# Fear Conditioning Enhances Different Temporal Components of Tone-Evoked Spike Trains in Auditory Cortex and Lateral Amygdala

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## Summary

**Single neurons were recorded in freely behaving rats during fear conditioning from areas of auditory cortex that project to the lateral nucleus of the amygdala (LA). The latency and rate of conditioning and extinction were analyzed, and the results were compared to previous recordings from LA itself. Auditory cortex neurons took more trials to learn, and they responded more slowly than LA neurons within trials. Short-latency plasticity in LA, therefore, reflects inputs from the auditory thalamus rather than the auditory cortex. Unlike LA cells, some auditory cortex cells showed late conditioned responses that seemed to anticipate the unconditioned stimulus, while others showed extinction-resistant memory storage. Thus, rapid conditioning of fear responses to potentially dangerous stimuli depends on plasticity in the amygdala, while cortical areas may be particularly involved in higher cognitive (mnemonic and attentional) processing of fear experiences.**

## Introduction

The fear memory system in the brain allows an organism to quickly assess and react to stimuli that, on the basis of past learning, predict danger. In humans, alterations in the fear memory system may contribute to various anxiety disorders (Wolpe, 1988; Ohman, 1992; Shalev et al., 1992; LeDoux, 1996; Charney and Deutch, 1996). Fear learning and memory is typically studied using fear conditioning, a procedure in which the subject is exposed to a conditioned stimulus (CS), such as a tone, in association with an unconditioned stimulus (US), often a footshock. After a few such pairings, the tone comes to elicit physiological and behavioral fear reactions (Blanchard and Blanchard, 1969; Bolles and Fanselow, 1980; LeDoux et al., 1984.)

It is well established that fear conditioning involves transmission of CS information to the amygdala (Kapp et al., 1992; Fanselow, 1994; Davis et al., 1994; LeDoux, 1995). For example, transmission of auditory CS inputs to the amygdala in the rat (and possibly other species) occurs over two pathways, both originating in the thalamus and terminating in the lateral nucleus of the amygdala (LA; Romanski and LeDoux, 1992). One involves direct transmission from the auditory thalamus to LA (LeDoux et al., 1985, 1990b; Turner and Herkenham,

1991), whereas the other involves multisynaptic pathways from the auditory thalamus to the auditory cortex to LA (Romanski and LeDoux, 1993; Mascagni et al., 1993).

The role of thalamic versus cortical inputs to the amygdala in fear conditioning has been debated. Animals can acquire fear conditioning normally following lesions of either the thalamo-amygdala (Romanski and LeDoux, 1992; Campeau and Davis, 1995) or cortico-amygdala (Romanski and LeDoux, 1992; Campeau and Davis, 1995; Armony et al., 1997a) pathways. However, if both pathways are damaged (Romanski and LeDoux, 1992), or LA itself is lesioned (LeDoux, et al., 1990a), conditioning is prevented. While lesions of the auditory cortex (defined broadly to include the perirhinal cortex) made before training have no effect, post-training lesions disrupt the expression of conditioned responses (Jarell et al., 1987; Rosen et al., 1992; Campeau and Davis, 1995; Corodimas and LeDoux, 1995). Some (Campeau and Davis, 1995) have suggested that post-training cortical lesions interfere with the expression of conditioned fear because the cortex is an obligatory relay for CS information to the amygdala. According to this hypothesis, if the cortical pathway is damaged, the thalamic pathway can be used; otherwise, the thalamic pathway contributes little to conditioning. Others, however, have suggested that during normal learning both pathways are used, and that lesions made after learning has taken place prevent the expression of learning by interfering with information storage or retrieval, contextual processing, or other factors that affect performance (Corodimas and LeDoux, 1995). In other words, information stored in a normal brain may not be readily accessible in a damaged brain.

Much of what we know about the contribution of thalamic and cortical CS pathways to fear conditioning comes from lesion studies. Though valuable, lesion results are limited by the fact that function is inferred from dysfunction. We have therefore turned to the use of recordings of single neurons in awake, behaving rats, in an attempt to obtain further details about information flow through the relevant circuits (e.g., Quirk et al., 1995, 1996). Previously, we found that fear conditioning enhances short-latency (10–20 ms after CS onset) single unit responses in LA (Quirk et al., 1995). In the present study, we recorded single unit activity during fear conditioning in auditory association cortex (area Te3/Te1v), which is the main source of auditory projections to LA (Romanski and LeDoux, 1993; Mascagni et al., 1993), and compared the results with our findings from LA. Our main goal was to assess the time course of conditioning across trials and the timing of conditioned responses within trials in Te3/Te1v and LA, for the purpose of determining whether the short-latency plasticity in LA might be accounted for by transmission from the auditory cortex. We tested two specific predictions. First, if the cortical route is essential for transmitting the CS to the amygdala, it follows that cortical tone responses would precede conditioned responses in LA in terms of response latency. Second, if the cortical route is essential to the plasticity in LA, conditioned responses should

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develop in fewer trials (or perhaps the same number of trials) but not in more trials than LA.

## Results

### Conditioning

Five to seven days after implantation of the electrodes, the microdrive was advanced several times each day until tone-responsive cells at a depth appropriate for Te3/Te1v could be discriminated. Fear conditioning then commenced. The procedures were identical to those used previously to study LA neurons (Quirk et al., 1995). The CS was a pure tone (5 kHz, 80 db, 2 s), and the US was a mild footshock (0.5 mA, 0.5 s). Initially, rats were exposed to a sensitization phase, which consisted of 10 unpaired tones and shocks. This was immediately followed by 20 conditioning trials, where the footshock was delivered 1500 ms after the onset of each tone; i.e., the US coterminated with the CS. A 1 hr rest period in the home cage followed. The rats were then returned to the test chamber for 30 extinction trials, where the tone was presented alone. The intertrial interval varied around 120 s in each phase (range, 60–180 s). The entire procedure took ~4 hr.

The rats learned the predictive value of the tone, as evidenced by the increase in tone-elicited freezing behavior observed following conditioning. Freezing is the cessation of all movement and is thought to allow the animal to escape detection by a potential predator (Blanchard and Blanchard, 1969). The time spent freezing during the 20 s test tone presented in a novel context increased from an average of $0.08 \pm 0.08$ s prior to conditioning to $8.46 \pm 2.10$ s after conditioning. This difference was statistically significant ($t[12] = 3.93$, $p < 0.01$) and is similar to previous findings from this laboratory for animals tested in a context different from the training context (Corodimas and LeDoux, 1995).

