Activity Profiles of Single Neurons in Caudal Anterior Cingulate Cortex During Trace Eyeblink Conditioning in the Rabbit

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Weible, Aldis P., Craig Weiss, and John F. Disterhoft. Activity profiles of single neurons in caudal anterior cingulate cortex during trace eyeblink conditioning in the rabbit. J Neurophysiol 90: 599–612, 2003. First published May 15, 2003; 10.1152/jn.01097.2002. Acquisition of trace eyeblink conditioning involves the association of a conditioned stimulus (CS) with an unconditioned stimulus (US) separated by a stimulus-free trace interval. This form of conditioning is dependent upon the hippocampus and the caudal anterior cingulate cortex (AC), in addition to brain stem and cerebellar circuitry. Hippocampal involvement in trace eyeblink conditioning has been studied extensively, but the involvement of caudal AC is less well understood. In the present study, we compared neuronal responses from rabbits given either paired (trace conditioning) or unpaired (pseudoconditioning) presentations of the CS and US. Presentation of the CS elicited significant increases in neuronal activity at the onset of both trace conditioning and pseudoconditioning. A robust CS-elicited neuronal response persisted throughout the first 2 days of trace conditioning, declining gradually across subsequent training sessions. In contrast, the magnitude of the CS-elicited excitatory response during pseudoconditioning began to decline within the first 10 trials. Neurons exhibiting excitatory responses to the CS during trace conditioning also exhibited excitatory responses to the US that were significantly greater in magnitude than US-elicited responses during pseudoconditioning. CS-elicited decreases in neuronal activity became more robust over the course of trace conditioning compared to pseudoconditioning. Reductions in activity during the CS interval consistently preceded excitation in both training groups, suggesting that the CS-elicited decreases in neuronal activity may serve to increase the signal-to-noise ratio of the excitatory response to the tone. Taken together, these data suggest that the caudal AC is involved early in trace eyeblink conditioning and that maintenance of the CS-elicited excitatory response may serve to signal the salience of the tone.

INTRODUCTION

Eyeblink conditioning (Gormezano et al. 1962) is an associative learning paradigm in which a neutral tone conditioned stimulus (CS) is paired with a behaviorally salient corneal air-puff unconditioned stimulus (US). After repeated pairings, presentation of the CS elicits a conditioned response (CR) prior to onset of the US. During delay eyeblink conditioning, the tone CS precedes, overlaps, and co-terminates with the air-puff US. Acquisition of delay conditioning is mediated by neural circuitry of the brain stem and cerebellum (Clark et al. 1984; Mauk and Thompson 1987). During trace eyeblink conditioning, the tone CS is separated temporally from the air-puff US by a stimulus-free trace interval. The temporal separation of the CS and US requires the input of forebrain structures, including the hippocampus (Kim et al. 1995; McEchron et al. 1998; Moyer et al. 1990; Solomon et al. 1986; Weiss et al. 1999) and the caudal region of the anterior cingulate cortex (Kronforst-Collins and Disterhoft 1998; Weible et al. 2000), for successful acquisition of the trace conditioned reflex.

Lesions of the hippocampus made prior to training reliably block acquisition of trace eyeblink conditioning (Moyer et al. 1990). Additional evidence indicates that hippocampal involvement occurs early in learning and contributes to consolidation of the conditioned response. Kim and colleagues (1995) demonstrated that, while lesions of the hippocampus made 1 day after learning blocked subsequent expression of the CR, similar lesions made 30 days after learning failed to produce a comparable deficit. In another study, modulation of hippocampal CA1 pyramidal neuron activity to both the CS and US during trace eyeblink conditioning was more robust on the day of initial CR performance as compared to either the day before or 2 days after initial CR performance (McEchron and Disterhoft 1997). The appearance of significant modulatory activity on the day of initial CRs was described as reflecting the coding of the temporal relationship between the CS and US (McEchron and Disterhoft 1997). These data are similar to those found in earlier studies of hippocampal neuronal activity during delay eyeblink conditioning which showed increases in responding to the CS and US as training progressed (Berger and Thompson 1978; Berger and Weisz 1987; Berger et al. 1983). The data clearly support the conclusion that the hippocampus is involved in trace eyeblink conditioning. However, as we will discuss in detail in the following text, the hippocampus may not be the first structure in the brain to form the association between the CS and the US. Instead, the initial association between the tone and air-puff may develop in cortical structures afferent to the hippocampus.

The prefrontal cortex (PFC) is composed of multiple subregions, definable by neuroanatomical and functional criteria (Fuster 2001), and is involved early in the acquisition of a variety of tasks. Imaging studies indicate that the lateral PFC in humans is activated early during the acquisition of motor tasks (Grafton et al. 1992; Jenkins et al. 1994) and that the lateral PFC is crucial for formulating and executing sequences of
actions (Fuster 2001). Primate physiological studies have identified a role for the dorsolateral PFC in a variety of working-memory tasks (Boussaoud 2001; Chafee and Goldman-Rakic 1998; Levy and Goldman-Rakic 1999; Vaadia et al. 1986). Human and non-human data indicate that the orbital PFC is involved in the exclusion of extraneous stimuli during learning (Fuster 1997). A major component of the medial PFC, the anterior cingulate, is involved in identifying behaviorally salient stimuli and mediating sustained attention (Mesulam 1981; Posner et al. 1988; Raichle 1994). The anterior cingulate is also involved in more complex orientation and selective-attention tasks through interactions with parietal (Bushnell et al. 1978) and dorsolateral PFCs (Coull et al. 1998), respectively. The data regarding the functional contributions of these regions have resulted in theories of attention in which the lateral, orbital, and medial regions of the PFC are integral components (Coull 1998; Mesulam 1981; Posner and Peterson 1990).

The medial PFC (mPFC) of the rabbit and rodent is commonly described as including the infralimbic, prelimbic, and anterior cingulate cortices and is located rostral to the genu of the corpus callosum. The anterior cingulate (AC; Brodmann’s area 24) also has a functionally distinct component that extends from the genu caudal to bregma (Brodmann 1909; Vogt et al. 1986; Weible et al. 2000). Previous work from our laboratory referred to the caudal portion of the AC as the caudal mPFC (cmPFC) (Kronforst-Collins and Disterhoft 1998; Weible et al. 2000). This name derived from the fact that the AC is itself considered a component of the mPFC in the rabbit and rodent and that Kronforst-Collins and Disterhoft (1998) and Weible and colleagues (2000) identified distinct behavioral effects of lesions including caudal or rostral components of the rabbit mPFC. Given the exclusive focus in the present study on the caudal portion of Brodmann’s area 24, we will refer to this region with the anatomically precise and more descriptive name caudal AC.

