

BRIEF COMMUNICATION

Choline Acetyltransferase mRNA Plasticity with Pavlovian Conditioning

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Choline acetyltransferase mRNA and somal area increased selectively in the ventral nucleus basalis of rats trained that a tone signals immediate shock (i.e., predicts danger). Retrograde tracing showed the affected cells correspond to those that project to the auditory cortex. Behavior responses and mRNA increased significantly above those of control groups trained with the tone not signaling immediate shock. In one of those control groups, animals learned that the same tone signaled a shock-free period before shock. These animals showed a visibly decreased riboprobe and a trend toward smaller somal areas. These results implicate transcriptional regulation of choline acetyltransferase in long-term memory storage. Selective attention and inattention to the tone are possible components of memory encoded by the molecular changes reported here. © 1996 Academic Press, Inc.

Encoding into long-term memory storage requires *de novo* cerebral protein synthesis, at least for many tasks (6, 23). A review of early studies employing protein synthesis inhibitors suggests that synthesis of the essential protein(s) must occur soon after learning for efficient long-term storage (1). Synthetic enzymes for the neurotransmitters increased by anti-amnesiac agents are possible candidates. Amphetamine, adrenergic blockers and acetylcholinesterase inhibitors, for example, reverse protein synthesis inhibitor-induced amnesia (19). All the amnesia-reversing compounds mentioned above increase acetylcholine (11, 12). If acetylcholine facilitates recovery, then the cholinergic synthetic enzyme, choline acetyltransferase (ChAT), quite possibly is necessary for long-term memory encoding. There is evidence that this is the case. Following protein synthesis inhibition by cycloheximide, the phar-

macological agent nefiracetam completely reverses amnesia in the same instances it completely restores ChAT activity to normal (22).

Although enhanced acetylcholine synthesis accompanies learning (16), enhanced synthesis of acetylcholine does not necessarily require enhanced transcription of the ChAT gene (11, 12). Only long-term control of acetylcholine synthesis is mediated through transcriptional regulation of ChAT (20). This contrasts with numerous nontranscriptional mechanisms exerting short-term control over acetylcholine synthesis (24). Signal transduction molecules that participate in the encoding of long-term memory do up-regulate ChAT gene expression. For instance, cAMP-dependent kinases contribute to protein synthesis-dependent, long-term memory of sensitization in *Aplysia* (5). These cAMP-dependent protein kinases also activate the human ChAT gene, most likely through its enhancer element (10).

In the present study, we assessed ChAT mRNA changes in forebrain cholinergic neurons following Pavlovian conditioning in rats. We expected changes for the reasons mentioned above. Namely, the ChAT gene is a potential site of regulatory control and feasible intermediary signal transduction molecules affecting ChAT expression are linked to long-term memory storage. The auditory cortex is reliably a site for electrophysiological and morphological indicators of plasticity associated with memory storage related to tone (2, 29). For these reasons, we identified the cholinergic neurons that project to the auditory cortex and assessed ChAT mRNA profiles for those neurons in groups of animals learning different outcomes to a tone signal.

We used Long Evans female rats (220–250 g) in the present experiments. Animals were randomly assigned to the fluorescent tracer study ($n = 4$) or to one of the three behavioral training groups ($n = 4$ in each group). All animals were humanely treated according to University of California and NIH guidelines.

In the first experiment, we infused fluorescent tracers into Te3 of the auditory cortex, a known site of

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maximal plasticity associated with the training employed in the present study (2, 29). True blue or fluorogold fluorescent tracers (0.1 μ l) were infused into Te3 at 5.8 mm posterior, 7.0 mm lateral, and 5.5 mm ventral to bregma (17) in four rats. Four days later, the animals were sacrificed by transcardial perfusion with phosphate-buffered saline (pH 7.6) followed by 0.04% paraformaldehyde in 0.1 M phosphate buffer with 0.2% picric acid. Tissue sections were processed with primary monoclonal antibodies to ChAT and fluorescein-conjugated secondary antibodies as described in previous reports (8, 28).

