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Fear Conditioning Enhances Short-Latency Auditory Responses of Lateral Amygdala Neurons: Parallel Recordings in the Freely Behaving Rat

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Summary

The lateral nucleus of the amygdala (LA) is the first site in the amygdala where the plasticity underlying fear conditioning could occur. We simultaneously recorded from multiple LA neurons in freely moving rats during fear conditioning trials in which tones were paired with foot shocks. Conditioning significantly increased the magnitude of tone-elicited responses (often within the first several trials), converted unresponsive cells into tone-responsive ones, and altered functional couplings between LA neurons. The effects of conditioning were greatest on the shortest latency (less than 15 ms) components of the tone-elicited responses, consistent with the hypothesis that direct projections from the auditory thalamus to LA are an important link in the circuitry through which rapid behavioral responses are controlled in the presence of conditioned fear stimuli.

Introduction

To survive, animals need to learn about places and things in their world that are likely to cause them harm. The primary experimental model for studying so-called aversive learning is classical fear conditioning, in which the subject is exposed to an innocuous conditioned stimulus (CS), such as a tone, in association with a noxious unconditioned stimulus (US), often foot shock. As a result of this CS–US pairing, the CS acquires aversive properties and comes to elicit defensive behaviors and autonomic nervous system responses.

A large and growing body of evidence points to the amygdala as a critical structure in the neural system that mediates fear conditioning (Davis, 1992; Kapp et al., 1992; Fanselow, 1994; LeDoux, 1995). This work suggests that the amygdala is situated between sensory systems involved in processing the conditioned fear stimulus and motor regions involved in controlling the conditioned fear responses. Although it is widely assumed that the amygdala is crucially involved in fear conditioning, mechanisms of neural plasticity within the amygdala that account for conditioned fear learning are not well understood.

Our approach to understanding amygdala plasticity has been to identify the anatomical circuits through which information about an acoustic CS flows into and through the amygdala in rats. The lateral nucleus (LA) is known to be the sensory-receptive region of the amygdala (LeDoux, 1992). Acoustic stimuli are transmitted to LA from auditory processing regions in the thalamus and cortex (LeDoux et al., 1985, 1990a; Turner and Herkenham, 1991; Mascagni et al., 1993; Romanski and LeDoux, 1993), and either of these pathways is sufficient for the mediation of fear conditioning to a pure tone CS (Romanski and LeDoux, 1992; LeDoux, 1995; but see Campeau and Davis, 1995). Furthermore, lesions of LA prevent fear conditioning (LeDoux et al., 1990b). LA is also a site of convergence of pathways transmitting the auditory CS and the somatosensory US (Romanski et al., 1993) and is the region of the amygdala with the shortest response latency to acoustic stimuli (Bordi and LeDoux, 1992). Information received by LA is then transmitted, over intra-amygdala pathways, to several additional nuclei (Price et al., 1987; Stefanacci et al., 1992; Pitkanen et al., 1995). Given the pivotal role of LA as the sensory interface of the amygdala, it thus seems important to determine whether plasticity occurs in this region during fear conditioning.

Most of the previous studies recording unit activity during fear conditioning have focused on the central (Applegate et al., 1982; Pascoe and Kapp, 1985; Muramoto et al., 1993) and basolateral (Maren et al., 1991; Muramoto et al., 1993) nuclei of the amygdala. While postulated to be sites of plasticity, conditioned responses observed in these structures may also reflect learning that occurs in LA, which is afferent to both other regions.

LA is not homogenous. Anatomical tracing studies indicate that the projections to LA from auditory processing regions of the thalamus and cortex terminate more extensively in dorsal (LAd) than in ventral (LAv) areas (LeDoux et al., 1990a; Turner and Herkenham, 1991; Mascagni et al., 1993; Romanski and LeDoux, 1993), and unit recording studies suggest that neurons in LAd respond to auditory stimuli with shorter latencies and with greater consistency than neurons in LAv (Bordi and LeDoux, 1992; Bordi et al., 1993; Romanski et al., 1993). Additionally, LAv, especially the medial part, gives rise to a more extensive set of intra-amygdala connections than LAd (Pitkanen et al., 1995).