Several studies indicate that the AC may be involved in eyeblink conditioning. During conditioning, event-related activation has been observed in the human AC using functional magnetic resonance imaging techniques (Preston et al. 2000). Lesions of the caudal AC in rabbit disrupt acquisition of trace eyeblink conditioning (Kronforst-Collins and Disterhoft 1998; Weible et al. 2000). However, the time frame during, and manner in which, the caudal AC contributes to the acquisition of trace eyeblink conditioning is unknown. To address this issue, we recorded the activity of multiple individual neurons during 10 days of trace eyeblink conditioning to determine how this structure may contribute to tasks of temporal associative learning.

METH ODS

Subjects

The subjects used in this study were 15 2.5 to 4.0-mo-old female New Zealand White rabbits weighing on average 2.8 kg. All subjects were housed individually, with a 12-h light-dark cycle.

Surgical procedures

All surgeries were performed using sterile procedures. Ketamine (60 mg/kg) and xylazine (10 mg/kg) were administered intramuscularly to anesthetize rabbits prior to surgery. Eyes were kept moist with a thin layer of antibacterial ophthalmic ointment. Rabbits were positioned in a stereotaxic frame, with lambda 1.5 mm below bregma. The skull was exposed and an opening 3.0 mm wide was made 1.0 mm laterally from the midline and extended from 0.0 to 5.0 mm anterior to bregma. In each animal, a fixed microwire electrode array was lowered to a depth of 3.0 mm beneath the dura. The microwires were oriented along the sagittal plane, 1.0 mm lateral to the midline, with the posterior-most microwire inserted 0.5 mm rostral to bregma. Five self-tapping screws (#2 × ¼”) were inserted into the skull to a depth of ~2 mm to anchor the electrode array and a headbolt assembly containing four nylon bolts. Dental cement was then used to cement the electrode array in place, and the headbolt was placed just anterior to the array. Rabbits were administered buprenex (0.3 mg/kg sc) to minimize discomfort after recovery from anesthesia.

Behavioral training

All subjects were given 5 days of post-operative recovery. During acclimation and subsequent training sessions, rabbits were restrained up to the neck using a cloth bag and Plexiglas restrainer, from which the head was allowed to protrude. The lids of the right eye were held open with a Velcro strap and two small stainless steel dress hooks. A lightweight aluminum assembly secured an infrared sensor and air-puff delivery tube to the head bolt for the duration of each training session.

Computers running in-house, custom-designed software controlled the delivery of all stimuli and acquired behavioral data (Akase et al. 1994). Each conditioning session consisted of 80 trials with an average intertrial interval (ITI) of 45 s. Each trial was 1,750 ms in duration and consisted of a 500-ms pre-CS baseline interval, 250-ms CS, and 150-ms US intervals separated by a 500-ms stimulus-free trace interval, followed by a 350-ms post-US interval. A binaural tone (6 kHz, 90 dB, 5-ms rise/fall time) served as the CS while an air-puff to the right eye (3.5 psi) served as the US. An infrared sensor suspended in front of the eye measured reflectance from the surface of the cornea. The computer recorded extension of the nictitating membrane across the surface of the cornea as an increase in signal voltage. This voltage was recorded by the computer at 1 kHz for a total of 1,750 data points per trial.

After the acclimation session, subjects received 10 consecutive days of either trace conditioning or pseudoconditioning. Trace-conditioned subjects were considered learners if they were able to perform eight CRs within any 10 consecutive trials on two consecutive days. Subjects undergoing pseudoconditioning received 80 presentations of the CS and 80 presentations of the US. The CS and US were pseudorandomly presented in an unpaired fashion (22.5 s mean ITI) so as to complete a training session within the same amount of time as trace-conditioned subjects.

After the 10 days of training, all subjects were given 4 days of delay conditioning. During delay conditioning, a 900-ms tone CS preceded, overlapped, and co-terminated, with the 150-ms air-puff US. Acquisition of delay conditioning by the previously pseudoconditioned subjects indicated that the basic brain stem/cerebellar circuitry mediating sensory input and integration was functionally intact.

Single neuron recording

The microwires used in the present study were Teflon-coated stainless steel (50 μm uncoated, 112 μm coated). An array of six 1-cm lengths of 26-gauge cannula tubing were attached in parallel to provide a template for positioning the individual wires of the recording array during fabrication. The wires were trimmed to a length of 4 cm and threaded through the cannulae to create an array of six parallel wires with a nominal separation of 500 μm. Epoxy was then used to cement the wires in this configuration. Amphenol pins soldered at one end of each wire were passed through an amphenol strip connector, and the whole array was cemented into position with epoxy. Imme-
diately prior to surgery, the recording end was trimmed to an appropriate length to ensure a clean recording surface.

Neuronal signals were amplified (10,000×), filtered (bandpass 300 Hz to 10 kHz) and collected with a DT 2821 Data Translation board (Marlboro, MA) attached to a 200-MHz Pentium computer that sampled channels at 30 kHz. Single-neuron data were collected for the duration of each training session using DataWave Technologies software (Longmont, CO). Data were collected for a 1.5-ms window each time a neuron fired an action potential that exceeded a threshold voltage set prior to the beginning of each training session. DataWave analysis routines incorporating template windows were used to cluster the waveforms of multiple neurons into the activity of single neurons (between 4 and 8 voltage settings were used to identify waveforms produced by individual neurons).

Because of alterations in responsivity to the stimuli across days and the possibility of electrode drift, it would be difficult to determine with certainty that a given neuron could be recorded across multiple days of training. Thus cells recorded from each day were treated as unique neurons. The total number of neurons analyzed represents the sum of the number of cells isolated per day for the total of 10 days of trace or pseudoconditioning.

