporting configural association learning (Butt & Bowman, 2002; Butt & Hodge, 1997; Butt, Noble, Rogers, & Rea, 2002), and in the acquisition of socially acquired food preferences (Berger-Sweeney, Stearns, Frick, Beard, & Baxter, 2000) and taste aversions (Miranda, Ferreira, Ramirez-Lugo, & Bermudez-Rattoni, 2003).

The release of ACh within the primary sensory cortices during conditioning may contribute to the storage of specific information

about the acquired behavioral importance of conditioned stimuli. For example, it has long been known that neural responses (e.g., as measured using electro-encephalogram) to conditioned stimuli increase in the primary sensory cortex corresponding to the conditioned stimulus (CS) modality during classical conditioning (reviewed in John, 1967; Morrell, 1961). Similarly, neurophysiological models of conditioning have shown that pairing a somatosensory stimulus with electrical stimulation of the NBM, which causes the release of ACh, produces non-transient facilitation of CS-elicited responses in the primary somatosensory cortex (Rasmusson & Dykes, 1988; Tremblay, Warren, & Dykes, 1990; Verdier & Dykes, 2001). Moreover, such cholinergically-induced long-term response enhancement is associative (Webster et al., 1991; reviewed in Dykes, 1990, 1997). Plasticity has also been demonstrated in the primary visual cortex in animals undergoing paired NBM stimulation and visual sensory stimulation, where this plasticity was blocked by administration of the muscarinic receptor antagonist scopolamine (Dringenberg, Hamze, Wilson, Speechley, & Kuo, 2007; see also Gu, 2003).

Most extensively studied in the primary auditory cortex, highly-specific CS plasticity has been demonstrated by observation of the changes in neuronal receptive fields before and after the formation

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of CS–US associations. For example, frequency tuning in the primary auditory cortex is shifted toward or to the frequency of a tonal CS (e.g., Bakin & Weinberger, 1990; Edeline & Weinberger, 1993), which causes an expansion in the representational area of the CS frequency band (Recanzone, Schreiner, & Merzenich, 1993; Rutkowski & Weinberger, 2005). Such “specific associative representational plasticity” (SARP) has the major attributes of behavioral memory: associativity, rapid development, specificity, consolidation over hours and days, and retention for weeks or months (reviewed in Weinberger, 1998). Such SARP can also be induced in the primary auditory cortex when a tone is paired with electrical stimulation of the NBM to activate the cortex (Bakin, South, & Weinberger, 1996; Bjordahl, Dimyan, & Weinberger, 1998; Kilgard & Merzenich, 1998).

It has recently been shown that pairing a tone with NBM stimulation not only induces CS-specific cortical plasticity as described above, but also causes CS-specific and associatively-dependent changes in conditioned behavior itself (McLin, Miasnikov, & Weinberger, 2002; Miasnikov, Chen, & Weinberger, 2006; Weinberger, Miasnikov, & Chen, 2006). Such memory, which depends on cholinergic activation of muscarinic receptors (Miasnikov, Chen, & Weinberger, 2008), has the major attributes of natural memory (Weinberger, 2003). Collectively, these findings provide support for a model stating that activation of cholinergic receptors is sufficient to induce associative plasticity in the primary auditory cortex, which is part of the substrate of specific behavioral auditory memory (Weinberger et al., 1990).

Although the evidence demonstrating involvement of the cholinergic system in learning and memory is compelling, there is currently a lack of direct evidence that ACh is released in the auditory cortex during natural associative learning. The goal of the current experiment is to test the hypothesis that ACh levels are elevated in the primary auditory cortex in parallel with the acquisition of an auditory association during classical conditioning. The relationship between ACh efflux in the primary auditory cortex and the acquisition of a Pavlovian conditioned approach response (CR) to a white noise CS paired with the delivery of a sucrose pellet unconditioned stimulus (US) is examined. White noise was chosen to serve as the CS in order to activate the maximum amount of the auditory cortex (e.g., Phillips, Orman, Musicant, & Wilson, 1985; Recanzone, 2000). It was expected that as rats in the conditioning group learned about the behavioral significance of the auditory CS, they would develop progressively greater levels of training-induced ACh release in the primary auditory cortex compared to rats in the control group. In contrast, for rats in the control group, where the auditory CS lacked predictive value, training-induced levels of ACh were not expected to increase across test days.

2. Materials and methods

2.1. Guidelines for animal use

The following procedures meet the requirements set forth by the Society for Neuroscience, the American Psychological Association, the National Research Council, and the California State University San Bernardino Animal Care and Use Committee.

2.2. Subjects

Subjects were 32 adult male Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing approximately 300 g upon arrival. One week prior to initiating behavioral procedures, rats were placed on a food-deprivation schedule to reduce weight to 85% of their free-feeding baseline weight. Rats were handled daily to reduce stress prior to microdialysis testing. Subsets of rats underwent microdialysis testing on either Day 1 (D1) or Day 3 (D3) of

the three days of classical conditioning or control procedures. Thus, individual rats were randomly assigned to either the conditioning D1 sub-group (COND-D1; $n = 9$), the conditioning D3 sub-group (COND-D3; $n = 9$), the control D1 sub-group (CTL-D1; $n = 7$), or the control D3 sub-group (CTL-D3; $n = 7$). The time line of experimental procedures carried on with these sub-groups is shown in Fig. 1 and described in detail below.