In the present study, we examined the effects of classical fear conditioning on LA neurons. Using parallel recording techniques, we simultaneously recorded from multiple single units in chronically prepared, freely moving rats during exposure to a tone CS and a foot shock US. We analyzed the responses of neurons in LAd and LAv separately, but were especially interested in the plasticity of neurons in LAd, the first locus of sensory processing in the amygdala. The use of parallel recordings of single units reduced the impact of nonstationary variables such as behavioral state (Nicolelis and Chapin, 1994) and also allowed the assessment of functional connectivity between pairs of LA neurons (using cross-correlation techniques).

Results

Electrode Placements

A total of 74 cells from 24 rats were included in the data analysis. The locations of the recording bundles (1 bundle

per rat) are shown in Figure 1. Thirty-eight of the cells were located in LAd and 36 in LAv. Although LAv is composed of medial and lateral divisions with separate connections (Pitkanen et al., 1995), we could not distinguish these on the basis of the locations of the bundles. Typically, 1–8 wires were active per bundle, with 1–3 cells isolated per wire.

Electrophysiological Characteristics of LA Neurons

As reported in previous studies, the spontaneous firing rates of LA neurons were low (e.g., Bordi and LeDoux, 1992). Firing rates ranged from 0.003 to 46.2 Hz, with an average of 2.3 Hz. However, 75% of the cells had rates that were less than 1 Hz, and the geometric mean was calculated to be 0.33 Hz. Spike widths (time between maximum and minimum voltage) ranged from 0.10 to 0.66 ms, with an average of 0.47 ms. Spike width and firing rate were inversely correlated ($r = -0.40$, $p < .001$), with a clustering of high width, low rate values. A width–rate correlation of −0.63 was calculated from data reported in a recent study of hippocampal place cells (Markus et al., 1994), where pyramidal cells have low rates and large spike widths. Like the hippocampus, LA contains pyramidal-type projection cells as well as nonpyramidal interneurons (MacDonald, 1982; Millhouse and DeOlmos, 1983). The preponderance of slowly firing cells with long spike widths suggests that many cells in our sample may be pyramidal-type neurons. Their selection is also favored by their large size relative to nonpyramidal cells.

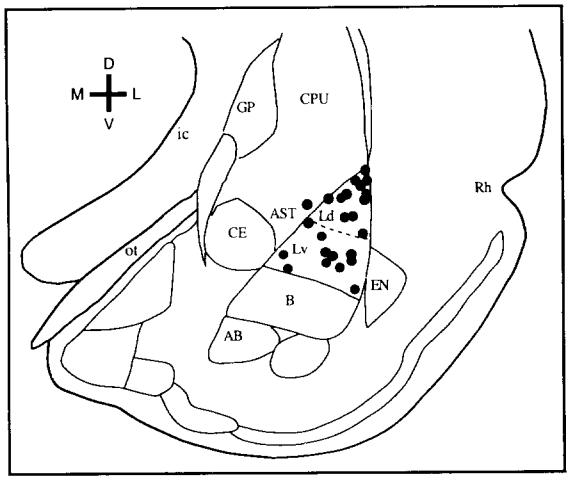

Figure 1. Electrode Placement in LA

Drawing of a coronal section through the rat brain showing the ventral half of the right cerebral hemisphere. Each circle represents the placement of an electrode bundle for the 24 rats in the study. A total of 74 single units were isolated from the 24 bundles. About half of the cells were in the dorsal part of LA (LAd; n = 38) and half in the ventral part (LAv; n = 36). One of the bundles was on the border of LAd and the caudate/putamen, in the amygdalostriatal transition area (AST). AB, accessory basal nucleus of the amygdala; B, basolateral nucleus of the amygdala; CE, central nucleus of the amygdala; CPU, caudate/putamen; EN, endopiriform nucleus; GP, globus pallidus; IC, internal capsule; Ld and Lv, dorsal and ventral parts of LA; OT, optic tract; RH, rhinal fissure.

Tone Responses Prior to Conditioning

The latency of tone responses was measured in 74 LA cells. Prior to training, 20 of 38 (53%) neurons in LAd and 18 of 36 (50%) neurons in LAv were tone responsive within 70 ms following tone onset. In LAd, 8 of 20 tone-responsive neurons (40%) responded with a latency under 20 ms, but in LAv, only 2 of 18 tone-responsive neurons (11%) had a latency under 20 ms. The average tone-response latencies prior to conditioning were 25.0 ms in LAd and 31.1 ms in LAv. Tone responses in LAd are thus earlier and probably precede those of LAv (Bordi et al., 1993).