Analyses

Neurons exhibiting a signal/noise ratio of <2:5:1 were excluded from analyses. Data from each of the remaining neurons were grouped into 70 25-ms bins, spanning the duration of the 1,750-ms trial interval, converted into ASCII format, and exported from DataWave for subsequent analyses. All analyses were performed with the aid of QBasic routines developed in our laboratory and commercial software packages StatView v5.0 (SAS Institute) and Microsoft Excel. Data recorded from individual neurons during the 80-trial training session were divided into eight blocks of 10 trials each. A non-parametric Wilcoxon matched-pairs signed-ranks statistical test compared baseline neuronal activity with neuronal activity recorded during CS, trace, US, and post-US (200 ms) intervals. The CS and trace intervals were each divided into two equal duration half intervals to identify discrete changes in activity after onset of the CS. Therefore, a total of six distinct time intervals were compared to baseline for each individual neuron. To achieve a P value of <0.05, a difference in firing rate between a trial interval and baseline had to occur in five of eight trial blocks. Neurons failing to exhibit any activity in a minimum of five separate blocks in at least one time interval (baseline through post-US) were excluded from further analyses (140/1,093).

Neurons exhibiting significant alterations in firing rate compared to baseline during one or more of the time intervals analyzed were grouped according to whether the increase or decrease appeared to be elicited by either the CS or US (occurring during either the 750-ms CS/trace interval or the 350-ms US/post-US interval). Classifications were determined by when the first significant change in activity was observed (e.g. neurons exhibiting excitatory responses to both the CS and the US were classified as CS-excitatory neurons). Data from each of these four groups were further divided into consecutive pairs of days and used to generate group histograms. Cell count comparisons between groups and across days of conditioning for each response type were made using a χ² analysis (Yate’s correction was employed when analyzing only 2 response categories).

Subsequent statistical analyses were performed on the group data and were based on the mean number of spikes/bin for each of the six time intervals described above for the single neuron analysis. T-tests were performed using these data to identify time intervals within the group histogram exhibiting significant increases or decreases compared to baseline. To determine the onset latency of the response within an interval exhibiting significant change, we identified the first bin with a change in firing rate greater than 4 SD from the mean of the baseline. To identify significant differences in response magnitude between neurons of trace and pseudoconditioned subjects during a given time interval, differences in baseline firing rate were controlled for by dividing the spikes/bin of the time interval in question by the mean firing rate of the baseline interval.

Histology

Marking lesions for the identification of electrode placement were made by passing DC current (+25 μA) for 30 s through each electrode from which data were recorded. Subjects were killed by intravenous administration of a lethal dose of pentobarbital sodium (97.5 mg/kg). Subjects were then perfused transcardially with 0.9% saline, followed by a 10% formalin solution. Brains were extracted and placed into a sucrose/formalin solution (300 g sucrose per 1 L 10% formalin) to cryo-protect the tissue. After 2 days, brains were removed from the sucrose/formalin solution, quick-frozen with crushed dry ice, and sectioned (75 μm thick) on a sliding microtome. Every fourth slice was mounted onto a gelatin-coated slide, stained with cresyl violet, and examined under the microscope at ×25 magnification. Only those data recorded from electrodes verified to be within the caudal AC (extending rostral from bregma to the genu of the corpus callosum) were included in the present study.

R E S U L T S

Trace eyeblink conditioning

After 10 days of behavioral training, 9 of 10 trace-conditioned subjects were able to achieve a criterion of 8 CRs within 10 consecutive trials on two consecutive days. This criterion was achieved, on average, on day 7 of trace conditioning for all nine subjects. No pseudoconditioned subjects (n = 5) demonstrated eight CRs following the tone during 10 consecutive tone-alone trials on any given day of pseudoconditioning. Trace and pseudoconditioned subjects performed comparably as of the third day of delay eyeblink conditioning, indicating that the basic brain stem/cerebellar circuitry of pseudoconditioned subjects was functioning normally. Learning curves for both trace-conditioned (n = 9) and pseudoconditioned (n = 5) subjects are illustrated in Figure 1.

Histology

The data from three microwires were excluded from the analyses because their electrode tips were found to be outside
of the caudal AC. All remaining microwires yielded usable data for analysis. Figure 2 illustrates the location of each microwire tip from which neuronal activity was recorded during training. The data presented here are discussed irrespective of hemisphere as analyses failed to detect hemispheric specificity of neuronal response patterns.

**Single-unit response profiles**

Eight-hundred-fourteen total neurons with a signal/noise ratio $>2.5$ and meeting the firing rate criterion specified in METHODS: Analyses were recorded from 15 subjects across 10 days of training. Seventy-three neurons from the one trace-conditioned subject that failed to reach behavioral criterion were excluded. The subsequent analyses were performed on a working population of 430 neurons recorded during trace conditioning and 311 neurons recorded during pseudoconditioning. The mean spike height ($y_2-y_1$), width ($x_2-x_1$), and signal/noise ratio of these 741 neurons were 170 $\mu$V, 0.52 ms, and 3.94, respectively (Fig. 3). The spike height, width, and signal/noise ratio did not differ significantly between training conditions.

**CS/trace interval excitatory activity**

The proportion of responsive cells changed significantly over the course of trace conditioning ($\chi^2 = 22.57, df = 4, P < 0.05$) but not pseudoconditioning (Fig. 4). The proportion of neurons exhibiting excitatory responses during the CS/trace interval during the first 2 days of training was significantly greater during trace conditioning as compared to pseudoconditioning ($\chi^2 = 10.01, df = 1, P < 0.05$). During trace conditioning, presentation of the CS elicited an increase in firing rate during the CS/trace interval from 25.5% of cells recorded on days 1 and 2 with the proportion of CS-excitatory responsive cells declining across subsequent days of training (Fig. 4). The proportion of CS-excitatory responsive cells never exceeded 9.7% (days 3 and 4) during pseudoconditioning sessions (Fig. 4). The spike frequency data from CS/trace interval excitatory neurons for each group were separated into pairs of days for analysis and are illustrated as rate histograms in Fig. 5. Table 1 lists mean firing rates for the different intervals of each histogram. Significant differences relative to baseline between grouped neuronal data of trace-conditioned and pseudoconditioned subjects were identified by t-tests.