Maximal numbers of ChAT cells (53–71%) projecting to Te3 were found in the ventral half of the nucleus basalis at 2.8 mm posterior to bregma in the coronal plane (Figs. 1A and 1B). Percentages of ChAT cells projecting to Te3 diminished anterior and posterior to that dense core. These maps identified the probable location of cells projecting to the auditory cortex in the following experiment.

In this second experiment, we trained three groups of unoperated, naive animals. A 2-kHz, 96-dB tone delivered for 30 s and a 1-mAmp grid shock delivered for 1 s served as the conditional and unconditional stimuli. Rats were trained on one of three training schedules: (i) the tone followed immediately by shock, (ii) the tone followed by a 5-min shock-free period and then shock, or (iii) the tone delivered with no shock. Training consisted of four trials lasting 10 min each for 2 consecutive days. We tested the animals on the third consecutive day, right after our measured conditional response of immobilized crouching reached asymptote.

On the testing day, we reduced conditional responding to the chamber by stimulating competing exploratory behavior (i.e., by adding small mouse toys to each cage, increasing the ambient illumination, and placing ammonia under the floor grids). High rates of conditional responding in the chamber uniformly decreased 18–25% across all groups by these changes. Along with conditional response rate decreases, exploratory behaviors such as sniffing, walking, and grooming increased. This suggests that “switching” occurred where one conditional response replaces another (16).

We devised a precisely regulated sampling procedure and the data collected during training and testing by this procedure were highly correlated ($\rho = 0.81$) with continuous behavioral scoring from videotapes of the same session. Animals in the experimental groups were shocked after the last test tone according to the contingencies used during training to circumvent extinction and to reinforce the newly laid memory trace just before sacrifice.

By design, the different groups learn to associate the tone with a particular outcome. The “tone followed immediately by shock” group learns that the tone is a danger signal. Learning that a stimulus predicts danger increases conditional responses and also increases

selective attention to the reinforced conditional stimulus (7, 13, 26). When the tone is followed by a shock-free period, the animal learns that the tone signals an immediate safe period even though shock will occur later. This training produces effects opposite to those described above, such as decreased conditional response rates and decreased attention to the nonreinforced conditional stimulus (7, 13, 26).

Since we delivered identical tone and shock stimuli to the first two groups, only the association, the prediction, or the level of attention association with the tone stimulus was altered. Thus, this design enabled us to determine how changes in ChAT mRNA expression are affected by the predictive nature of the tone and the consequential effects on attention or inattention. We anticipated functional changes in cholinergic neurons for the first two groups, since basal forebrain neurons adapt their responses along with associative learning, but not according to the sensory parameters of stimuli (9, 18, 27). Thus, the presentation of tone without shock, in which there is no predictive learning involving the tone, served as the control condition in which we expected no functional changes in cholinergic basal forebrain neurons.

Within 1 h after testing, animals were transcardially perfused, brains were sectioned, and tissue was reacted with digoxigenin-labeled ChAT RNA probe at 50°C overnight. The basic method for preparing digoxigenin-labeled ChAT RNA probe is described in detail in previous publications (4, 15).

We detected the hybridized reaction product using a solution of 1:1000 anti-digoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN). Microscopic analyses were performed with an Olympus Vanox microscope and Bioquant image analysis and biometric software (R & M Biometrics, TN). We made somal measurements directly from tissue labeled with the ChAT riboprobe, as we knew from previous studies that our staining method fills the entire somal extent of cholinergic forebrain cells (4, 15).