### Firing Properties of Auditory Cortex and LA Cells

A total of 40 cells in auditory cortex from 15 rats was studied. The electrode bundles were located in auditory association cortex, primarily in area Te3, with three of the placements being in Te1v (see Figure 1). Both of these areas (Te3/Te1v) project directly to LA (Romanski and LeDoux, 1993; Mascagni et al., 1993). The 40 cells from Te3/Te1v were compared to 30 cells recorded in the dorsal subdivision of lateral amygdala and described in our previous study (Quirk et al., 1995). Te3/Te1v neurons fired spontaneously at an average of 4.9 spikes/s (geometric mean, $2.50 \pm 1.28$ Hz), with a range of 0.1–16.0 Hz. This was faster than the LA cells, which fired at an average of 2.41 Hz (geometric mean, $0.40 \pm 1.51$ Hz).

### Latency of Tone Responses

Before the start of conditioning (i.e., during sensitization trials), cells in Te3/Te1v and LA both responded to tones at short latencies. Tone responsivity was measured for each 10 ms bin after tone onset from 0–100 ms. A cell was counted as tone responsive in a bin if it was significantly more active in that bin than in the 500 ms period
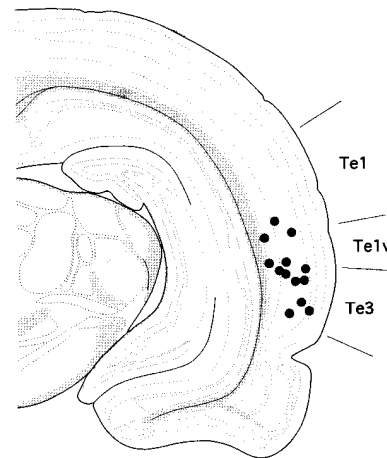


Figure 1. Electrode Placements in Auditory Cortex

Drawing of a coronal section through the rat brain at ~5 mm posterior to bregma. Each circle represents the placement of one electrode for each rat. Placements were located in auditory cortex, specifically in areas Te3/Te1v and the ventral part of Te1 (Zilles and Wree, 1985), both of which project to the lateral nucleus of the amygdala. Adapted from Swanson, 1992.

prior to tone onset (see Experimental Procedures). Representative post-stimulus time histograms of tone responses for Te3/Te1v and LA are shown in Figure 2A. The percent of tone-responsive cells in each 10 ms bin following tone onset is plotted in Figure 2B. Cells in both areas started responding at 10–20 ms, and the number peaked at 20–30 ms following tone onset. Responses as early as 10–20 ms in both structures are most likely driven by direct input from the auditory thalamus (Li et al., 1996; Quirk et al., 1996). LA contained a somewhat higher proportion of tone-responsive cells than Te3/Te1v (53% versus 38%, respectively), and onset tone responses were more prolonged in LA than in Te3/Te1v.

A conditioned response was defined as a significant increase in the rate of a 10 ms bin over what was observed for that bin during the sensitization trials. Using these criteria, conditioning increased the magnitude of tone responses in 9 out of 40 Te3/Te1v cells (22%). A somewhat higher percentage was observed in LA (10 out of 30 cells, 33%). The time course of conditioned responses is shown in Figure 2C. The latency of cortical conditioning was typically 20–40 ms, considerably later than the 10–20 ms plasticity observed in LA (Quirk et al., 1995). In fact, few cells showed plasticity in Te3/Te1v at the earliest latencies at which Te3/Te1v cells were tone responsive (10–20 ms). Thus, the latency of LA plasticity preceded the latency of plasticity in Te3/Te1v. These data suggest that LA cannot depend on the cortex for the expression of plasticity, since LA plasticity occurs earlier in time.

In addition to conditioning of tone onset responses, there were also conditioned tone offset responses. Defined in the same way as for conditioned onset responses, the fraction of cells showing offset conditioning were 23% and 37% for Te3/Te1v and LA, respectively.

Similar to previous studies of auditory cortex (Disterhoft and Stuart, 1976; Diamond and Weinberger, 1984),
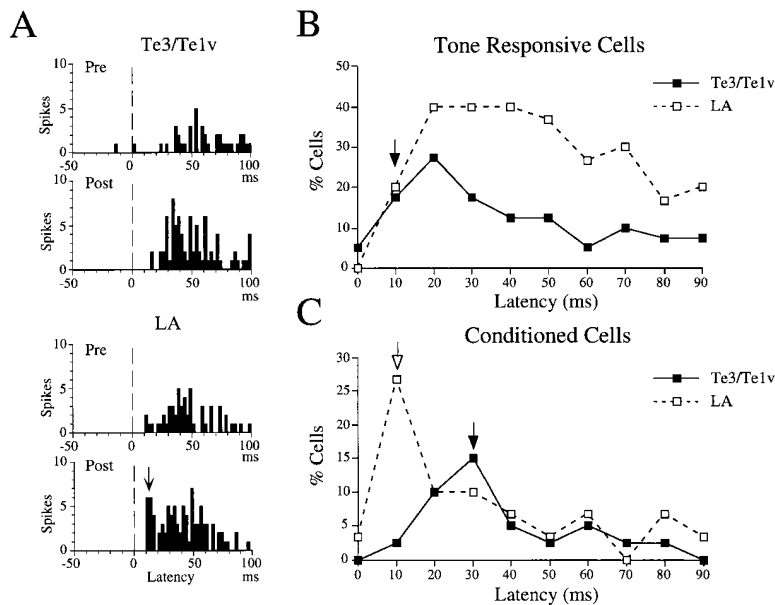
Figure 2. Early Tone Response of Neurons in Auditory Association Cortex and the Lateral Nucleus of the Amygdala

(A) Post-stimulus time histograms showing the tone responses of representative neurons from auditory association cortex (area Te3/Te1v, top) and the dorsal subnucleus of the lateral amygdala (LA, bottom), before and after fear conditioning. Tone onset occurs at 0 ms. The magnitude of the tone response increased in both structures after (post) relative to before (pre) the start of conditioning (pre-conditioning refers to the sensitization phase of training; see Experimental Procedures). The conditioned increase began ~35 ms after tone onset in Te3/Te1v and ~15 ms in LA. Each histogram represents 10 trials; bin width is 2.5 ms.

(B) The percent of cells significantly tone responsive (see Experimental Procedures) in each 10 ms bin following tone onset, for auditory association cortex (Te3/Te1v) and LA. The measurements were made prior to conditioning. Note that both areas show early tone responses (10–20 ms) before conditioning.

(C) The percent of cells that significantly increased their firing rate following fear conditioning in each 10 ms bin after tone onset, for recordings in Te3/Te1v and LA. Note that while LA showed maximal conditioned increases prior to 20 ms, Te3/Te1v did not maximally condition until 20–40 ms. Arrows mark peak conditioning latencies.

conditioning decreased the spontaneous firing rates of some Te3/Te1v cells. Seven out of forty cells decreased, while two increased (two-tailed t test, p < 0.05). These effects did not account for the increases in tone responses described above, as only one of the conditioned cells also showed an increase in spontaneous rate.