Analyses of CS/trace interval data indicated a significant increase in firing rate during the second half of the CS interval compared to baseline during trace conditioning. The mean onset latency of the CS-elicited excitatory neuronal response was 125 ms. The amplitude of the CS-elicited excitatory neuronal response was greatest on days 1 and 2 with neurons exhibiting a mean firing rate during the second half of the CS interval 196% of the baseline firing rate. On days 3 and 4, the firing rate during the second half of the CS interval declined to 168% of the baseline interval. Significant CS-elicited increases in neuronal activity persisted into the trace interval during the first 4 days of trace conditioning (Table 1). The increases in firing rate during the second half of the CS interval for days 5–10, while still significant, had declined to a mean amplitude of 148 $\pm$ 1% (means $\pm$ SE) of baseline.

Analysis of the grouped CS/trace interval excitatory response data recorded during pseudoconditioning did not reveal a significant increase in firing rate compared to baseline during either half of the CS interval. Though the Wilcoxon test did identify individual neurons exhibiting a significant increase in firing rate at some point during the 750-ms CS/trace period, the excitation was not consistently synchronized to the CS and
Group differences in CS-elicited excitatory response magnitude were observed within the first 10 training trials (Fig. 6A). The cells from trace-conditioned animals had a greater than twofold increase in firing rate during the second half of the CS interval, and this increase was maintained throughout the first day of trace conditioning. In contrast, the initial excitatory neuronal response to the CS during pseudoconditioning declined rapidly, returning to baseline within the first 30 training trials.

Table 1. CS-elicited increases in single-neuron activity

<table>
<thead>
<tr>
<th>Trace Conditioned</th>
<th>Pseudo Conditioned</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td><strong>CS (1–125 ms)</strong></td>
</tr>
<tr>
<td>Trace, Hz</td>
<td>Pseudo, Hz</td>
</tr>
<tr>
<td>D1/2</td>
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</tr>
<tr>
<td>D3/4</td>
<td>2.8</td>
</tr>
<tr>
<td>D5/6</td>
<td>5.3</td>
</tr>
<tr>
<td>D7/8</td>
<td>2.0</td>
</tr>
<tr>
<td>D9/10</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Bold print in Trace or Pseudo columns represents periods of significant modulation in neuronal activity compared to the corresponding mean baseline firing rate (t-test, P < 0.05). Significant differences in response magnitude compared to baseline between neurons of trace-conditioned and pseudoconditioned subjects are listed in the ΔT·ΔP column for each time interval. Baseline differences for comparisons between neurons of trace conditioned and pseudoconditioned subjects were controlled for as described in Analyses. CS, conditioned stimulus.

FIG. 5. Mean rate histograms illustrating CS/trace interval excitatory neuronal responses recorded during trace conditioning (black) and pseudoconditioning (gray). Each histogram represents data collected over two consecutive days of training and illustrates the mean firing rate for neurons exhibiting excitatory responses during the 750-ms CS/trace interval. Cell counts are reported in the top left corner of each histogram.

FIG. 6. A: response magnitudes compared to baseline for neurons of trace conditioned (•) and pseudoconditioned (□) subjects exhibiting excitatory responses during the 2nd half of the CS interval on the 1st day of conditioning. The CS/trace interval has been divided into 8 blocks of 10 trials each. The y-axis represents the percent of the mean neuronal firing rate during the 2nd half of the CS interval compared to the mean baseline firing rate. B: trial-by-trial response magnitudes for all neurons exhibiting a cumulative increase in neuronal activity over the 1st 10 trials during the CS interval compared to baseline. Proportion of responsive neurons was similar between trace conditioning (41%) and pseudoconditioning (45%). Group differences for trials 1–5 were not significant. Group differences for trials 6–10 were significant.
trials. The significant difference in trace-conditioned and pseudoconditioned excitatory response magnitude during the CS interval was maintained through the first 6 days of training (Table 1).

Data were analyzed for all neurons exhibiting a cumulative increase in firing rate compared to baseline during the first 10 trials of trace conditioning and pseudoconditioning. The results indicate similarities during the first 10 training trials with regard to both proportion of responsive neurons (trace conditioning: 41%; pseudoconditioning: 45%) as well as neuronal response magnitudes (Fig. 6B). No significant group difference was detected during the first five training trials, using a repeated-measures ANOVA \([F(1,48) = 0.533; P = 0.4691]\). The same test identified a group effect on trials 6–10 \([F(1,48) = 4.145; P = 0.0473]\), indicating a drop in neuronal responsiveness during pseudoconditioning relative to neuronal activity during trace conditioning during trials 6–10.

**CS/trace interval inhibitory activity**

The greatest proportion of neurons exhibiting CS-elicited decreases in neuronal activity during both trace conditioning and pseudoconditioning was on days 1 and 2 (31.1 and 33.8%, respectively). The proportion of neurons exhibiting decreases in activity during each pair of days for trace-conditioned and pseudoconditioned subjects is illustrated in Fig. 7. The spike frequency data from CS-inhibitory neurons were separated into 2 groups for each pair of days. *, days with a significantly different percentage of responsive neurons between training conditions. The proportion of neurons exhibiting inhibitory responses following tone onset was comparable across the remaining days of training between the 2 groups for each pair of days. * days with a significantly different percentage of responsive neurons between training conditions.

FIG. 7. Percentages of CS/trace interval inhibitory neurons recorded during trace conditioning (■) and pseudoconditioning (□) grouped by pairs of days. Each bar represents the percentage of neurons responding of the total recorded during a 2-day interval for each training condition. The proportion of neurons exhibiting inhibitory responses following tone onset was significantly greater during days 5 and 6 of trace conditioning compared to pseudoconditioning \((\chi^2 = 6.66, df = 1, P < 0.05)\). The proportion of neurons exhibiting inhibitory responses was comparable across the remaining days of training between the 2 groups for each pair of days. * days with a significantly different percentage of responsive neurons between training conditions.

FIG. 8. Mean rate histograms illustrating CS/trace interval inhibitory neuronal responses recorded during trace conditioning (■) and pseudoconditioning (□). Each histogram represents data collected over 2 consecutive days of training and illustrates the mean firing rate for neurons exhibiting inhibitory responses during the 750-ms CS/trace interval. Cell counts are reported in the top left corner of each histogram.
the CS interval was unexpected, given the group differences in response magnitude compared to baseline. However, the non-significant inhibition observed on days 7 and 8 and days 9 and 10 during pseudoconditioning resulted in differences in response magnitude between groups of only 18% and 20.5% on those days.