At 2.8 mm posterior to bregma, ChAT mRNA was visibly changed in the ventral half of the nucleus basalis for animals trained that the tone predicted either danger or safety compared to control animals never shocked (see Figs. 1C–1E). Following training that the tone signaled shock, ChAT hybridization label in the ventral nucleus basalis was increased over that of control (Fig. 1C cf. 1D). The somal areas of these ventral nucleus basalis cells were significantly increased (Table 1). In contrast, the animals trained with the tone signaling a safety period had visibly decreased riboprobe in the ventral basal forebrain (Fig. 1D cf. 1E). The same number of ventral nucleus basalis cells were detected, however, the cells were very lightly stained, and there was a trend toward decreased somal size (Table 1). No visible or measurable differences across

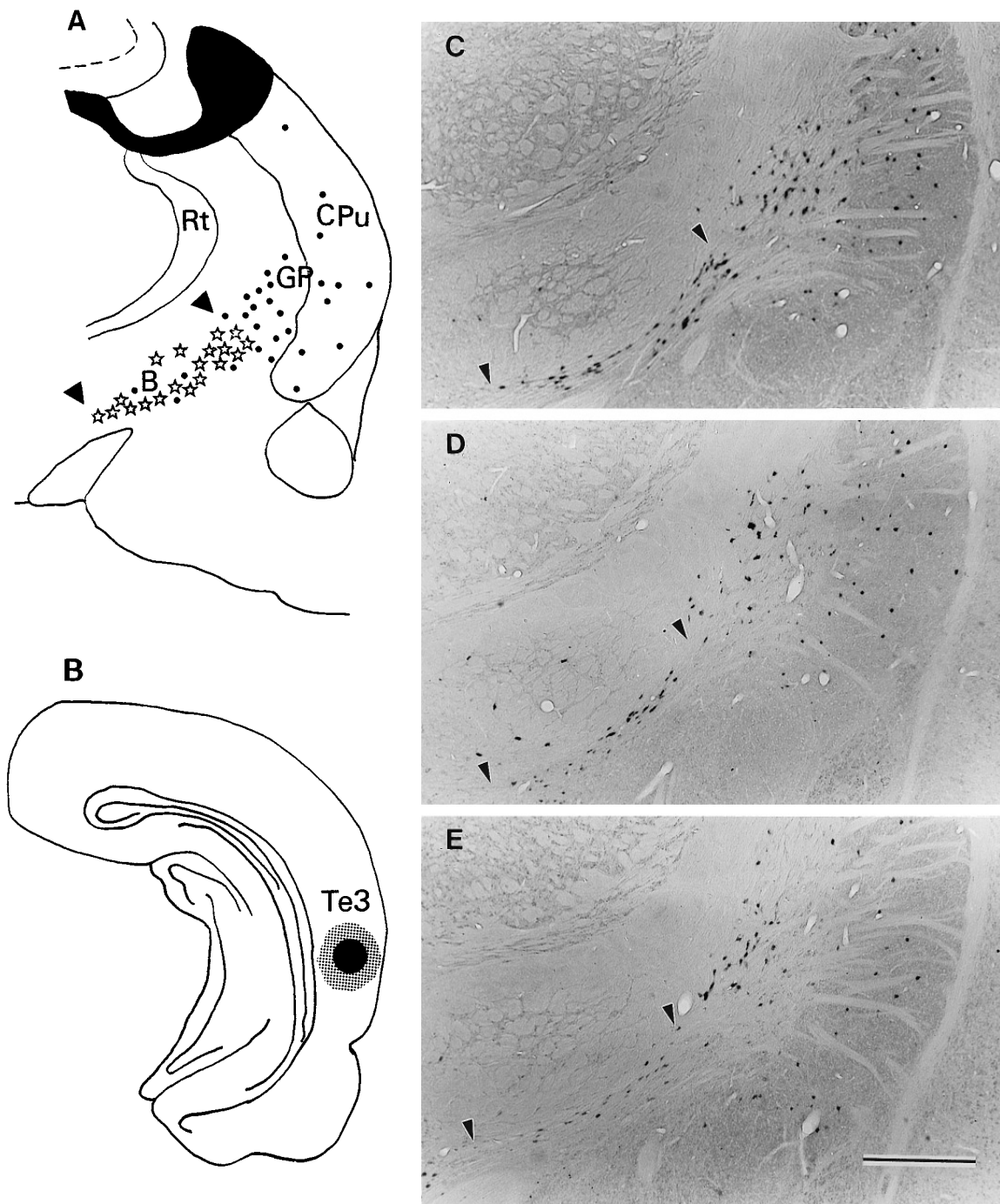


FIG. 1. (A) Distributions of cholinergic cells that project to auditory cortex Te3 are maximal at the level 2.8 mm posterior to bregma (17). Cholinergic projection cells are depicted as stars (each star represents one cell) and ChAT cells not retrogradely labeled are depicted as dots (each dot represents three cells). Arrowheads point to the densest aggregate of projection cells located in the ventral half of the nucleus basalis. Abbreviations: B, nucleus basalis; CPu, caudate-putamen; GP, globus pallidus; Rt, reticular nucleus of the thalamus. (B) The locus of retrograde tracer and the surrounding region of diffusion were wholly contained within region Te3 of auditory cortex. (C–E) Photomicrographs through the coronal plane at 2.8 mm posterior to bregma showing ChAT mRNA in typical animals trained with tone signaling immediate shock (C), tone without shock (D), or tone signaling a safe shock-free period (E). Arrowheads correspond to the ventral half of the nucleus basalis where a majority of cells project to Te3 of the auditory cortex (cf. A).

TABLE 1
Cholinergic Somal Area Changes Parallel Behavioral Changes

Training*	ChAT mRNA somal areas ($\mu\text{m}^2 \pm \text{SEM}$)			Conditioned response (percentage + SEM)	
	Ventral n. basalis	Dorsal n. basalis	Striatum	Pretone	Tone
Tone signals shock	239 \pm 22**	202 \pm 15	284 \pm 14***	78 \pm 11***	100 \pm 0**
Tone signals wait before shock	144 \pm 6	221 \pm 22	217 \pm 35	75 \pm 15	50 \pm 20
Tone with no shock	179 \pm 11	216 \pm 14	262 \pm 12	0 \pm 0	44 \pm 16

Note. Results of two-way ANOVA.

* Main effects: $P < 0.01$ (P values range, 0.0001–0.01).