## Rate of Conditioning

The latency argument made above for precedence of LA over Te3/Te1v is incomplete. Late responses in cortical neurons could influence early responses in LA if cortical plasticity developed in fewer trials than LA (Gabriel, 1976). To address this, onset responses of Te3/Te1v and LA neurons occurring between 0–50 ms were analyzed on a trial-by-trial basis to determine the minimum number of tone–shock pairings necessary to observe significant plasticity. This analysis was not performed in the previous study of LA cells (Quirk et al., 1995).

In Te3/Te1v, 15 out of 40 cells (38%) showed one or more significantly conditioned trials (onset response greater than 2 standard deviations [SD] above sensitization) during the conditioning phase of training. A similar proportion was observed in LA (10/30 cells, 33%). LA, however, learned in fewer trials than Te3/Te1v. A histogram of the first significant trial for each neuron is shown in Figure 3A. Whereas half of the LA neurons developed plasticity within the first three trials, Te3/Te1v neurons did not usually exhibit plasticity until the sixth to ninth trials. Figure 3B shows the averaged onset response (0–50 ms) for all conditioned neurons throughout sensitization and conditioning trials. The onset of tone–shock pairing caused increases in both areas, with LA preceding Te3/Te1v by several trials. Thus, it is unlikely that LA depends on the cortex for the expression of plasticity, since amygdala plasticity preceded the cortex both

within and across trials. An unexpected finding was that toward the middle of conditioning, the firing rate of LA cells began to return to their pre-conditioning level, while Te3/Te1v cells, though variable in the second half, remained generally elevated over the 20 conditioning trials (Figure 3B).

## Extinction

One hour after conditioning, rats were returned to the test chamber for 30 extinction trials (tone alone). The average onset response (0–50 ms) of cells that significantly conditioned is shown for sensitization and extinction trials, for both Te3/Te1v and LA, in Figure 4. As previously reported for LA data (Quirk et al., 1995), extinction training caused LA onset responses to return to their original levels. In contrast, Te3/Te1v cells maintained the conditioned increase in firing rate throughout the 30 trials. The difference between the two groups was significant (ANOVA, main effect, F = 8.81, p < 0.01). While some Te3/Te1v cells decreased their responses during extinction, several cells actually increased their responses as extinction training progressed. This was never observed in LA. Thus, extinction training distinguishes LA and Te3/Te1v, suggesting that they play different roles in the extinction process.

## Delayed Conditioned Responses

The analyses presented so far have concentrated on the short-latency (0–50 ms) onset responses of Te3/Te1v neurons. Onset responses of this type have been the focus of most previous studies of auditory cortex (e.g., Weinberger, 1995). In addition to onset plasticity, however, we observed delayed conditioned responses that started hundreds of milliseconds after tone onset. The raster plots of Figure 5 show examples of delayed

A

## First Significant Trial
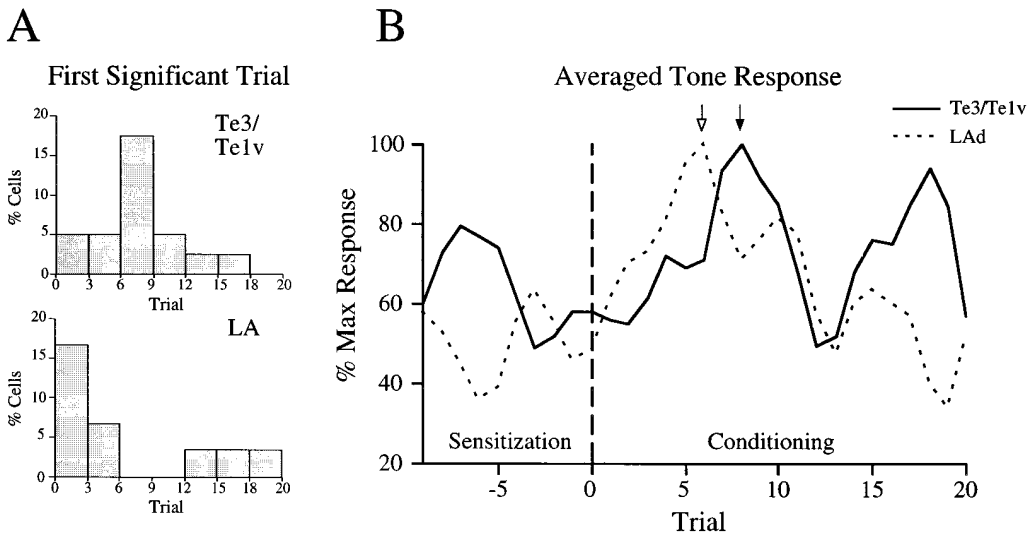


Te3/
Te1v

LA

B

## Averaged Tone Response



Figure 3. Rate of Conditioning Across Trials for Auditory Association Cortex and Lateral Amygdala

(A) Histograms show the occurrence of the first trial during the conditioning phase in which a significantly increased tone response was exhibited by neurons in auditory association cortex (Te3/Te1v, top) and LA (bottom). This occurred for 15 out of 40 Te3/Te1v cells and 10 out of 30 LA cells. Note that LA tended to condition earlier in the trial sequence than Te3/Te1v.

(B) The averaged tone response during pre-conditioning trials (i.e., sensitization) and during conditioning for the neurons represented in (A). The firing rate during the first 50 ms after tone onset was averaged across cells, smoothed with a weighted, nearest neighbor average, and normalized relative to the maximal value, in order to facilitate comparison between Te3/Te1v and LA. Note that the changes in LA preceded changes in Te3/Te1v. The peak firing rates were 32 Hz and 40 Hz for Te3/Te1v and LA, respectively.

conditioning in Te3/Te1v neurons, as well as onset conditioning in LA and Te3/Te1. Sensitization (pre-conditioning) and extinction (post-conditioning) trials are shown. Similar to LA, the onset cell in Te3/Te1 only showed conditioning in the initial bin after tone onset. Later components of the tone response were unaffected by conditioning. The delayed cell in Te3/Te1, on the other hand, never exhibited an onset response, before
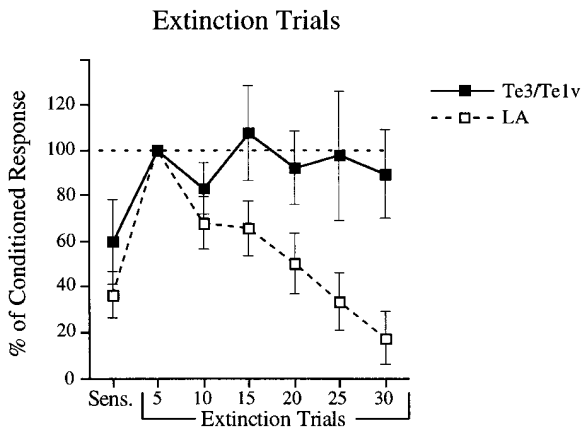
## Extinction Trials



Figure 4. Extinction of Tone Responses in Auditory Association Cortex and Lateral Amygdala

The average firing rate (0–50 ms) of neurons in auditory association cortex (Te3/Te1v) and LA that significantly conditioned are plotted for the last block of five trials in sensitization and successive five-trial blocks during extinction. Extinction caused LA tone responses to return to their pre-conditioning levels, while Te3/Te1v maintained its conditioned response throughout extinction. Error bars indicate SEM.

or after conditioning. It did, however, develop a late conditioned response starting at ~700 ms after tone onset and peaking at 1500 ms. Interestingly, 1500 ms was the latency of the footshock during conditioning trials (see arrow in Figure 5), suggesting that this delayed response may anticipate the US.