2.3. Surgery

Surgery was conducted two days before beginning behavioral training. Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Sigma) and placed in a stereotaxic frame (Kopf, Tujunga, CA). The scalp was incised and a craniotomy was made over the left primary auditory cortex at -4.8 mm posterior to Bregma and 2.7 mm lateral to midline (Paxinos & Watson, 1998). An electrode (model SNEX-100x, 13 mm; Rhodes Medical Instruments, Inc., Summerland, CA) was lowered at a 24° angle through the craniotomy ventro-laterally away from midline towards the auditory cortex in the left hemisphere. The auditory cortex was located by mapping auditory evoked potentials along the trajectory of the electrode. Auditory clicks (500 μ s, 90 dB SPL; stimulator model SD9, Grass Technologies, West Warwick, RI) were presented at 1 s intervals to the contralateral ear via a calibrated miniature speaker. Electrophysiological recording of the amplified (model DP-301; Warner Instrument Corp., Hamden, CT) signal continued as the electrode traveled ventro-laterally from the site of the first auditory evoked potential onset to the site where no further evoked potentials were detected. A minimum of 2 mm of cortex showing auditory responsiveness was located using this method. The 2 mm range of cortical tissue containing the largest evoked potentials was selected for subsequent placement of the 2 mm

Day	Procedure
P1	Sucrose Pre-exposure
P2	Food cup & tether training
P3	Surgery
P4	Recovery
D1	Microdialysis testing: Day 1 Sub-groups
D2	Behavioral testing: All sub-groups
D3	Microdialysis testing: Day 3 Sub-groups

Fig. 1. Experimental time line. On the first pre-test day (P1) rats were fed sucrose pellets in their home cage to reduce neophobic responses to this novel food. On the second pre-test day (P2), rats were tethered and placed in the testing chambers for 1 h with 10 sucrose pellets in the food cup to habituate animals to the tether and the testing environment and to allow them to discover the location of the food cup and consume the sucrose pellets. Food cup training was conducted in order to facilitate subsequent acquisition of conditioned food cup approach. Rats were then surgically implanted with microdialysis probe guides on the third pre-test day (P3) and allowed approximately 48 h to recover (P4–D1) prior to initiating behavioral training. Classical conditioning or control procedures were carried out on all sub-groups for three consecutive days (D1–D3; grey panels). The conditioning and control D1 and D3 sub-groups underwent microdialysis testing procedures on first and third day of behavioral training, respectively.

tip of the microdialysis probe. Mean latencies for evoked potentials from the 2 mm target range for the microdialysis probe did not differ between sub-groups ($F_{(1,3)} = 0.47, p > 0.05$). The grand mean latency for evoked potentials across all rats used in this study was 7.3 ms. Representative auditory evoked potentials are shown in Fig. 2.

Following the trajectory of the electrode used for evoked potential recordings, microdialysis probe guides (MAB 4.15.IC; SciPro Inc., Sanborn, NY) were inserted under stereotaxic control into the cortex until the end of the microdialysis probe guide came to rest at the top of the 2 mm range of the auditory cortex identified for subsequent probe placement. Probe guides were secured using dental acrylic anchored to the skull via three small stainless steel screws. The incision was cleaned and sutured and rats were immediately administered an antibiotic (Baytril 2.5 mg/kg, s.c.; Western Medical Inc., Anaheim, CA) to guard against infection and an analgesic (Ketaprofin 2 mg/kg, s.c.; Western Medical Inc., Anaheim, CA) for pain management.

2.4. Apparatus

Experiments were conducted in four computer-controlled operant chambers housed in sound-attenuating enclosures (model H12-24A; Coulbourn Instruments, Allentown, PA). Each chamber was equipped with a calibrated speaker (model H12-O1R, Coulbourn Instruments, Allentown, PA) attached to an audio signal generator (model A12-23; Coulbourn Instruments, Allentown, PA) for presenting the 10 s white noise CS (10 ms sine-shaped rise, 80 dB). Sucrose pellets (45 mg; Bio-Serv, Frenchtown, NJ) were delivered into a magazine (model ENV-200R3 M; MedAssociates, Lancaster, NH) located in one corner of the testing chamber at floor level. Snout entries into the food magazine were recorded by photo-beam response detectors (model ENV-254; MedAssociates, Lancaster, NH) positioned at the entrance to the food magazine. An infrared motion sensor (model H24-61; Coulbourn Instruments, Allentown, PA) was placed on a side wall of the chamber near the food magazine to detect whole body movement. The presentation of white noise, delivery of sucrose pellets, detection of snout entries into the food cup, and measures of whole body movement were computer-controlled (Graphic State software; Coulbourn Instruments, Allentown, PA).

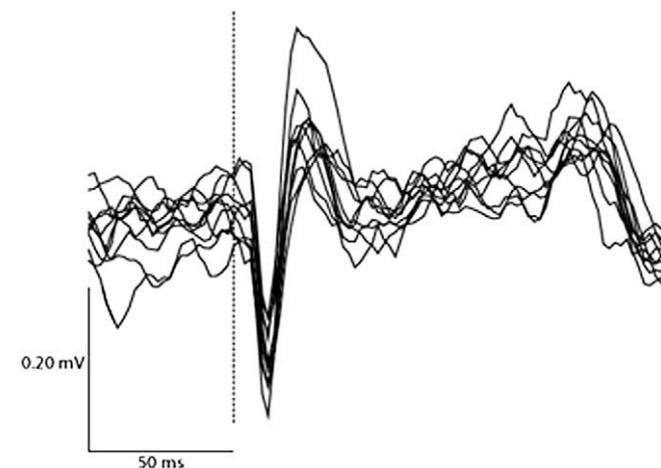


Fig. 2. Representative auditory evoked potentials recorded during microdialysis probe guide implantation surgery. Evoked potentials with short latencies (dashed line indicates stimulus onset) were recorded at multiple depths, with a minimum of 2 mm of cortical tissue showing reliable evoked potentials. This range was used to determine subsequent microdialysis probe placement so that when fully inserted into the probe guide, the probe membrane extended directly into the primary auditory cortex.