**US/post-US interval excitatory and inhibitory activity**

The greatest proportion of neurons exhibiting US-elicited excitatory responses during trace conditioning was on days 1 and 2 (28.7%; Fig. 9). During pseudoconditioning, the greatest proportion of US-elicited excitatory responses was on days 5 and 6 (23.6%; Fig. 9). These percentages include neurons that may also have exhibited significant changes in firing rate to presentations of the CS. A significantly greater proportion of neurons exhibiting US-elicited excitatory responses was observed during the first 2 days of trace conditioning compared to pseudoconditioning ($\chi^2 = 6.02, df = 1, P < 0.05$; Fig. 9). Across 10 days of training, presentation of the US elicited excitatory responses during the US/post-US interval from 65 of 430 (15.1%) neurons during trace conditioning and 52 of 311 (16.7%) neurons during pseudoconditioning, according to the Wilcoxon matched-pairs signed-ranks test. The histograms in Fig. 10 illustrate the mean spike frequency data for US-excitatory responses during the US/post-US interval from 65 of 311 (15.1%) neurons during trace conditioning and 52 of 311 (16.7%) neurons during pseudoconditioning, according to the Wilcoxon matched-pairs signed-ranks test. The histograms in Fig. 10 illustrate the mean spike frequency data for US-excitatory responses during the US/post-US interval from 65 of 311 (15.1%) neurons during trace conditioning and 52 of 311 (16.7%) neurons during pseudoconditioning, according to the Wilcoxon matched-pairs signed-ranks test.
tatory neurons of trace-conditioned and pseudoconditioned subjects grouped by pairs of days. Table 3 lists firing rates for the different intervals of each histogram, including intervals of significant decrease compared to baseline and significant difference in activity between neurons of trace-conditioned and pseudoconditioned subjects. The trace-conditioning data represented in Fig. 10 and Table 3 are based on neurons exhibiting responses only during the US/post-US interval.

No significant excitatory activity was observed for neurons of trace-conditioned or pseudoconditioned subjects during the presentation of the 150-ms US (Table 3). Neurons of subjects from both training groups exhibited significant increases in firing rate during the post-US interval early in training (Table 3). These increases in neuronal activity were less robust as training progressed across days (Table 3). The only significant difference between groups in response magnitude during the US/post-US interval was a significantly greater increase in firing rate on days 3 and 4 during trace conditioning.

The greatest proportion of neurons exhibiting US-elicited decreases in activity during trace conditioning and pseudoconditioning was on days 1 and 2 (27.7 and 30.1%, respectively). The proportion of neurons exhibiting US-elicited decreases in activity did not differ significantly between groups across days (Fig. 11). The histograms in Fig. 12 illustrate the mean spike frequency data for US-inhibitory neurons of trace-conditioned and pseudoconditioned subjects separated by pairs of days. Table 4 lists firing rates for the different intervals of each group histogram, including intervals of significant decrease compared

**TABLE 3. US-elicted increases in single-neuron activity**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>US (1–150 ms)</th>
<th>Post-US (1–200 ms)</th>
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<tbody>
<tr>
<td></td>
<td>Trace, Hz</td>
<td>Pseudo, Hz</td>
<td>Trace, Hz</td>
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<tr>
<td>D1/2</td>
<td>4.7</td>
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<td>D3/4</td>
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<td>D5/6</td>
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<td>D9/10</td>
<td>2.7</td>
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</tbody>
</table>

Bold print in Trace or Pseudo columns represents periods of significant modulation in neuronal activity compared to the corresponding mean baseline firing rate (t-test, \( P < 0.05 \)). Significant differences in response magnitude compared to baseline between neurons of trace-conditioned and pseudoconditioned subjects are listed in the ΔT:ΔP column for each time interval. Baseline differences for comparisons between neurons of trace conditioned and pseudoconditioned subjects were controlled for as described in Analyses. US, unconditioned stimulus.

**FIG. 11.** Percentages of US/post-US interval inhibitory neurons recorded during trace conditioning and pseudoconditioning grouped by pairs of days. Each bar represents the percentage of neurons responding of the total recorded during a 2-day interval for each training condition. Proportions for US-specific (●) as well as CS-US (●) responsive neurons recorded during trace conditioning are shown. The percentage of neurons exhibiting inhibitory responses to the US only was significantly greater during pseudoconditioning compared to trace conditioning across all 10 days of training (\( \chi^2 = 13.28 \), \( df = 4 \), \( P < 0.05 \)). No significant difference between groups was detected when US-inhibitory responsive neurons also exhibiting inhibitory responses to the CS (●) were included in cell proportion comparisons. The asterisk identifies days with a significantly different percentage of responsive neurons between training conditions.

**FIG. 12.** Mean rate histograms illustrating US/post-US interval inhibitory neuronal responses recorded during trace conditioning (●) and pseudoconditioning (●). Each histogram represents 2 consecutive days of training and illustrates the mean firing rate for neurons exhibiting inhibitory responses during the 350-ms US/post-US interval. Cell counts are reported in the top left corner of each histogram.
to baseline and significant difference in response magnitude between neurons of trace-conditioned compared to pseudoconditioned subjects. The trace-conditioning data represented in Fig. 10 and Table 3 are based on neurons exhibiting reduced activity only during the US/post-US interval.

The proportion of neurons exhibiting significant decreases in firing rate during the US interval did not differ significantly across days for trace-conditioned and pseudoconditioned subjects. However, significant differences in response magnitude were observed between groups. Significant reductions of neuronal activity during trace conditioning persisted into the post-US interval on days 1 and 2 and 7 and 8. Neuronal activity during the post-US interval was significantly decreased on days 3 through 8 of trace conditioning compared to pseudoconditioning. This difference is attributed more to a small post-US increase in neuronal activity during pseudoconditioning rather than the overall amplitude of inhibition exhibited by neurons of trace-conditioned subjects during the post-US interval (Table 4).