The time course of delayed conditioning is shown for group data in the "ribbon" plots of Figure 6. The fraction of cells that were significantly tone responsive throughout the 2 s tone is plotted, before and after conditioning, for LA and Te3/Te1v. In both regions, there was a high percentage of tone responses at tone onset and offset. Long after tone onset, however, the LA plot is flat, while Te3/Te1v showed a progressive increase in the number of late tone responses as a result of conditioning. Similar to the example shown in Figure 5, the delayed response for the group data started around 700 ms, increased to a maximum at 1500 ms, and returned to baseline by the end of the tone.

Based on the plots of Figure 6, a region of interest was identified between 500 ms after tone onset (the end of all onset responding) and 1500 ms (onset of footshock). Student's t tests were used to identify the number of cells that were significantly tone responsive during this period (two-tailed t test, $p < 0.05$). After conditioning, 11 out of 40 Te3/Te1v neurons showed significant delayed tone responses. The data from these 11 neurons are combined in a single raster plot, which has been color-coded for average firing rate (Figure 7). After conditioning (within the first 10 trials of extinction), there is a clear high-rate region in the 700–1500 ms range. The peak of this response appears as red pixels in Figure 7. Since the cells were selected to be tone responsive in this region, this alone is not surprising.
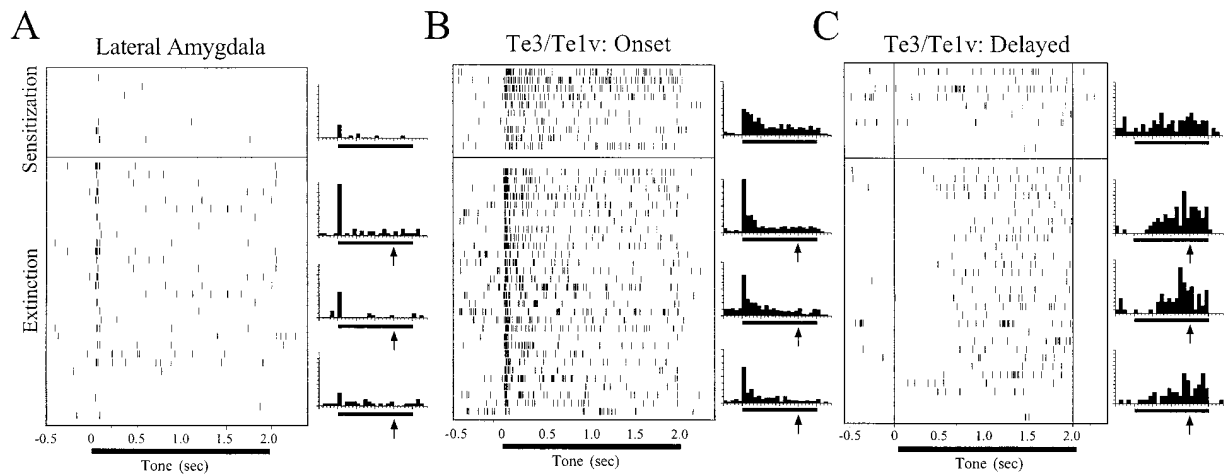
Figure 5. Examples of Conditioned Tone Responses in Auditory Association Cortex (area Te3/Te1v) and Lateral Amygdala

(A) Conditioned onset response in an LA neuron. Raster plot showing the occurrence of individual spikes before conditioning (10 trials of sensitization) and after conditioning (30 trials of extinction). The bar indicates the duration of the tone. Unit firing is summarized for 10-trial blocks in the post-stimulus time histograms (PSTHs) shown to the right of the raster plot (bin width, 100 ms). The LA onset response increased with conditioning and decreased to original levels following 30 extinction trials. No changes were observed later in the tone. The arrow marks the time that footshock occurred during conditioning trials.

(B) Conditioned onset response in a Te3/Te1v neuron. Raster and PSTHs are the same as in (A). Unlike in the LA cell, conditioned increases in the onset response persisted following 30 extinction trials.

(C) Conditioned delayed response in a Te3/Te1v neuron. Conditioning caused an increase in firing rate that began ~700 ms and peaked ~1500 ms after tone onset, which was the time at which the footshock occurred during conditioning (arrow), suggesting that these cells anticipated the unconditioned stimulus. No onset response was present in these cells.

However, no trace of this delayed tone response is visible prior to conditioning (Figure 7, top). The delayed response, then, was entirely induced by the pairing of the tone and shock during conditioning. Figure 7 also shows that there was relatively little indication of an onset response in these cells, suggesting that onset conditioning and delayed conditioning may be exhibited by different cell classes in auditory cortex. Finally, it appears from Figure 7 that delayed conditioned responses did not survive extinction training to the extent that onset conditioned responses did. Thus, while both LA and Te3/Te1v showed onset conditioning, only Te3/Te1v exhibited an additional firing correlate that anticipated the US event.

## Discussion

We have recorded from neurons in auditory association cortex area Te3/Te1v in the rat during auditory fear conditioning and compared the responses to lateral amygdala (LA) cells previously recorded under similar conditions (Quirk et al., 1995). While this is the first description of the effects of fear conditioning on neurons in the auditory association cortex of rats, homologous structures have been studied in cats (Diamond and Weinberger, 1984, 1986) and rabbits (Kraus and Disterhoft, 1982). Additionally, numerous recording studies during aversive conditioning have been performed in the primary auditory cortex of several species (Oleson et al., 1975; Weinberger et al., 1984; Edeline et al., 1990; Edeline et al., 1993; Weinberger et al., 1993; reviewed by Weinberger, 1995). Fear conditioning has also been

shown to increase evoked potentials (Adey, 1967), oxygen utilization (Gonzalez-Lima and Scheich, 1986), and blood flow (LeDoux et al., 1983) and to activate microtubule-associated protein-2 (Woolf et al., 1994) in the auditory cortex. Given these many previous reports of plasticity in the auditory cortex, the focus of the present study was not to demonstrate neuronal plasticity per se. Nor was the purpose to identify the brain areas critical for conditioned fear behavior; rather, our aim was to evaluate the potential contribution of Te3/Te1v to lateral amygdala plasticity. Specifically, we were interested in whether plasticity in Te3/Te1v, the cortical area that provides most of the auditory input to LA, could account for the early plasticity of LA cells, based on the latency of plastic responses and their development across training trials.