Conditioned Increases in Tone Responses

A total of 59 LA neurons were recorded during conditioning (the remaining 15 cells were from rats given prolonged sensitization training, described below). For the conditioned rats, tone responses (0–70 ms after tone onset) during the preconditioning sensitization period (during which tones and shocks were unpaired) were compared with those at the beginning of extinction (after completion of tone–shock pairings) to determine the effects of conditioning (see Experimental Procedures). Neurons were judged to have conditioned if the tone responses during extinction were significantly greater than those during sensitization ($p < .05$, one-tailed with Bonferroni correction). With these criteria, 16 of 59 LA neurons (27%) conditioned. Of cells that were tone responsive prior to conditioning, 12 of 32 (38%) conditioned. There was no difference between LAd and LAv in the fraction of cells that conditioned.

Figure 2A shows perievent time histograms for 5 simultaneously recorded neurons in LAd at three points during training. The tone response increased during the early extinction trials (relative to sensitization) and then returned to preconditioning levels in late extinction. The greatest changes occurred in the shortest-latency components of the tone response, as seen in the difference histogram (early extinction–sensitization) in Figure 2B. All 5 neurons show a conditioned response within 16 ms of tone onset. As described below, the potentiation of early tone responses was consistently seen in LAd.

A learning curve showing the increase in tone responses with training for LA cells that significantly conditioned is shown in Figure 2C. The average tone response during blocks of 10 trials is expressed as a percentage of responses during sensitization trials. Note the increase in responsivity in the early extinction trials relative to the last block of conditioning trials (these 2 blocks of trials were separated by a 1 hr rest period in the home cage). Tone–shock pairing increased the tone responses of conditioned cells to an average of 5.1 times sensitization levels (n = 16). For all LA cells recorded (those that conditioned and those that did not; n = 59), this factor was 2.5. The magnitude of the changes was similar in LAd and LAv. The conditioned tone responses reverted to preconditioning (sensitization) levels after 3 blocks (30 trials) of extinction training.

To determine when in the training sequence conditioned responding appeared, we analyzed tone responses in blocks of 3 trials during training. Of 15 conditioned cells, 5 significantly conditioned by the first block of training (trials 2–4). Interestingly, all 5 of these cells were in LAd (5