**Enhanced US responses following CS presentation**

The preceding analyses were based on data grouped according to the time interval during which individual neurons first exhibited significant modulation in activity. However, 72% (44 of 61) of CS-excitatory neurons recorded during trace conditioning also exhibited US-elicited excitatory responses (dual CS-US neurons). The US-elicited response of dual CS-US neurons differed significantly in magnitude from that of neurons exhibiting responses only during the US/post-US interval (US-specific) (Fig. 13). The magnitude of change compared to baseline during the presentation of the US itself was comparable between dual CS-US response neurons and US-specific neurons. However, during the post-US interval, dual CS-US response excitatory neurons exhibited a significant increase in response magnitude compared with US-specific excitatory responses ($t = 5.209, df = 7, P = 0.0012$). The response magnitude of US-specific excitatory neurons from trace-conditioned and pseudoconditioned subjects was comparable during the US and post-US intervals. Of the neurons from trace-conditioned rabbits exhibiting learning-related inhibitory responses to the CS exhibited inhibition to the US as well, whereas 50% (31 of 62) of neurons exhibiting inhibitory responses to the US exhibited inhibition to the US as well.

**Baseline variability across training sessions**

To identify changes in baseline neuronal activity over the course of training, the firing rates of individual neurons recorded during the first 2 and last 2 days of training were compared. First, neurons recorded during the first two (early) and last two (late) days of training were sorted according to training condition and response type, as described for Figs. 5, 8, 10, and 12. The mean baseline firing rate for each neuron was then determined. Comparison of the mean baseline firing rates of individual neurons in the early and late groups was performed using an unpaired $t$-test. This test revealed that those neurons exhibiting a significant increase in firing rate during the 750-ms CS/trace interval (as illustrated in Fig. 5) also exhibited a significant decrease in baseline firing rate on late compared to early training days. This reduction in mean baseline activity late in training for CS-excitatory neurons was evident during both trace conditioning ($t = 1.820, df = 32, P = 0.0391$) and pseudoconditioning ($t = 2.428, df = 7, P = 0.0228$). No significant differences in mean baseline firing rate were observed between early and late training days for groups of neurons exhibiting CS-inhibitory, US-excitatory, or US-inhibitory responses, regardless of training condition. The absence of significant changes in baseline firing rate in the other cell groups suggests that the significant decrease in baseline firing rate observed in CS-excitatory neurons over the course of training is specific to neurons exhibiting the CS-excitatory response profile rather than a general decline in neuronal

### Table 4. US-elicited decreases in single-neuron activity

<table>
<thead>
<tr>
<th>Baseline (Trace, Hz)</th>
<th>US (1–150 ms)</th>
<th>Post-US (1–200 ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1/2</td>
<td>4.1</td>
<td>2.8</td>
</tr>
<tr>
<td>D3/4</td>
<td>2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>D5/6</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>D7/8</td>
<td>3.2</td>
<td>2.0</td>
</tr>
<tr>
<td>D9/10</td>
<td>2.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**Note:** Bold print in Trace or Pseudo columns represents periods of significant modulation in neuronal activity compared to the corresponding mean baseline firing rate ($t$-test, $P < 0.05$). Significant differences in response magnitude compared to baseline between neurons of trace-conditioned and pseudoconditioned subjects are listed in the $\Delta f/\Delta P$ column for each time interval. Baseline differences for comparisons between neurons of trace conditioned and pseudoconditioned subjects were controlled for as described in Analyses.
activity or signal quality attributable to extended use of chronically implanted electrodes.

DISCUSSION

CS-elicited excitatory activity

The purpose of the present study was to characterize caudal AC single-neuron activity during acquisition of trace eyelink conditioning in the rabbit. The data suggest that during trace-conditioning neurons of the rabbit caudal AC may promote the salience of the tone-CS by maintaining the amplitude of the initial CS-elicited excitatory response. CS-elicited excitatory neuronal activity was observed on trial 1 of both trace conditioning and pseudoconditioning (see Fig. 6A). The greater than twofold increase in CS-elicited neuronal activity relative to baseline observed on the first trial of trace conditioning persisted throughout the first day of training (see Fig. 6A). In contrast, CS-elicited excitatory neuronal responses began to decrease in magnitude within the first 10 trials of pseudoconditioning (see Fig. 6A). When considering all neurons recorded on the first day of training, the proportion of neurons exhibiting a CS-elicited increase in activity over the first 10 training trials was similar between trace conditioning and pseudoconditioning (41 and 45%, respectively). The activity exhibited by these neurons during the first five trials did not differ with respect to group (see Fig. 6B). However, when data were analyzed across all 80 trials of the first training session, a greater proportion of neurons remained significantly responsive to the CS during trace conditioning (36%) compared to pseudoconditioning (6%). Taken together, these data suggest that the disparity between groups regarding CS-elicited response magnitudes and proportions of CS-excitatory neurons is, at least partially, attributable to a rapid habituation to the non-salient CS by caudal AC neurons during pseudoconditioning. The persistence of the CS-elicited excitatory response during trace conditioning presumably then reflects the associative significance of the tone, and in view of the previous lesion data (Kronforst-Collins and Disterhoft 1998; Weible et al. 2000), is likely to be functionally relevant to acquisition of the conditioned reflex.

Data from the present study also suggest that sensory information concerning the CS and US may converge upon individual caudal AC neurons to affect the observed modulations of activity. First, neurons recorded during trace conditioning maintain a robust CS-elicited excitatory response throughout the first training session compared to a rapidly diminishing neuronal response to the tone during pseudoconditioning. This is presumably attributable to the fixed temporal relationship of the CS and US during trace conditioning. Second, during the first 2 days of trace conditioning, neurons that show excitatory responses to both the CS and US exhibit a significantly greater response after US presentations compared to neurons that show excitatory responses specifically to the US (Fig. 12). In contrast, US-specific excitatory neuronal responses during trace conditioning are comparable to neuronal responses to the US during pseudoconditioning. It is important to note that individual neurons exhibiting excitatory responses to both the tone and the airpuff during the first 10 trials of pseudoconditioning do not show a similar maintenance of CS-responsivity across the first day of training as CS-responsive neurons recorded during trace conditioning. The increase in response magnitude exhibited by CS-US responsive neurons suggests that explicitly paired CS/US presentations do not simply increase stimulus response magnitudes of the caudal AC neuronal population in general. Instead, the nature of the responses to both CS and US presentations may be indicative of the convergence of temporally related sensory information upon a subset of individual caudal AC neurons.