### Tone Responses before Conditioning

Previous work has shown that the shortest response latencies in LA occur in the dorsal subdivision (Bordi and LeDoux, 1992; Romanski et al., 1993). Following tone onset, the earliest tone responses in LA and Te3/Te1v occurred at 12 ms. These early responses are most likely due to direct projections from the auditory thalamus to both structures. This follows from known response latencies of units in the auditory pathway. The earliest tone responses in the medial subdivision of the medial geniculate nucleus/posterior interlaminar nucleus (MGm/PIN) occur 7–9 ms after tone onset (Bordi and LeDoux, 1994), and electrical stimulation of MGm/PIN activates cells in LA as early as 5 ms after onset (Clugnet and LeDoux, 1990; Li et al., 1995). Thus, the

## A

### Area Te3/Te1v
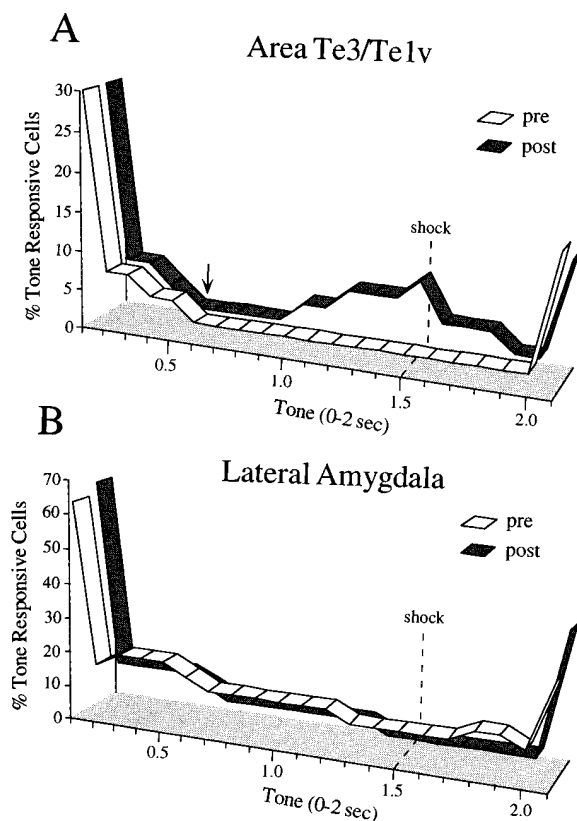


## B

### Lateral Amygdala



Figure 6. Delayed Tone Responses of Auditory Association Cortex Neurons

(A) Ribbon plots showing the percentage of cells that were significantly tone responsive before (white) and after (black) fear conditioning in area Te3/Te1v. While there were prominent onset and offset responses before and after conditioning, conditioning caused cells to show a late tone response that began at ~900 ms and peaked at 1500 ms (the time of shock occurrence in conditioning). This late response was not observed prior to conditioning. Bin width was 100 ms. The tone occurred between 0 and 2 s. The arrow marks the start and the dashed line the end of the "region of interest" used to analyze these delayed responses in Figure 7 (see text).

(B) The same plot as in (A) but for LA neurons. Note that no delayed conditioned response occurred in LA.

minimum conduction delay for activation of LA by thalamo-amygdala fibers is 12 ms. Response latencies of 12 ms were also observed in Te3/Te1v, suggesting that the 10–20 ms response in Te3/Te1v is probably due to thalamic activation rather than transmission from Te1. While responses prior to 20 ms in LA must be thalamic, responses after 20 ms could reflect either thalamo-amygdala or thalamo-cortico-amygdala transmission. These latencies are summarized in Figure 8.

A similar proportion of cells in LA and Te3/Te1v were tone responsive in the 10–20 ms latency range (20% and 18%, respectively). Between 20 and 100 ms, however, more LA cells responded to the tone than Te3/Te1v cells (53% versus 38%). The robustness of LA onset responses compared to Te3/Te1v may be due to the stimulus used as a CS (a 5 kHz pure tone). Past studies have found that LA cells respond briskly to pure tones (Bordi and LeDoux, 1992). In contrast, cells in

auditory association cortex tend to prefer more complex stimuli, such as FM sweeps, over pure tones (Tian and Rauschecker, 1994; Rauschecker et al., 1995). Another explanation for the difference between LA and Te3/Te1V may be the high degree of convergence of auditory-responsive cortical areas onto LA. In addition to receiving thalamic input, LA receives input from Te1v, Te2, Te3/Te1v, and perirhinal cortex (Romanski and LeDoux, 1993), whereas Te3/Te1v only receives auditory information from the medial geniculate body (MGB) and Te1. The convergence of these various auditory inputs in LA could lead to spatial and temporal summation and thus to more intense responses to sensory stimulation.

### Tone Responses after Conditioning

As reported in our previous study (Quirk et al., 1995), fear conditioning caused an increase in the shortest latency tone responses of LA neurons (10–20 ms). In the present study, Te3/Te1v cells were also tone-responsive in the 10–20 ms range, but did not condition until markedly later, 20–40 ms after tone onset. Given that early responses in LA and Te3/Te1v reflect thalamic input, these data suggest that conditioning increased thalamic responses in the LA, but not in Te3/Te1v. Conditioned responses later than 20 ms in Te3/Te1v are probably due to cortico-cortical connections (e.g., Te1 to Te3/Te1V projections), although delayed activation by the thalamus cannot be ruled out. Furthermore, conditioned onset responses in LA developed in fewer training trials (one to three trials) than Te3/Te1v responses (six to nine trials).

Thus, there was no indication that tone responses (conditioned or unconditioned) in Te3/Te1v preceded conditioned responses in LA, in terms of latency or trials, as would be expected if cortico-amygdala transmission were the key to plasticity in LA. In fact, the reverse was true. These data suggest that the cortical input is not required, that fear conditioning modifies thalamic input in the LA, and that the thalamic input is entirely responsible for the early plasticity observed in LA units (LeDoux, 1995; Quirk et al., 1995). Given that we focused on area Te3/Te1v, we cannot rule out a contribution of other cortical structures, such as perirhinal cortex, to subnuclei of the amygdala downstream from LA.

Rapid plastic responses in LA may drive rapid behavioral reactions to threatening stimuli. In fact, fear-potentiated behavioral reactions have been observed as quickly as 50 ms after CS onset (Davis et al., 1989). Later components of the LA tone response could reflect cortically transmitted tone responses or conditioned responses. Given the latency of plasticity in Te3/Te1v (20–40 ms) and the conduction delay between Te3/Te1v and LA (11 ms; Li et al., 1996), we estimate that conditioned responses between 30 and 50 ms in the LA would be the earliest that could reflect cortical processing.