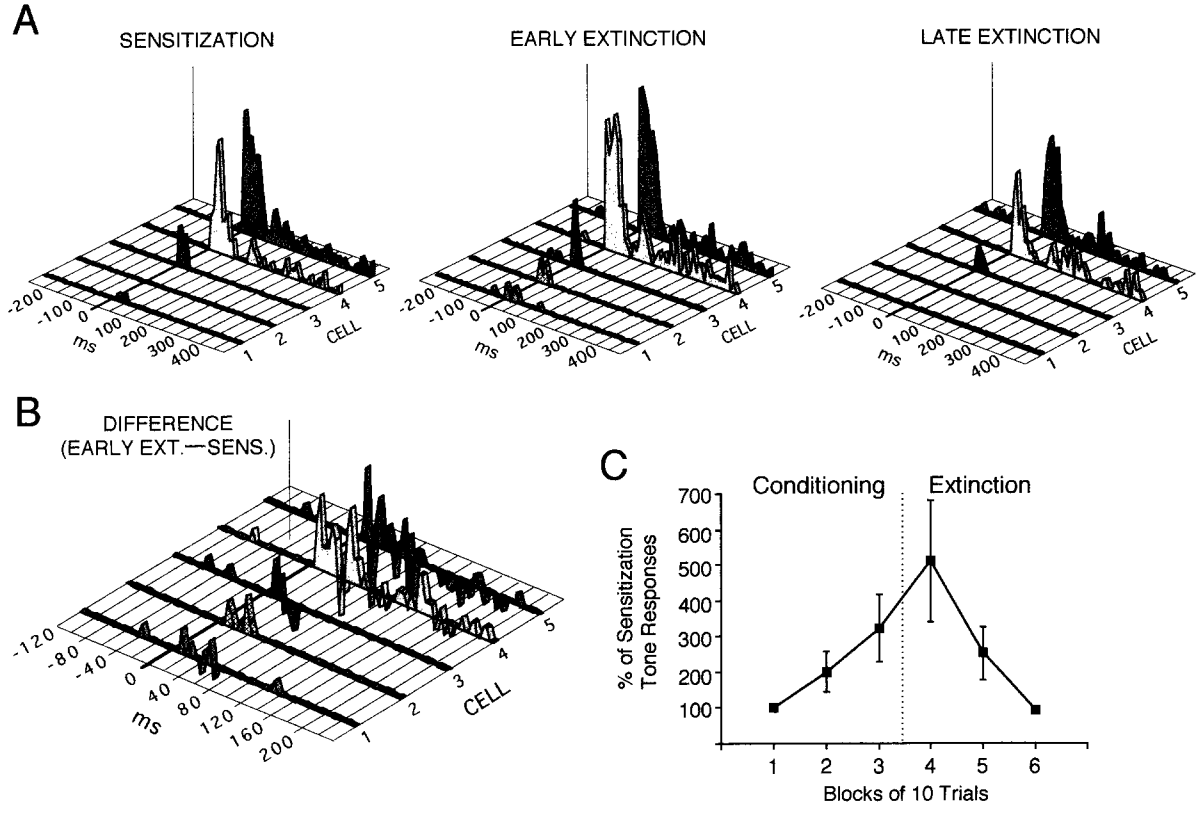

Figure 2. The Tone Responses of Simultaneously Recorded Neurons in LA at Different Points during Training

(A) Representative perievent time histograms showing 5 neurons from LAd simultaneously recorded prior to conditioning (sensitization), 1 hr after conditioning (early extinction), and following 30 extinction trials (late extinction). The start of the tone is marked with a heavy line at t_0. Note the short-latency increases in tone response at the beginning of extinction and the return to preconditioning levels by late extinction. Bin width is 10 ms, 10 trials are summed at each phase, and the peak spike counts for the cell with the greatest response (#5) in the three phases were 15, 18, and 12, respectively.

(B) A difference perievent time histogram showing the conditioned responses of the 5 cells. Spike counts during sensitization were subtracted from counts during early extinction. Note the increase in short-latency conditioned responses (present in all 5 cells) that terminate by the fourth bin (16 ms) following tone onset. Bin width is 4 ms, 10 trials are summed, and peak count for cell #5 was 6 spikes.

(C) A learning curve showing the modulation of tone responses in significantly conditioned LA neurons across training. Tone responses at all points in the experiment are expressed as a percentage of the tone response during sensitization trials (block 1). The tone response is the difference between the firing rate during the first 70 ms following tone onset and the 500 ms preceding the tone (bars show SEM). After completion of conditioning (first extinction block), tone responses increased by a factor of 5.1. Blocks 1–4, n = 16 cells; blocks 5–6, n = 9 cells.

of 9 cells), whereas none were in LAv (0 of 6). Thus, plasticity in LAd, but not LAv, developed within 4 trials.

Cells with fast spontaneous rates (>1 Hz; n = 14) conditioned in greater numbers (6 of 14) than slower cells (<1 Hz; n = 37; 8 of 37 conditioned; $\chi^2 = 6.9$, $p < .01$). This suggests that fast cells (interneurons?) may be particularly plastic.

Prolonged Sensitization Training

In addition to the conditioning group described above, we also studied the tone responses of LA cells in 6 rats that received unpaired tones and shocks throughout training. We recorded from a total of 15 LA neurons in these rats. There were no differences between responses of cells in LAd and LAv, and these data were pooled. On average, prolonged sensitization training decreased the tone responses of these cells, but 2 of the 15 cells showed small but significant increases. In the first block of extinction trials, the average tone response of these 6 cells was 97% of the value during sensitization. In contrast, as described above for animals given paired training, the average tone response in the early extinction trials significantly increased by a factor of 2.5 ($t = 2.5$, $df = 58$, $p < .05$).