Microdialysis probes were perfused using an infusion pump (model MD 1001; Bioanalytical Systems Inc, West Lafayette, IN) located outside the chamber housing. A motor-assisted liquid swivel (model MCS/5A, Instech Laboratories, Plymouth Meeting, PA) was attached to a counter-balanced arm near the top of the testing chamber, with the rat tether hanging down from the swivel. Two channels on the liquid swivel were utilized in the current experiment, with one input and one output channel attached to the microdialysis probe on microdialysis test days. The tethering system allowed rats to move freely while placing only minimal strain on the microdialysis tubing on microdialysis testing days.

The original operant chambers were modified by adding 30 cm high aluminum wall extensions, an aluminum roof fitted with a 5 W white light bulb to provide ambient illumination, and an extended (30 cm additional height) front Plexiglas door in order to allow room for the microdialysis equipment described above. The enlarged test chamber provided enough room for the motorized swivel and counter-balanced arm and created an enclosed testing environment from which the rat could not escape. The side wall of each chamber had a small hole to allow passage of microdialysis tubing and to allow the counter-balanced arm to extend outside of the enclosed chamber.

2.5. Behavioral procedures

Two days prior to surgery, rats were pre-exposed to 20 sucrose pellets in their home cage to reduce neophobic responses to the pellets during subsequent training. The following day, each rat was habituated to the tether, motorized liquid swivel, and counter-balanced arm by placing a light-weight collar around the rat's neck, attaching the collar to the tether, and placing the rat in the operant chamber for 1 h. Over the course of this food cup and tether training session, rats were allowed to freely consume 10 sucrose pellets placed in the food cup, leading them to expect food in the food cup during subsequent conditioning or control procedures. The following day, rats underwent microdialysis probe guide implantation surgery. Rats were then allowed 48 h to recover from surgery prior to beginning behavioral training.

During behavioral training, rats were tethered in the operant chambers daily, although microdialysis procedures were conducted only on either D1 or D3 according to their sub-group assignments. Prior to commencing conditioning or control procedures, rats in each group remained in the chambers under dim illumination for 1–4 h on microdialysis testing days as described below) to acclimate them to the testing environment. Preliminary observations show that CR is acquired more reliably when this acclimation period precedes the beginning of the conditioning session. Additionally, the acclimation period reduces variability in procedure on microdialysis test days when rats must spend an additional 3 h undisturbed in the chambers in order to allow ACh levels around the newly inserted microdialysis probe to stabilize prior to microdialysis sampling (see Bruno, Sarter, Arnold, & Himmelheber, 1999).

The training protocols for the conditioning and control groups are illustrated in Fig. 3. The COND-D1 and COND-D3 sub-groups received two blocks of 30 CS-US pairings (60 total trials) on each of three consecutive days. CS-US pairings were separated by an average inter-trial-interval (ITI) of 50 s. The CTL-D1 and CTL-D3 sub-groups similarly received two blocks of 30 presentations each of the CS and US (60 trials in total) on each of three consecutive days. Controls were exposed to an equal number of CS and US presentations, but with these stimuli being presented pseudo-randomly and independently such that the CS was not a reliable predictor of the US (average ITI of 25 s). The control condition had a restriction of no more than three successive presentations of the same stimulus type, with stimuli occurring no closer than 10 s apart,

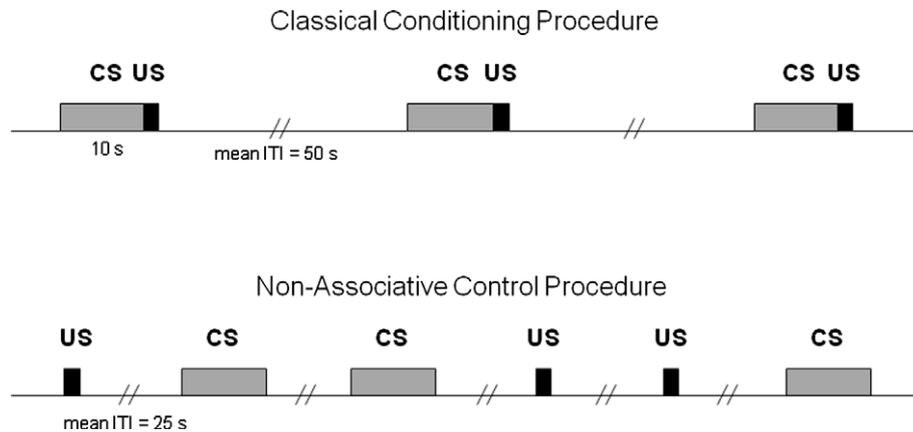


Fig. 3. Training protocols for animals in the conditioning and control groups. Rats in the conditioning protocol received two blocks of 30 trials where a 10 s white noise CS was paired with a sucrose pellet US delivery on each of three consecutive days of training. The control group received the same number of CS and US presentations, but these stimuli were presented at random such that the CS was not a reliable predictor of the US.

and with an equal number of CS and US presentations within each half hour of the training session. Upon completion of daily training, rats were returned to their home cages and fed a restricted amount of standard rat chow as described above. Water was freely available in the home cage but unavailable during behavioral training in the operant chambers.