The rapid acquisition of LA plasticity (within one to three trials) suggests that the amygdala is well suited to signaling stimulus–danger associations that could increase an animal's chances of survival (LeDoux, 1996). On average, cortical neurons conditioned much later (after six to nine trials), although there were two cortical cells in our study that showed early changes. The relatively late acquisition rate of neurons in auditory cortex
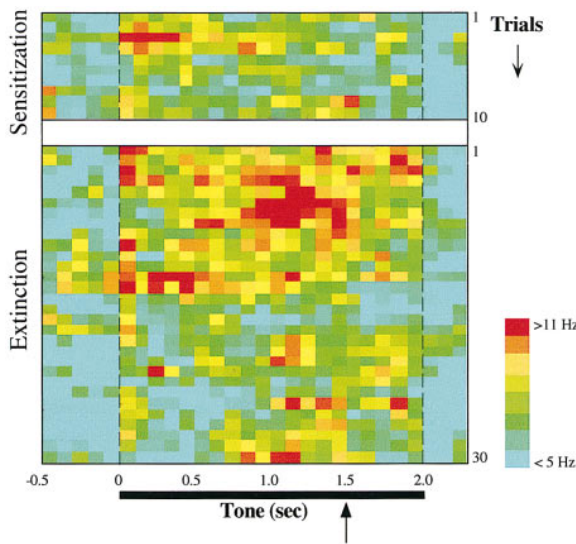
Figure 7. Averaged Delayed Conditioned Response of Auditory Association Cortex Neurons

Color plot represents a raster showing the average firing rate of Te3/Te1v cells that showed a significant delayed tone response during extinction (n = 11). Each row is one trial and each bin is 100 ms. The upper half of the figure shows sensitization trials and the lower half shows extinction trials. The duration of the tone is indicated by the bar (0–2 s). Firing rates were first averaged across cells for each bin and then averaged with nearest neighbors and color-coded (lowest to highest rates: blue, green, yellow, orange, and red). Note that no trace of the delayed response was present prior to conditioning, and that the conditioned response is maximal just prior to the time of footshock during conditioning (arrow).
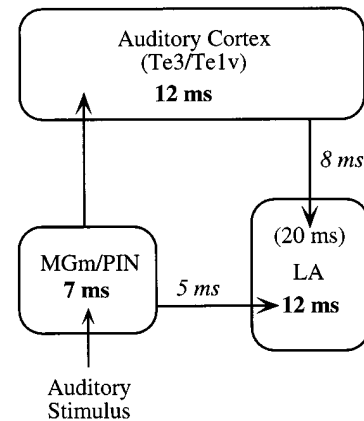


Figure 8. Minimum Response Latencies to Auditory Stimuli

The numbers inside each nucleus (boldface) give the minimum response latency of neurons in that nucleus to auditory stimuli. The numbers next to the arrows (italicized) are minimum latencies resulting from electrical stimulation of the pathway. The number in parentheses is the minimum possible latency for activation of LA via the thalamo-cortical-amygdala pathway.

has been previously noted in comparisons with unit activity in the auditory thalamus (Disterhoft and Stuart, 1976; Edeline et al., 1990), and with conditioned behaviors (Oleson et al., 1975; Kraus and Disterhoft, 1982). Thus, while the auditory cortex may encode important information about the learning event, it may not be responsible for generating simple conditioned behaviors or early plasticity in subcortical structures.

### Extinction-Resistant Memories

An interesting difference between LA and Te3/Te1v was observed during the extinction phase of the training protocol. Units in LA, as we reported earlier, lost their conditioned onset responses after 30 extinction trials (Quirk et al., 1995). Te3/Te1v cells, on the other hand, did not show appreciable extinction of the onset response. While we do not know the rate of behavioral extinction in these animals, the dissociation between LA and Te3/Te1v units suggests that they may have different roles in extinction. Extinction does not erase CS–US associations, because conditioned responses abruptly return following presentation of the US, change of context, or the passage of time (reviewed by Bouton, 1994). Therefore, during extinction, fear memories are preserved while fear responses are temporarily inhibited.

The extinction-resistant onset responses we observed in Te3/Te1v cells suggest that Te3/Te1v may be a site of extinction-resistant fear memories. Once established, these associations could modulate fear behavior via connections to the amygdala, as suggested by the deficits caused by post-training lesions of the auditory cortex (Campeau and Davis, 1995). While tone responses in LA extinguished, spontaneous firing of LA showed changes in the degree of synchrony that persisted throughout extinction (Quirk et al., 1995). Synchronous firing between two cells can be caused by activation of a common input to the cells. Te3/Te1v is a candidate for such an input, since its cells show evidence of persistent memory. Thus, long-term fear memories appear to be "present" in the Te3/Te1v–LA circuit. Interestingly, extinction-resistant conditioned onset responses of single neurons have also been observed in the cingulate cortex and subiculum during appetitive conditioning (Segal, 1973). It is tempting to speculate that sensory cortex, together with polymodal processing regions such as the subiculum and cingulate cortex, might participate in long-term storage processes. These regions may also be involved in the formation of cognitive representations that underlie explicit or declarative memories of the experience, mediated by interactions between the sensory cortex and the medial temporal lobe memory system (Squire and Zola, 1996; Eichenbaum et al., 1996).

The persistence of conditioned onset responses in Te3/Te1v also suggests that it may contribute to the inhibition of LA activity and consequently behavior during extinction trials. Previous studies have shown that lesions of the auditory cortex (Teich et al., 1989) or visual cortex (LeDoux, et al., 1989) impair extinction to auditory or visual CSs, respectively. Using the 2-deoxyglucose method, Gonzalez-Lima and Scheich (1986) showed an increase in Te3/Te1v metabolism during extinction trials. During extinction, sensory cortices may act individually, or together with prefrontal areas (Morgan and LeDoux, 1995) or perirhinal cortex, to reduce fear responses when no longer appropriate. Extinction appears to involve plasticity in the amygdala, because injections of the NMDA antagonist APV into the amygdala prevented extinction (Falls et al., 1992). Thus, normal function of

LA and its cortical inputs appears to be critical for extinction of conditioned fear responses.

## Anticipatory Responses of Neurons in Auditory Association Cortex

An unexpected difference between Te3/Te1v and LA was seen late in the tone-elicited spike train. Our previous analysis of LA tone responses (Quirk et al., 1995) was restricted to the first 70 ms, while in the present study we analyzed the entire 2 s of tone presentation plus the time just after tone offset. Conditioning in LA cells occurred only during the onset response, while some Te3/Te1v cells showed a delayed conditioned response which started at 700 ms and peaked at 1500 ms after tone onset. As shown in Figure 7, this delayed signal was not present prior to conditioning. In general, these cells showed no tone responses (onset or delayed) prior to conditioning but developed a delayed conditioned response as a result of tone–shock pairing.