Latency of Plasticity

Conditioning increased the magnitude of the earliest tone responses, especially in LAd. The average latency of the earliest conditioned responses was 21 ms in LAd and 42 ms in LAv. Figure 3A shows the latency of conditioned responses for the 10 LAd neurons that conditioned (see above): 7 of these neurons (70%) conditioned prior to 20 ms after tone onset, and 6 of the 7 conditioned prior to 15 ms. Thus, 6 of the 10 LAd cells (60%) conditioned prior to 15 ms. These latencies are consistent with the latency of activation of LAd neurons by afferents from the auditory thalamus (Bordi and LeDoux, 1994; Romanski et al., 1993). Figure 3B shows the number of cells that conditioned in each bin expressed as a percentage of all cells

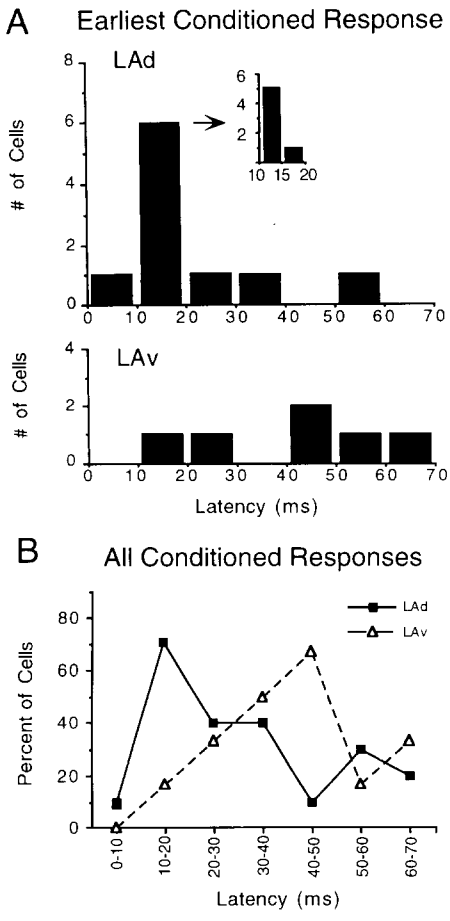

Figure 3. The Latency of Conditioned Responses in LA Neurons

(A) Histograms show the latency of the earliest significant conditioned response of cells in LAd and LAv, as measured during the first block of extinction trials (see Experimental Procedures for determination of significance). Bin width was 10 ms. Inset shows the results from the 10–20 ms bin at 5 ms resolution. Note the preponderance of cells with conditioned responses prior to 15 ms in LAd.

(B) The latencies of all significant conditioned responses from 0–70 ms are plotted (a given cell may be represented more than once). Note that conditioning in LAd peaks at 10–20 ms and decreases thereafter, whereas plasticity in LAv peaks at 40–50 ms.

that conditioned. After peaking at 10–20 ms, LAd neurons showed few conditioned responses by 40 ms, whereas the plasticity in LAv neurons peaked at 40–50 ms.

The Effect of Training on Latency of Tone Responses

As suggested from the early latency of conditioned responses, the proportion of cells in LAd exhibiting early tone responses was increased following training. Figure 4A shows the distribution of tone response latencies in LAd cells before and after conditioning. Two effects of conditioning are apparent. The percentage of tone-responsive cells increased from 56% to 69%, and the number of responses occurring prior to 20 ms increased significantly relative to other bins ($\chi^2 = 4.0$, $p < .05$). The increased number of short-latency responses was due to two factors: the addition of newly tone responsive neurons (cells that did not respond to tones prior to conditioning) with response latencies below 20 ms (3 cells) and a decrease in the latency of tone-responsive neurons to below 20 ms (4 cells). Two examples of conditioning at the early part of the tone response are shown in Figure 4B. Most of these changes were reversed during extinction trials.

Conditioned Decreases in Tone Responses

Owing to the low firing rates of most LA neurons, it was difficult to measure inhibitory tone responses and also difficult to assess inhibitory effects of training on tone responses. Visual inspection of the perievent time histograms revealed that the tone responses of 4 LA cells were inhibited by conditioning and returned to preconditioning levels after extinction. Adding these 4 cells to the 16 that exhibited conditioned increases (see above) brings the total number of LA cells that learned to 20 of 59 (34%).