2.6. Microdialysis procedures

On either D1 or D3 of training, microdialysis probes (model MAB 4.15.2.PES; SciPro Inc., Sanborn, NY) were inserted into the probe guides and rats were tethered and placed in the chambers as usual. Using an externally-located, motorized infusion syringe (model MD-1000 N; Bioanalytical Systems Inc., West Lafayette, IN), probes were continuously perfused (1 μ l/min) with artificial cerebrospinal fluid (148 mM NaCl, 4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, pH 6.9) containing a low dose (50 nM) of the acetylcholinesterase inhibitor neostigmine bromide (see Chang, Savage, & Gold, 2006). Following probe insertion, ACh levels were allowed to equilibrate for 3 h prior to collecting microdialysis samples (see Bruno et al., 1999). Immediately after this equilibration interval, two 30 min baseline ACh samples were collected via an automated, refrigerated fraction collector (model CMA 470; CMA Microdialysis, North Chelmsford, MA). No stimuli were presented during this baseline interval. Finally, conditioning or control procedures began with two 30 min ACh samples being collected over the course of the training session. Upon completion of training, microdialysis samples were immediately frozen and stored at -80°C .

2.7. Acetylcholine assay and quantification procedures

Quantification of ACh in the dialysates was accomplished using high-performance liquid chromatography with electrochemical detection (Coulechem II; ESA Inc., Chelmsford, MA). Briefly, 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 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. Absolute levels of ACh in the microdialysis samples were quantified by integrating the area under the ACh peak and comparing it to a standard curve based on the areas for a range of known standard values of ACh.

2.8. Histology

Upon completion of behavioral training, rats were euthanized by lethal dose of sodium pentobarbital (80 mg/kg, i.p.; Sigma, St. Louis, MO) followed by cardiac perfusion with 0.9% saline followed by 10% buffered formalin. Brains were extracted and placed in a 10% buffered formalin and 25% sucrose solution for 48 h prior to sectioning (80 μ m) and thionin staining for subsequent examination of probe placements.

2.9. Data analyses

2.9.1. Motor behavior

Previous studies have shown a relationship between ACh release in cerebral cortex and locomotor activity, although others have reported contradictory findings (see Pepeu & Giovannini, 2004). In order to rule out potential locomotor activity-dependent group differences in ACh efflux in the current experiment, the amount of movement exhibited during training was assessed in each sub-group by quantifying the number of "movement units" (arbitrary but fixed distance of movement of center of body heat profile across an internal detector grid) registered by the infrared motion detector located on the side of the testing environment. Movement measures were compared using repeated measures analysis of variance (ANOVA) to confirm that no differences in the amount of movement existed between the sub-groups in the conditioning or control conditions, respectively. Upon satisfying this condition, repeated measures ANOVAs were performed on the combined movement data from the D1 and D3 sub-groups in the conditioning and control conditions, respectively.

2.9.2. Conditioned responding

Rats in the conditioning sub-groups were subjected to performance analysis on the final day of training to confirm acquisition of the CR. Rats with cumulative (i.e., combined Blocks 5 and 6) D3 conditioning ratios (CS response duration/(CS response duration + Pre-CS response duration) less than 0.5 (i.e., chance perfor-

mance, where responding during the CS was no more likely than responding in the absence of the CS) were removed from subsequent behavioral or biochemical analyses. Conditioned responding in the remaining conditioning and control animals was then assessed using difference scores calculated by subtracting the total duration of responding (i.e., snout entry into the food magazine) during the 10 s pre-CS intervals from the total duration of responding during the 10 s CS intervals across each of the two 30-trial blocks on each day of training. Difference scores for each sub-group were compared via repeated measures ANOVA to confirm that no differences in behavior occurred between the D1 and D3 sub-groups in the conditioning or control conditions, respectively. Upon satisfying this criterion, repeated measures ANOVAs were performed on the combined behavioral data from the D1 and D3 sub-groups in the conditioning and control conditions.

2.9.3. Biochemistry

On microdialysis testing days, two 30-min ACh samples were collected during the hour immediately preceding behavioral training (i.e., baseline). Comparisons of absolute levels of ACh released during the two 30-min periods during the baseline interval in the conditioning and control groups on D1 and D3 were conducted using ANOVA to test for potential pre-existing differences in basal ACh release among the four sub-groups.

Absolute levels of training-induced ACh release were also obtained from samples collected during the two 30-min, 30-trial blocks for both the conditioning and control D1 and D3 sub-groups. Mean absolute training-induced ACh levels across the two blocks of training on D1 or D3 were compared to mean absolute baseline ACh levels on the corresponding day for each group using two-tailed Student's paired *t*-tests in order to determine whether behavioral training and exposure to the auditory CS caused a within-group increase in absolute levels of ACh release relative to baseline release.

Training-induced levels of ACh were also expressed as a percentage of the mean level obtained during the baseline interval

for each sub-group. Thus, the percent of baseline ACh release obtained during training was calculated as follows: Percent baseline ACh release = $100 \times (\text{training-induced ACh release} / \text{mean baseline ACh release})$. Separate comparisons were made between training-induced percent of baseline ACh release across the two 30-min training blocks in the conditioning and control sub-groups tested on D1 (Blocks 1 and 2) or D3 (Blocks 5 and 6) using ANOVA. Finally, repeated measures ANOVA was used to assess potential changes in ACh release across test days; training-induced ACh release, expressed as the percent of baseline, was compared between D1 and D3 sub-groups within the conditioning and control conditions, respectively.

3. Results

3.1. Histology

Probe placement was determined based on the auditory evoked potential measures obtained during surgical implantation of microdialysis probe guides. Histological verification confirmed that probes were located in the primary auditory cortex in all animals (see Fig. 4).