The time course of the delayed response suggests that it may be anticipating the footshock US. The response starts long after tone onset and peaks at the time of shock onset. This delayed conditioned response is reminiscent of the response described by Berger and colleagues in single hippocampal cells of rabbits undergoing eyelid conditioning (Berger et al., 1983). There, the tone CS came to elicit closure of the nictitating membrane, after it was paired with an air puff to the cornea. As in our study, the CS and US coterminated, but the interval between the CS onset and the US was much shorter in their study (250 ms) than in ours (1500 ms). Neither auditory cortex nor hippocampus is required for either fear conditioning or eyelid conditioning. Therefore, it appears that cortical areas may encode certain aspects of the conditioning experience (such as interval timing), even though they are not an essential part of the circuitry that generates the conditioned behavior. Anticipatory firing of cortical neurons could underlie the accurate behavioral timing of CS–US intervals observed during bar pressing for food (Gallistel, 1990) and fear conditioning (Davis et al., 1989). Finally, anticipatory responses of cortical neurons may reflect increased attention during the CS, perhaps triggered by the amygdala (Armony et al., 1997b). It is interesting to note that, unlike cortical onset responses, delayed conditioned responses partially extinguished with extinction trials, suggesting a possible role in generating the conditioned behavior.

## The Role of the Lateral Amygdala in Fear Conditioning

Our present data on latency and trials indicates that onset plasticity in LA during fear conditioning is due to thalamo-amygdala activation (LeDoux, 1995; Quirk et al., 1995). The question remains, however, whether this plasticity is generated locally in LA or in the auditory thalamus. Either possibility is consistent with the latencies we observed. There are numerous reports of plasticity in MGm neurons during fear conditioning (Gabriel et al., 1976; Ryugo and Weinberger, 1978; Birt et al., 1979; Edeline et al., 1990; McEchron et al., 1995). However, the latency of conditioned responses (e.g., 20–40

ms) was often later than would be expected from potentiation of inferior colliculus inputs (reviewed by Quirk et al., 1996). Moreover, it is PIN rather than MGm that gives rise to most thalamoamygdala fibers (LeDoux et al., 1990b). Conditioned responses of PIN cells have not yet been studied, but stimulation results suggest a role in conditioning (Cruikshank et al., 1992). The latency and trial development of plastic responses in both the MGm and PIN need to be accurately measured and compared to LA and Te3/Te1v before the contribution of MGm/PIN to fear conditioning can be fully evaluated.

Behavioral studies suggest that MGm/PIN plasticity without LA plasticity is not sufficient to produce fear conditioning. Temporary disruption of the lateral amygdala with local injections of APV (Miserendino et al., 1990) or the GABA agonist muscimol (Muller et al., 1997) during the acquisition phase of fear conditioning blocks the expression of learned behaviors at a subsequent time when the drug has worn off. These deficits cannot be attributed (as lesion deficits can) to the loss of circuitry critical for the expression of conditioned responses. Thus, LA seems to serve a key role during the acquisition phase, when plasticity is developing.

Another clue to the role of LA comes from the time course of changes in LA onset responses across conditioning trials. While LA cells rapidly signaled the pairing of the CS and US within three conditioning trials, their tone responses returned to pre-conditioning levels after ~12 trials (see Figure 3B). Te3/Te1v cells maintained their conditioned response, more or less, throughout the conditioning phase. These data suggest that LA cells signal changes in the degree of CS–US association rather than the magnitude of association. Hence, the LA signal is high when the CS–US relationship is changing (at the beginning of conditioning and the beginning of extinction) and low when the CS–US relationship is constant (at the end of conditioning and the end of extinction). In other words, the LA tone response seems to act as a differentiator of the CS–US association. This could serve as the error signal between predicted and experienced outcomes that has been proposed by classical learning theory (Rescorla and Wagner, 1972) and modeled in adaptive networks (Sutton and Barto, 1981). In such networks, the differentiator is often followed by an integrator, which maintains the CS–US signal throughout conditioning, which is similar to observed behavior. Considering that LA is the first processing station of the amygdala, subsequent nuclei in the amygdala may show correlates closer to what is observed in conditioned behavior. Following a change in CS–US association, the alerting signal of LA is transmitted to other amygdaloid subnuclei for rapid initiation of defensive behaviors. Cortical areas, as well as amygdala subnuclei downstream from LA, may be involved in further mnemonic and attentional processing of traumatic events.

In summary, an analysis of the effects of fear conditioning on onset tone responses in auditory association cortex (area Te3/Te1v) and LA has shown that changes in LA precede those in Te3/Te1v both within and across trials. These findings provide strong support for the hypothesis that plasticity during auditory fear conditioning to a single, simple tone does not depend on the auditory

cortex but instead involves direct projections from the auditory thalamus to the lateral amygdala. Surely, the auditory cortex is required for fear learning in situations involving more complex stimuli but not for the tasks typically used in simple fear conditioning. At the same time, the persistence of conditioned onset responses in auditory association cortex during extinction training suggests that this region is a site of long-term storage of some aspects of the fear memory, and it may also play a role in the inhibition of conditioned fear responses when danger is no longer present. The presence of shock-anticipatory conditioned responses in auditory association cortex suggests that this area may contribute to higher cognitive processes such as timing and increased attention, while LA has an essential but temporally limited, role in signaling changes during a potentially dangerous situation.

## Experimental Procedures

The procedures were essentially the same as those used to study LA neurons (Quirk et al., 1995). All procedures were in accordance with Public Health Service guidelines and were approved by the animal use committee of New York University.

### Animals and Surgery

Male Sprague-Dawley rats (300–350 g) were anesthetized with nembutal (50 mg/kg intraperitoneally) following pretreatment with atropine (0.24 mg/kg). Additional doses of anesthetic were administered when the respiratory rate increased or there was movement in response to a tail pinch. Body temperature was maintained with a gel heating pad. While in a stereotaxic headholder, the cranium was exposed, burrholes were drilled, and self-tapping, stainless steel screws were implanted anterior to bregma and posterior to lambda for anchoring the implant to the cranium.

A movable drive of 20 microwires (adapted from Kubie, 1984) was implanted in the auditory cortex, using the following coordinates from Paxinos and Watson (1986): 5.0 mm posterior to bregma, 5.1 mm lateral to the midline, and 6.0 mm ventral to bregma. The drive consisted of nichrome wires (25 $\mu$m), insulated except for the cut tip (impedance, ~2–3 M$\Omega$) contained in a stainless steel tube (diameter, 0.018 in). The brush of wires had a diameter of ~0.5 mm. The wires were attached to two 10-pin connectors (Augat), which were embedded in a triangular acrylic platform. The platform could be advanced by turning three screws (one turn, 460 $\mu$m), each of which entered threaded, nylon cuffs that were cemented to the skull. To improve placement accuracy, an additional guide tube (diameter, 0.032 in) was implanted just above the cortex, in which the guide tube and wires were inserted. The tips of the electrode bundle were positioned just dorsal to area Te3/Te1v in area TE1. At the end of surgery, the incision was sutured around the acrylic block, a topical antibiotic was applied, and rats were allowed 5 days to recover.