CS-elicited decreases in activity

The relative timing of decreases and increases in neuronal firing rate is suggestive of an important functional interaction between neurons exhibiting these two response profiles. Figure 14 illustrates the mean responses of neurons exhibiting increases and decreases in firing rate during the CS/trace interval for the first 2 days of trace conditioning. CS-elicited reductions in firing rate during trace conditioning occur with a mean onset latency of 25 ms. The mean onset latency of the CS-elicited excitatory response was 125 ms. CS-elicited decreases in neuronal activity may thus serve to increase the signal-to-noise ratio for CS-elicited excitatory responses through a reduction in firing rate immediately preceding the CS-elicited increase in firing rate, and/or facilitate the maintenance of the excitatory response observed during trace eyelink conditioning.

The data suggest that the reduction in firing rate is integral to caudal AC neuronal activity, as CS-elicited decreases in activity early in training are quite similar between the two conditioning protocols. First, the proportion of neurons exhibiting decreases in activity in response to the CS during the first 4 days of training is comparable between trace-conditioning and pseudoconditioning groups (see Fig. 7). Second, the magnitudes of the CS-elicited decreases in activity during the first day of trace conditioning and pseudoconditioning were comparable (38 and 40% below baseline, respectively). Third, the mean onset latency of the decrease in activity for both groups during the first 4 days of conditioning is 25 ms. Fourth, similarly timed decreases in activity are observed after US onset during both trace conditioning and pseudoconditioning. Given these observations, it seems likely that the short-latency reduction in firing rate is inherent to the function of a subset of caudal AC neurons and that it serves to facilitate the excitatory response elicited by stimuli that are either directly, or through association, behaviorally relevant, predictive stimuli.

Functional implications

Maintenance of the CS-elicited excitatory response in the caudal AC early in training, combined with a learning deficit.
after caudal AC lesions (Kronforst-Collins and Disterhoft 1998; Weible et al. 2000), suggests a possible functional interaction between the caudal AC and the hippocampus during acquisition of the trace-conditioned reflex. Kim and colleagues (1995) demonstrated that the hippocampus, which is essential for learning the trace-conditioned eyelblink reflex (Moyer et al. 1990; Solomon et al. 1986), was selectively involved in acquisition and consolidation, but not retention, of the conditioned response. The response of hippocampal neurons to the presentation of conditioning stimuli changes over the course of both delay and trace conditioning in a manner reflecting the development of the conditioned reflex (Berger and Thompson 1978; Berger et al. 1983; McEchron and Disterhoft 1997; Munera et al. 2001). McEchron and Disterhoft (1997) demonstrated significant modulation of hippocampal CA1 neuronal activity during trace conditioning on the day of initial CR performance compared to the day before initial CRs. These changes in hippocampal neuronal activity were not observed in subjects that failed to acquire the trace-conditioned reflex (McEchron et al. 2001). The behavioral (Kim et al. 1995) and in vivo (McEchron and Disterhoft 1997) data are supported by post-training, in vitro data obtained using hippocampal slice preparations that indicated a learning-related, transient modulation of hippocampal CA1 and CA3 neuronal excitability (Moyer et al. 1996; Thompson et al. 1996). These results support the theory that the hippocampus acts as a temporary storage buffer and/or analyzing CS-US associative strength (McEchron and Disterhoft 1997; Munera et al. 2001).

By maintaining the excitatory response to the tone, neurons of the caudal AC signal the behavioral significance of the tone prior to the development of learning-related activity in the hippocampus. Early in delay conditioning, hippocampal pyramidal cells exhibit only basal or slightly enhanced firing rates to presentation of the CS and US (Berger et al. 1983). By trial 10 of delay conditioning, enhanced activity was reported during the CS period, though of lesser magnitude than activity observed during the US period (Berger et al. 1983). The magnitude of the responses during the CS and US periods continued to increase over the course of delay conditioning (Berger et al. 1983). McEchron and Disterhoft (1997) described increases in hippocampal neuronal activity during trace conditioning to presentations of the US but not the CS on the day before initial CRs. Munera and colleagues (2001) described increases in hippocampal neuronal activity to the CS during the first day of both trace conditioning and pseudoconditioning. However, the response to the CS did not appear to differ appreciably between groups during the first day of training (Munera et al. 2001).

In the present study, the initial CS-elicited excitatory response observed in caudal AC neurons in both groups was maintained throughout the first session of trace conditioning. During pseudoconditioning, the initial CS-elicited excitatory response began to decline in amplitude within the first 10 training trials (see Fig. 6 A and B). McEchron and Disterhoft (1997) described learning-related increases in CS-elicited hippocampal neuronal activity either immediately preceding or during the days of initial conditioned responses. Maintenance of the CS-elicited excitatory response by caudal AC neurons may provide information to the hippocampus regarding the behavioral salience of the tone and therefore facilitate the development of the association between the two stimuli in the hippocampus. No direct connections between the caudal AC and hippocampus have previously been described. However, interaction between the two structures is conceivable via multisynaptic pathways (Weible et al. 2001).

The maintenance of the CS-elicited excitatory response may influence components of different sensory systems. Neurons of the caudal AC respond to both the tone and the airpuff at the onset of training. The preferential maintenance of the CS-elicited excitatory response during trace conditioning is evident prior to learning-related changes observed in structures associated with the auditory system, such as the auditory association cortex and related thalamic nuclei (Buchwald et al. 1966; Disterhoft and Stuart 1976; Kraus and Disterhoft 1982; Oleson et al. 1975). Maintenance of the CS-elicited excitatory response may therefore facilitate the development of learning-related changes in activity observed in those regions.

The activity in the caudal AC may also influence the lateral pontine nucleus (LPN). The LPN is a component of the auditory pathway involved in the circuitry mediating eyelblink conditioning (Berger and Bassett 1992; Steinmetz et al. 1989). However, the LPN is believed to be a relay for auditory-CS information during delay conditioning rather than a site of plasticity for the learned response (Tracy et al. 1998). Electrical stimulation of the LPN serves as an effective CS (Castro-Alamancos and Borrell 1993; Steinmetz et al. 1986; Tracy and Steinmetz 1998). Direct projections from the caudal AC to the LPN (Weible et al. 2001) may be of functional importance to the conditioning of auditory stimuli given the proposed role for the LPN as a relay for auditory-CS information. Hippocampotomized rabbits are able to acquire trace conditioning when the interval between the CS and US is ≤300 ms, indicating that a 300-ms gap between stimuli is sufficiently brief for the circuitry of the brain stem and cerebellum to mediate acquisition of the CR (Moyer et al. 1990). CS-excitatory neurons in the caudal AC of trace-conditioned subjects maintain significantly elevated firing rates during the first 250 ms of the 500-ms trace interval throughout the first 4 days of training. By maintaining elevated firing rates into the trace interval, neurons of the caudal AC exhibiting CS-elicited excitatory responses could prolong the effect of the CS by continuing to stimulate the LPN after tone offset. In this way, the LPN could still function as a relay signaling CS presentation to the rest of the brain stem/cerebellar circuit.