** Pairwise contrasts: tone signals shock different from either control group, $P < 0.05$ (P values range, 0.0001–0.05).

*** Pairwise contrasts: tone signals shock different from one control group, $P < 0.005$ (P values range, 0.0001–0.005).

training groups appeared in the dorsal half of the nucleus basalis at this coronal plane, whereas the adjacent striatum (caudate–putamen) showed training-related changes similar to those in the ventral nucleus basalis (Table 1).

As expected, the training significantly affected the measured conditional response of an immobilized crouching posture (Table 1). This posture has been defined both as a conditioned fear response (3) and as a defense behavior (21). The different behavioral rates produced by the tone depended on the predictive outcome of the tone (Table 1, last column). In animals trained that the tone signaled immediate shock, crouching was much greater than that of the other two groups of animals (Table 1, last column). Moreover, crouching during the tone was increased above the pretone response rate when the predictive outcome of the tone was danger, whereas crouching was decreased below the pretone rate when the predictive outcome of the tone was a safe period (Table 1, columns 4 and 5). This bidirectional change in behavioral responding paralleled the bidirectional effects on ChAT mRNA in basal forebrain cells projecting to the auditory cortex.

What the tone predicted differentially affected ChAT mRNA, indicating that these changes reflect something more specific than a general associative learning process. The plasticity shown here may be related to a specific aspect of associative learning, such as selective attention. The recent literature describing the attentional function of the basal forebrain supports this notion (14, 25). The up-regulation of ChAT synthesis could be a step in encoding selective attention or inattention to the tone.

In the present study, altered ChAT gene expression occurred in cells with a specific cortical projection area. The cells projecting to surrounding cortical areas were left unaffected. We did not assess the entire basal forebrain, however, and a number of other cortical and limbic areas participate in fear conditioning (3). Further study of ChAT mRNA fluctuations may elucidate how selective or focused attention related to the overall

associative memory is encoded. The ChAT mRNA expression changes reported here may represent a part of the basic molecular substrate underlying long-term memory storage.

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