### Unit Recording

Rats were placed in a test box (25 × 30 × 35 cm) with an electrifiable grid floor (Coulbourn Instruments) and an open top, which was enclosed in a sound attenuating chamber (Industrial Acoustics, Bronx, NY). The electrode was connected to a headstage consisting of field-effect transistors (FETs) with unity gain in source-follower configuration, attached to a cable that was allowed to freely rotate via a slip-ring commutator (Plastics One, Roanoke, VA). The commutator was connected to a patch panel, which allowed the outputs of the FETs to be connected to differential amplifiers (AM Systems, Everett, WA). Signals were amplified (10,000× gain), passively filtered (300–5000 Hz passed), digitized at 16 kHz (DataWave Technologies, Longmont, CO), and displayed on a computer monitor and digital oscilloscopes. Signals exceeding a selected voltage threshold were captured in 2 ms epochs.

The electrode was advanced in 40 $\mu$m steps, usually three to four times per day. When discriminable single units were encountered,

they were tested for tone responsiveness by delivering 50 ms tone pips at various frequencies, including white noise. Tones were generated with a DSP board running on a PC microcomputer, which was controlled by a clocked sequence (MOZART) running within the spike acquisition software (Discovery, DataWave Technologies). A speaker placed in the ceiling of the sound-attenuating chamber emitted tones that were measured to be 80 db ($\pm$ 5 db) at all locations in the test box. Spike waveforms were stored on disk and sorted off-line with DataWave software, using cluster plots of different waveform parameters (e.g., spike height, time to peak, etc.). Spike clusters were defined as single cells if they were separate from other clusters and from background activity. Usually, cellular activity could be recorded on several wires, and typically one to three single cells could be discriminated on active wires.

### Conditioning

Once several tone-responsive cells were identified in what was believed to be cortical area Te3/Te1v, a conditioning experiment commenced. The protocol was identical to our previous study of LA cells (Quirk et al., 1995). The conditioned stimulus (CS) was a pure tone (5 kHz, 80 db, 2 s, 5 ms rise time), and the unconditioned stimulus (US) was a mild footshock (0.5 mA, 0.5 s) delivered through the grid floor of the test box. A sensitization phase consisting of 10 tones and 10 shocks that were explicitly unpaired was immediately followed by a conditioning phase where the tone and the shock coterminated (20 trials). Trials were separated by a variable interval of 1–3 minutes. After conditioning, the rat was placed in its home cage for 1 hr, after which it was returned to the test chamber for 30 extinction trials (tone alone) on the same schedule as conditioning. Freezing to the CS was measured before sensitization and after conditioning, in a different context from the testing chamber. In this behavioral test, rats were placed in the chamber and a single 20 s tone at the CS frequency (5 kHz, 80 db) was delivered. The cumulative time the rat spent freezing (defined as the absence of all movements except for respiration) was measured with a digital timer. The entire conditioning procedure took ~4 hr. Each rat was conditioned only once.

### Data Analysis

Files containing the time of spike occurrences and tone onsets and offsets were written to disk, and subsequently analyzed on a PC with commercially available software (STRANGER, Biographics, Winston-Salem, NC). Post-stimulus time histograms (PSTHs) showing the tone response of neurons were generated with different bin widths (10–100 ms), depending on the particular question. Binned spike counts were transferred to a spreadsheet (Excel) for statistical analyses of the tone responses.

#### Onset Tone Responses

Responses during sensitization trials 1–10 were used to determine whether a cell had a response to tone stimuli before conditioning. Past work on LA and preliminary studies on Te3/Te1v indicated that onset responses (0–50 ms) were typical of both areas. To measure onset responses, 5 bins of 10 ms each were analyzed. Significant onset tone responses were defined by bins with a firing rate greater than 4 SD above the average rate in the time (500 ms) just before tone onset.

#### Conditioned Onset Responses

To test whether long-term changes in onset responses were present at least 1 hr after conditioning, onset responses before conditioning (sensitization trials 1–10) were compared with responses after conditioning (early extinction trials 1–10). During early extinction, a bin was classified as conditioned if it had a firing rate greater than two times the sensitization rate in that bin. The difference in spike count had to be two or more spikes; a difference of one spike was never classified as significant. A cell was classified as conditioned if one or more of the 10 ms bins (in the first 50 ms following tone onset) satisfied the criterion.

#### Acquisition Rate across Trials

Rate of acquisition of conditioned responses across trials was determined for onset responses using a single 50 ms bin. Responses were analyzed trial by trial. Trials with responses greater than 1.96 SD (95% confidence interval) above the sensitization rate were considered to be conditioned.

### Delayed Tone Responses

Although most cells showed onset responses before and after conditioning, some cells developed, after conditioning, late or delayed tone responses that began several hundred milliseconds after tone onset. These delayed tone responses were analyzed with 100 ms bins. Two-tailed t tests ($p < 0.05$) comparing the rate during the tone to pre-tone rates were used to identify significant tone responses. This analysis revealed a clear "region of interest," bordered by the onset of the delayed activity (typically ~500 ms after tone onset) and the point of occurrence of the shock during conditioning (1500 ms after tone onset). This region (500–1500 ms) was analyzed with Student's t tests ($p < 0.05$, two-tailed) to determine tone responsiveness (comparing tone to pre-tone rates) as well as conditioned responsiveness (comparing rates during early extinction trials to rates during sensitization trials). The rate of acquisition (across trials) of conditioned responses in the delayed region of interest could not be measured, because inputs to the amplifiers were grounded during shock presentation (in conditioning trials) to avoid shock artifact contamination of the unit recordings.

### Color-Coded Firing Rate Plots

In order to show averaged conditioned responses for group data across training trials, we used a colorized raster plot, where rows indicate single trials and columns indicate time bins. The pixels were color-coded for average firing rate. Data from multiple cells were pooled for each trial. After smoothing with a weighted, nearest neighbor algorithm, the array of averaged rates was converted to a color plot by a Macintosh-based program (Mathematica). The range of rates was linearly mapped onto a color scale (lowest to highest rates: blue, green, yellow, orange, and red). Bin widths were 100 ms.

### Histology

At the conclusion of the conditioning experiment, the rats were given an overdose of sodium pentobarbital (100 mg/kg). Anodal current (8 μA for 10 s) was passed through one of the recording wires in order to deposit iron. The rats were transcardially perfused with buffered formalin. The brains were removed and stored in a formalin–sucrose solution with 2% nitroferrocyanide added to visualize the iron deposit (Prussian blue reaction). Frozen sections (40 μm thick) were cut on a microtome, and sections were stained for Nissl bodies and examined with a light microscope. The site of the Prussian blue reaction was used to locate the region of the recorded cells.

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