**Anterior cingulate cortex activity and attention**

The data from the present study suggest that neurons of the caudal AC are involved in attending to stimuli with associative significance. Initial presentations of the tone-CS and airpuff-US elicited excitatory responses from caudal AC neurons regardless of training condition. During pseudoconditioning, the excitatory neuronal response to the CS rapidly declined as the tone became a familiar, non-significant component of the environment. However, paired presentations of the CS and US during trace conditioning resulted in the maintenance of the initial excitatory response to the tone. The capacity of caudal AC neurons to reflect the associative significance of the tone supports the theory that these neurons may be part of an
attentional mechanism necessary for the subsequent acquisition of the trace-conditioned reflex.

An early, attentional role of the AC in rabbit has been proposed previously (Freeman and Gabriel 1999). Gabriel and colleagues (1991) demonstrated retarded acquisition rates after AC damage. Orona and Gabriel (1983) demonstrated elevated firing rates in response to a predictive tone-CS on the first day of training during discriminative avoidance conditioning. In addition, the timing of the peak CS-elicited excitatory response they reported (100–200 ms) is similar to that reported in the present study. The more attentionally demanding the CS (e.g. brief vs. longer-duration tones), the greater the response magnitude of AC neurons to that CS (Sparenberg and Gabriel 1990). When using more attentionally demanding stimuli, impairments in avoidance learning, with accompanying alterations in AC neuronal processing of the CS, have also been described in rabbits after exposure in utero to cocaine (Gabriel and Taylor 1998), a procedure demonstrated to produce various alterations in AC neurons (Levitt et al. 1997). The data from the present study are consistent with these earlier findings (Gabriel and Taylor 1998; Sparenberg and Gabriel 1990) and expand upon the work of Orona and Gabriel (1983) by identifying a robust initial response to the tone during eyeblink conditioning, regardless of training protocol, that is maintained only during trace conditioning when the tone-CS is paired with the airpuff-US.

In a previous study, we hypothesized that the caudal AC in the rabbit is homologous to the caudal half of the AC in the primate (Weible et al. 2000). Data from the present study appear to support this view. Devinsky and colleagues (1995) differentiate between rostral “affective” and caudal “cognitive” components of the anterior cingulate in human. An affective role for the pregenual medial PFC of the rabbit has been described by Powell and colleagues (1996), who identified learning-related changes in the prelimbic area of the mPFC during eyeblink conditioning when using a strongly aversive peri-orbital shock US but not a mildly aversive corneal airpuff US. The present study supports the theory that neurons of the rabbit caudal AC are part of an early attentional system involved in the detection of potentially significant associations between novel environmental stimuli, regardless of their initial affective strength.

In their review of human neuropsychological and primate single-neuron recording data, Posner and Peterson (1990) proposed an anterior attentional system, reliant upon efficient functioning of the AC, that is involved in the detection of targets. Furthermore, according to Mesulam’s model of attention (1981), the AC is involved in determining the motivational salience of environmental stimuli. The corneal air-puff can be regarded as a behaviorally salient stimulus because its presentation reflexively elicits a behavioral response. In contrast, the tone can be regarded as a neutral stimulus in the naive subject because it does not normally possess motivational attributes. In the present study, neurons responding specifically to the behaviorally salient US exhibited similar changes in excitatory activity regardless of the training condition. However, the neuronal response to the behaviorally neutral CS was significantly greater when the CS and US were presented in a specifically paired fashion. Therefore the consistent temporal relationship between presentations of the CS and US during trace conditioning resulted in the attribution of salience to the previously neutral stimulus. Strengthening the nascent association between these two stimuli is critical for the development of the behaviorally adaptive eyeblink reflex that is observed in the well-trained subject.

Further support for the proposed homology between the human AC and the rabbit caudal AC may be found in a recent study incorporating in vivo single-neuron recording in the human. Davis and colleagues (2000) identified neurons in the AC cortex that were modulated by attentionally demanding tasks. In their study, the firing rate of neurons exhibiting inhibition dropped to 1–2 Hz, while that of excitatory neurons demonstrated a two- to fivefold increase from 0–10 to 10–50 Hz. The modulation of excitatory and inhibitory responses presumably augments the communication with efferent structures with regard to the significance of a given stimulus. The neurons they recorded were described as being within a region that is activated, according to functional magnetic resonance imaging data, during other attentionally demanding tasks (Davis et al. 1997). The similarities in both frequency and response magnitude between neurons of the rabbit caudal AC and the human AC cortex lend further support for the argument of homology between these two structures.

Those changes observed in human single-neuron activity during performance of an attentionally demanding task presumably serve a similar function as those observed in the rabbit caudal AC during trace eyeblink conditioning. The excitatory response of caudal AC neurons to the CS is maintained when the CS is predictive of the airpuff-US (i.e., trace conditioning). If the significance of the CS is not effectively established, efferent structures involved in elements of timing, behavioral response, and long-term retention, will presumably not have adequate information to form the CR.

In conclusion, we recorded the activity of caudal AC neurons across 10 days of training and identified patterns of neuronal activity specific to the associative nature of the CS and US early in training. A robust response to the CS is maintained across the first 2 days of trace conditioning, declining gradually during subsequent training sessions. In contrast, a comparable initial response to the tone during pseudotraining begins to decline within the first 10 training trials. These changes occurred prior to learning-related changes in activity in the hippocampus, a forebrain structure critical to acquisition of the trace-conditioned eyeblink reflex, and appear to support the development of the association between the neural tone CS and the behaviorally salient air-puff US. Similar to the role of the AC in the primate, the caudal AC of the rabbit appears to be important in determining the motivational salience of external stimuli. By increasing the behavioral salience of the tone CS early in the learning process, the caudal AC could trigger a neural cascade of events that results in the acquisition of the behaviorally adaptive conditioned response.

DISCLOSURES

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