3.2. Motor behavior

No significant differences in motor behavior were observed between the COND-D1 and CTL-D1 sub-groups on their day of microdialysis testing ($F_{(1,13)} = 0.65$, $p > 0.05$). A significant within-group effect ($F_{(1,13)} = 10.41$, $p < 0.01$), however, reflected the increase in motor activity seen in both groups across the first two blocks of training on D1. The COND-D3 and CTL-D3 sub-groups also did not differ in motor behavior on their day of microdialysis testing ($F_{(1,12)} = 2.30$, $p > 0.05$). Comparisons of the combined groups across all six blocks of training revealed a significant Group \times Block interaction ($F_{(5,130)} = 4.40$, $p < 0.01$), where the combined control group showed progressively greater levels of motor activity across

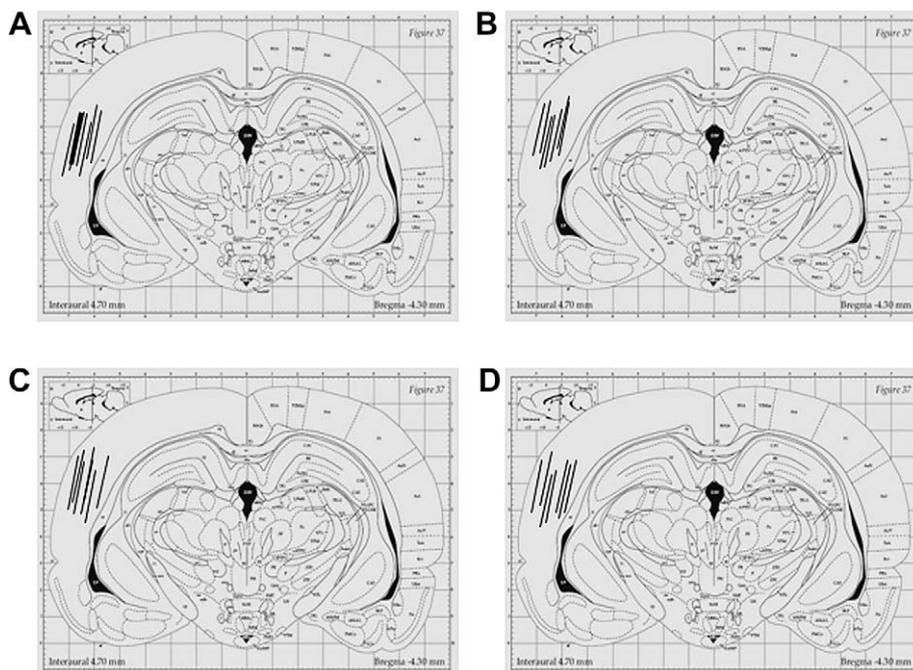


Fig. 4. Microdialysis probe locations for the COND-D1 (A) and COND-D3 (B) sub-groups, and CTL-D1 (C) and CTL-D3 (D) sub-groups of rats. No systematic differences in probe placement were observed between sub-groups. Probes were consistently located at approximately AP -4.8 from Bregma (Paxinos and Watson, 1998). Probe placements were confined to neocortex.

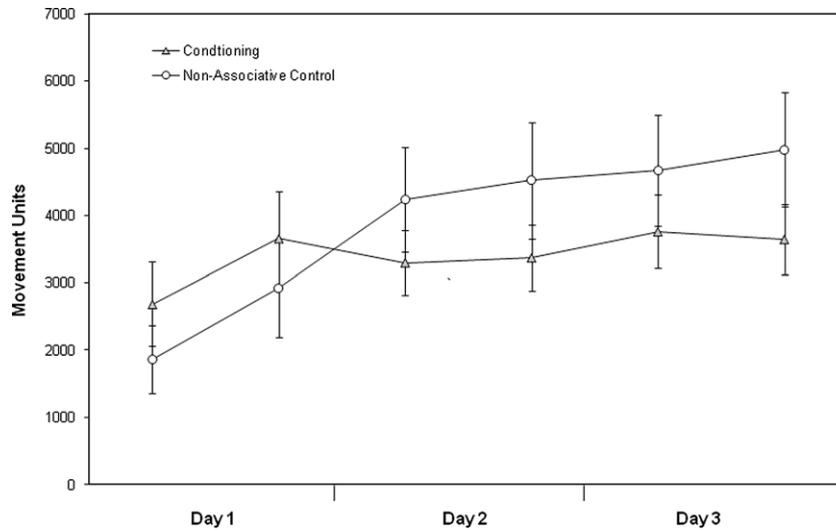


Fig. 5. Movement (mean \pm SEM) in the combined conditioning and control groups across all three days (six blocks) of behavioral training. Both groups showed increased movement across the first two blocks of training on D1 ($p < 0.01$), where these changes may reflect increased arousal associated with both auditory stimulation and the availability of sucrose pellets in the food cup. Perhaps as a reflection of contextual (rather than discrete CS) conditioning, the combined control group also showed a greater increase in movement levels across blocks compared to the combined conditioning control group ($p < 0.01$).

blocks of training compared to the combined conditioning group (see Fig. 5).

3.3. Conditioned responding

Three rats (one from the COND-D1 sub-group, and two from the COND-D3 sub-group) failed to demonstrate a reliable CR to the auditory CS on D3, as confirmed by conditioning ratios of less than 0.5. These animals were removed from subsequent behavioral and biochemical analyses. Behavioral data from all remaining rats in the conditioning and control sub-groups are described below.

CR difference scores in the COND-D1 and COND-D3 sub-groups did not differ when compared across all three days of training ($F_{(1,13)} = 2.72$, $p > 0.05$). Similarly, CR difference scores in the CTL-D1 and CTL-D3 sub-groups did not differ across the three days of training ($F_{(1,11)} = 0.01$, $p > 0.05$). Behavioral data from each sub-

group in the conditioning and control conditions were therefore combined for subsequent behavioral data analyses.

Behavioral performance in the combined conditioning and control sub-groups is shown in Fig. 6. The combined conditioning group showed significantly greater conditioned responding across the six training blocks on Days 1–3 compared to the combined control group; ANOVA yielded a Group \times Block interaction effect ($F_{(5,130)} = 10.65$, $p < 0.001$) and a between-group effect ($F_{(1,26)} = 182.72$, $p < 0.001$). Follow-up comparisons between the combined conditioning and control groups across the first two blocks of training on D1 revealed no significant between-group differences ($F_{(1,12)} = 3.54$, $p > 0.05$) in CR difference scores. Combined group comparisons of CR acquisition across the final two blocks of training on D3, however, revealed significantly greater CR difference scores in the conditioning group than in the control group ($F_{(1,12)} = 20.88$, $p < 0.001$).

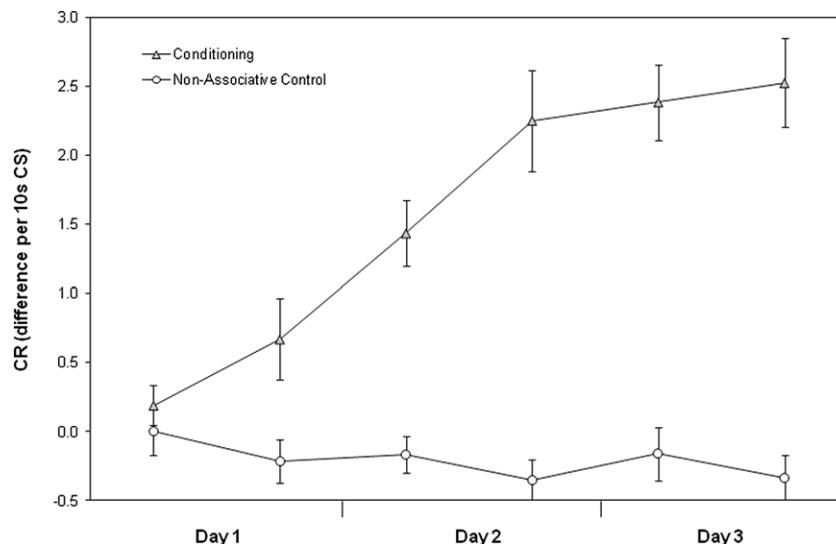


Fig. 6. CR difference scores (mean \pm SEM) in the combined conditioning and control groups across all three days (six trial blocks) of behavioral training. Although significant differences were not yet observed at the end of the first two blocks of training on D1, the combined conditioning group showed significantly greater conditioned responding than controls on Blocks 5 and 6 on D3 ($p < 0.001$). The combined conditioning group also showed progressively greater increases in conditioned responding across the three days of training compared to the combined control group, as revealed by a significant Group \times Block interaction ($p < 0.001$).

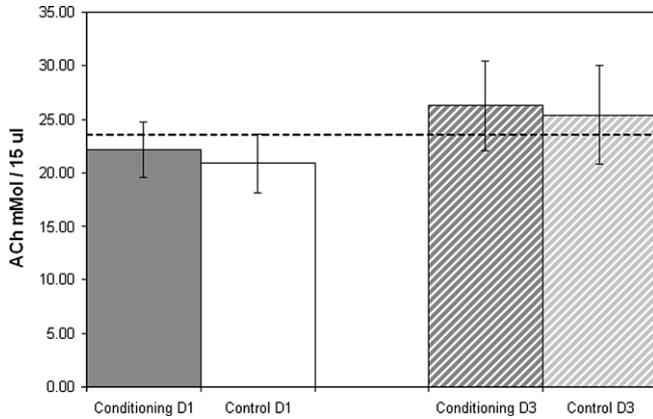


Fig. 7. Average absolute levels (mean \pm SEM) of ACh (fMol/15 μ l sample) released during the two 30 min baseline periods in the conditioning (COND) and control (CTL) D1 and D3 sub-groups (dashed line represents the grand mean across all groups). There were no significant differences in mean basal ACh release during baseline among the four sub-groups, regardless of microdialysis test day or training condition.

3.4. Biochemistry

The mean absolute levels of ACh during the two 30-min periods of the 1-h baseline interval on the microdialysis test day were COND-D1 = 22.18 f, CTL-D1 = 20.91, COND-D3 = 26.31, and CTL-D3 = 25.42 fMol/15 μ l (see Fig. 7). Comparisons of mean ACh release during baseline in the COND- and CTL-D1 and D3 sub-groups yielded no between-group differences ($F_{(3,29)} = 0.53$, $p > 0.05$). Given that mean absolute levels of ACh released during baseline did not differ among COND and CTL sub-groups, subsequent comparisons of training-induced ACh release (expressed as a percentage of mean baseline release) was warranted. Because ACh levels were ultimately converted to a percentage of baseline release, potential differences in microdialysis probe yield were controlled for. Additionally, the finding that absolute levels of baseline ACh

release did not differ suggests that there were no systematic differences in probe yield in the two groups.

Two-tailed paired student's t -tests comparing the mean absolute amount of ACh released during baseline and the mean training-induced absolute level of ACh were conducted for each sub-group. Training-induced levels of ACh exceeded baseline levels in the CTL-D1 ($t_{(6)} = 2.53$, $p < 0.05$) and CTL-D3 ($t_{(6)} = 4.05$, $p < 0.01$) sub-groups, and in the COND-D3 sub-group ($t_{(6)} = 7.33$, $p < 0.001$). Training-induced ACh levels did not exceed baseline levels in the COND-D1 sub-group ($t_{(7)} = 1.69$, $p > 0.05$).

Comparisons of training-induced ACh release expressed as a percent of baseline release in the conditioning and control sub-groups are shown in Fig. 8, along with corresponding behavioral data from each group. Training-induced ACh release (expressed as a percent of baseline release) in the conditioning and control D1 sub-groups did not differ across the first two blocks of training on D1 ($F_{(1,13)} = 0.11$, $p > 0.05$). Between-group comparisons of training-induced ACh release (expressed as a percent of baseline release) across the two blocks of training on D3, however, yielded significant differences between the conditioning and control D3 sub-groups ($F_{(1,12)} = 5.09$, $p < 0.05$); rats in the COND-D3 sub-group showed greater levels of training-induced ACh release than the CTL-D3 sub-group. This between-group difference was maintained when training-induced ACh release data (expressed as a percent of baseline release) were collapsed across Blocks 5 and 6; one-way ANOVA yielded a significant overall differences between COND-D3 and CTL-D3 ($F_{(1,26)} = 9.27$, $p < 0.01$).

As can be seen in Fig. 8, variability in the CTL-D3 group was comparatively high in Block 6. Although repeated measures ANOVA yielded significant between-group differences across repeated Blocks 5 and 6, and when collapsing across Blocks 5 and 6 as described above, further analysis of ACh release on individual training blocks on D3 were conducted to determine potential group differences on a block-by-block basis. Results from a one-way ANOVA comparing ACh release (expressed as a percent of baseline release) in the COND-D3 and CTL-D3 groups on Block 5 show significant between-group differences ($F_{(1,12)} = 7.78$, $p < 0.05$),

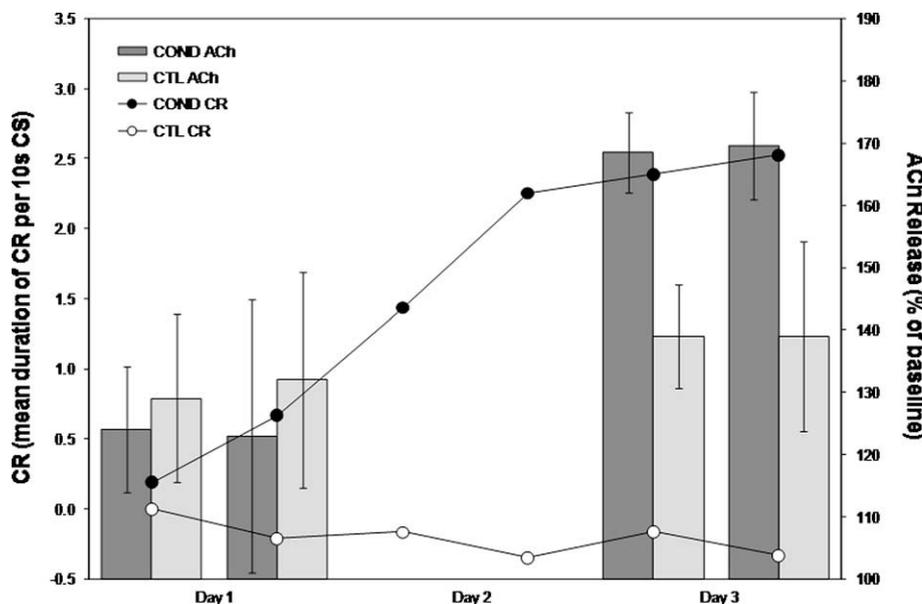


Fig. 8. Mean (\pm SEM) conditioned responding (left axis; line graph) and mean (\pm SEM) training-induced ACh release (right axis; bar graph) across test days (two trial blocks per day) in the conditioning (COND) and control (CTL) groups. The combined conditioning group shows a progressive increase in conditioned food cup approach across days while the combined control group does not ($p < 0.001$). The conditioning sub-groups also show a corresponding progressive increase in ACh release in the primary auditory cortex from D1 to D3 ($p < 0.05$), while the control sub-groups do not. Training-induced levels of ACh across blocks in the COND-D3 sub-group were also significantly greater than in the CTL-D3 sub-group ($p < 0.05$), demonstrating the influence of classical conditioning on ACh release in the primary auditory cortex.

whereas the same comparison on Block 6 shows no significant between-group differences ($F_{(1,12)} = 3.01, p > 0.05$), largely because of variability in the CTL-D3 group.

Comparisons of ACh release on the first (D1) and last (D3) days of training revealed different effects within the COND and CTL groups. Repeated measures ANOVA comparing ACh release in the COND-D1 and COND-D3 sub-groups showed that training-induced ACh release (expressed as a percent of baseline release) was greater on D3 than on D1 of conditioning ($F_{(1,14)} = 8.42, p < 0.01$). In contrast, training-induced ACh release expressed as a percent of baseline in the CTL-D1 and CTL-D3 sub-groups did not differ across test days ($F_{(1,13)} = 0.24, p > 0.05$).

4. Discussion

Auditory Pavlovian conditioning caused a systematic increase in the amount of ACh released in the primary auditory cortex, where the level of ACh release was clearly related to the acquired behavioral significance of the auditory CS. Across days of Pavlovian conditioning, the level of training-induced ACh release in the auditory cortex increased along with the strength of the CR. Additionally, on the third and final day of training, where the highest levels of conditioned food cup approach were observed, ACh levels were significantly greater in conditioning animals than in the control animals. In contrast, ACh release in the control condition did not change across test days. Training-induced ACh release in both the conditioning and control groups was greater than its baseline levels on both microdialysis test days (i.e., D1 and D3). The training-induced increase in ACh release in the control group is attributed to sensory stimulation resulting from exposure to the white noise CS. The greater levels of training-induced ACh release observed in the conditioning group compared to controls group, on the other hand, reflects not only the effects of sensory stimulation, but also uniquely reflects the influence of associative learning on sensory cortical ACh release. Collectively, these data provide strong support for the hypothesis that cholinergic efflux increases in the primary auditory cortex during natural memory formation, where such cholinergic facilitation is known to contribute to specific associative representational memory formation (Weinberger, 2004).

The associative learning-dependent enhancement of sensory cortical ACh release observed in the conditioning group cannot be attributed merely to the influence of sensory stimulation. This is because the conditioning and control groups were exposed to identical auditory sensory stimulation, but only the conditioning group showed progressive increases in training-induced ACh release across training. The increase in training-induced ACh release seen following acquisition of the CR in the conditioning group is not due to increased motor activity either, as the conditioning and control groups did not differ in terms of gross motor activity on either day of microdialysis testing. Instead, the greater levels of ACh release observed in the COND-D3 conditioning group compared to the CTL-D3 controls are argued to result specifically from the accumulation of associative strength by the auditory CS.

4.1. Sensory-evoked versus learning-evoked ACh release

The observation of training-induced increases in ACh release relative to baseline release in the control group, which was exposed to auditory stimuli along with random presentations of sucrose pellets, is consistent with findings of sensory stimulation-induced increases in ACh release in the primary sensory cortex reported for a number of different sensory modalities. For example, presentation of visual (Collier & Mitchell, 1966; Fournier, Semba, & Rasmusson, 2004; Laplante, Morin, Quirion, & Vaucher, 2005; Rasmusson, Smith, & Semba, 2007; Rasmusson & Szerb, 1976), auditory (Rasmusson et al., 2007), or somatosensory

(Fournier et al., 2004; Kurosawa, Sato, & Sato, 1992; Rasmusson et al., 2007) stimuli causes an increase in ACh release in the corresponding primary sensory cortical areas in anesthetized rats. The presentation of gustatory stimuli similarly causes a sensory stimulation-evoked release of ACh in the cortical gustatory area in waking rats (Shimura, Suzuki, & Yamamoto, 1995).

Although in the present experiment auditory sensory stimulation was associated with an increase in ACh release in the primary auditory cortex in both groups on D3, the level of ACh released in the conditioning group at the end of training exceeded the level observed in control animals. These findings are compatible with previous research (Miasnikov et al., 2006) demonstrating that conditioned changes in heart rate and respiration and CS-specific changes in auditory evoked potentials do not occur following tone presentations in the absence of electrical stimulation of the NBM. Instead, specific associative behavioral memory occurred only following electrical stimulation of the NBM in conjunction with auditory stimulation. Another experiment demonstrates that the amount of NBM stimulation controls the specificity of associative memory for auditory cues paired with NBM stimulation-induced release of cortical ACh (Weinberger et al., 2006). These earlier findings, along with the current results, suggest that in order to induce specific associative memory in sensory cortex, the amount of ACh released during associative learning must exceed some critical threshold, above that normally seen in response to sensory stimulation alone.

4.2. Global versus regional ACh release in neocortex

Results from the current study demonstrate that cholinergic activation is enhanced in the sensory cortex corresponding to the modality of the training CS during associative learning. Because ACh efflux was measured from only a single cortical area, it remains to be determined whether the observed associative learning-dependent increases in ACh release occurred only in the primary auditory cortex or occurred more globally throughout the neocortex (see Himmelheber, Fadel, Sarter, & Bruno, 1998; Sarter & Bruno, 1997). Previous research has shown that ACh can be released in a regionally specific manner, with greater levels of ACh release occurring in task-relevant sensory cortices than in other cortical areas. For example, Pavlovian conditioning with a visual CS causes associative learning-dependent increases in primary visual but not primary auditory cortex (Butt, Chavez, & Flesher, 2006; see also Butt, Testylier, & Dykes, 1997).

A growing body of evidence suggests that ACh can be differentially released in various cortical regions, depending on the nature of sensory stimulation (e.g., Fournier et al., 2004), the level of motor activity (Jiménez-Capdeville & Dykes, 1996; Laplante et al., 2005; Rasmusson & Szerb, 1976; Rasmusson et al., 2007), or the type of learning task (Butt, Testylier, & Dykes, 1997; Butt et al., 2006; but see Himmelheber et al., 1998). For example, sensory modality- and region-specific ACh release in visual and somatosensory cortices has been previously demonstrated in an experiment where visual stimulation in anesthetized rats evoked a large increase in visual but not somatosensory cortex, while skin stimulation produced the opposite pattern of ACh release (Fournier et al., 2004). These demonstrations of sensory modality-dependent increases in sensory cortical ACh release, along with previous findings showing sensory modality-specific and associative learning-dependent release of ACh (Butt et al., 2006) suggest that ACh levels in the conditioning group in the current study were selectively enhanced in the primary auditory cortex and not in other sensory cortical areas.

5. Conclusions

The current results demonstrate that associative learning causes a specific increase in ACh release in the auditory cortex,

where the level of ACh efflux is related to the learned significance of the CS. These data are consistent with the view that ACh promotes the long-term storage of specific associative memory traces in the primary auditory cortex during natural learning. In addition to the NBM's role in providing regionally specific (Jiménez-Capdeville, Dykes, & Myasnikov, 1997), modality-dependent, sensory stimulus-evoked ACh release as demonstrated by others (e.g., Fournier et al., 2004), the data reported herein suggest that the NBM is further capable of providing differential cholinergic modulation within sensory cortex as a function of the behavioral significance of the sensory stimulus presented. This level of sophistication in the modulatory capability of the NBM could allow for exceptional control of sensitivity to sensory inputs of varying behavioral significance, where regionally specific increases in cholinergic efflux would permit highly selective representational memory formation for behaviorally-relevant stimuli in associative learning.

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