The Effect of Training on Spontaneous Firing Rate

Spontaneous activity was recorded at four points during the training sequence: immediately prior to sensitization, immediately following conditioning, 1 hr following conditioning, and following the extinction trails. All spontaneous recordings were made while the rats were in the test chamber. The geometric means of spontaneous rates throughout conditioning ranged from 0.59 to 0.66 for all LA cells ($n = 25$) and from 0.59 to 1.12 for those LA cells that conditioned ($n = 11$). No significant effect of training on these rates was observed ($p = .96$ for all LA cells and $p = .52$ for conditioned LA cells, repeated measures ANOVA). Analysis of spontaneous rates on a cell by cell basis showed that the firing rates of 11 of 41 neurons (27%) were altered immediately following conditioning (8 decreased and 3 increased; $p < .05$, one-way ANOVA repeated measures comparison). This fraction was greater for neurons that exhibited a conditioned increase in response to the tone (45%) than for cells that did not (15%; $\chi^2 = 7.16$, $p < .01$). This difference was no longer significant 1 hr after conditioning, when the effects of conditioning on tone responses were measured. Thus, while there was no overall change in mean firing rate as a result of conditioning, short-lasting changes were observed in a proportion of cells that conditioned.

The Effects of Training on Functional Connectivity

The effects of conditioning on functional connectivity between LA neurons were examined by performing cross-correlations between spike trains of pairs of simultaneously recorded neurons and inspecting the distributions for peaks or valleys close to t_0 (the time of occurrence of reference cell spikes). Periods of spontaneous activity (5 min) were examined at different points during training.

Two classes of cell–cell interactions were most commonly observed: synchrony of firing (peak at t_0), suggesting common input, and direct interactions (peak shortly after t_0), suggesting local monosynaptic excitation. Of 113 cross-correlograms generated, 41 contained an adequate number of spikes to interpret. Of these, 3 showed direct interactions with latencies of 1–4 ms after

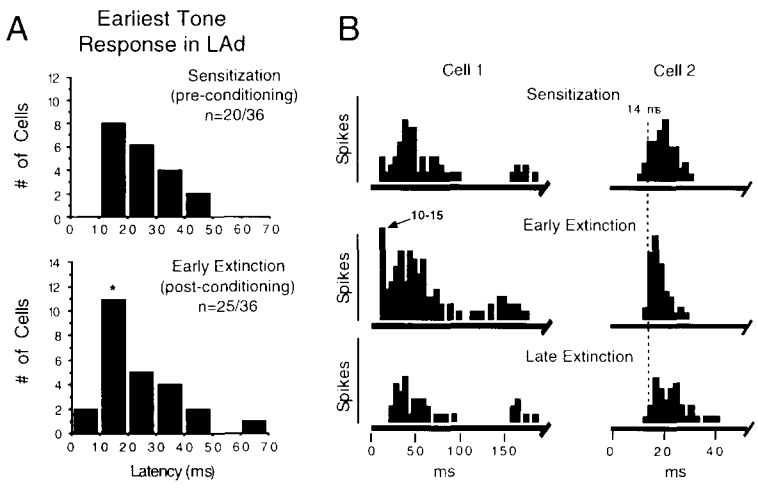

Figure 4. Training Reduces the Latency of Tone Responses

(A) Latency of the earliest significant tone responses in LAd before and after conditioning. Note the disproportionate increase in cells with tone responses prior to 20 ms in early extinction.

(B) Perievent time histogram for representative LAd neurons showing the increase in short-latency (<15 ms) tone responsivity. Bin width was 5 ms for cell 1 and 2 ms for cell 2.

t_0. Cross-correlograms and efficacy values (see Experimental Procedures) for 2 of these cases are shown in Figure 5A. In the first pair (upper), no interaction was evident prior to training; however, training induced an interaction, as indicated by an efficacy value of 0.185. This efficacy value indicates that the target neuron fired a greater than expected number of spikes following 18.5% of the spikes of the reference cell. The difference between pre- and postconditioning was significant ($\chi^2 = 24.5$, $p < .001$). In the second pair (Figure 5A, lower), the latency was much shorter (1.5 ms), but the increase in efficacy was not significant. In addition to the 2 cases illustrated here, a third pair showed direct interactions, and its efficacy was unchanged by training.

In addition to the direct interactions, effects of training on synchrony of cell firing were also observed. Eleven of 41 cross-correlograms showed a peak at t_0 with distributions confined to ± 40 ms. Two of these cases showed training-induced changes and are illustrated in Figure 5B. In the first cell pair (Figure 5B, upper), the loose synchrony evident before conditioning was not observed after conditioning, suggesting a conditioned loss of synchrony. The re-

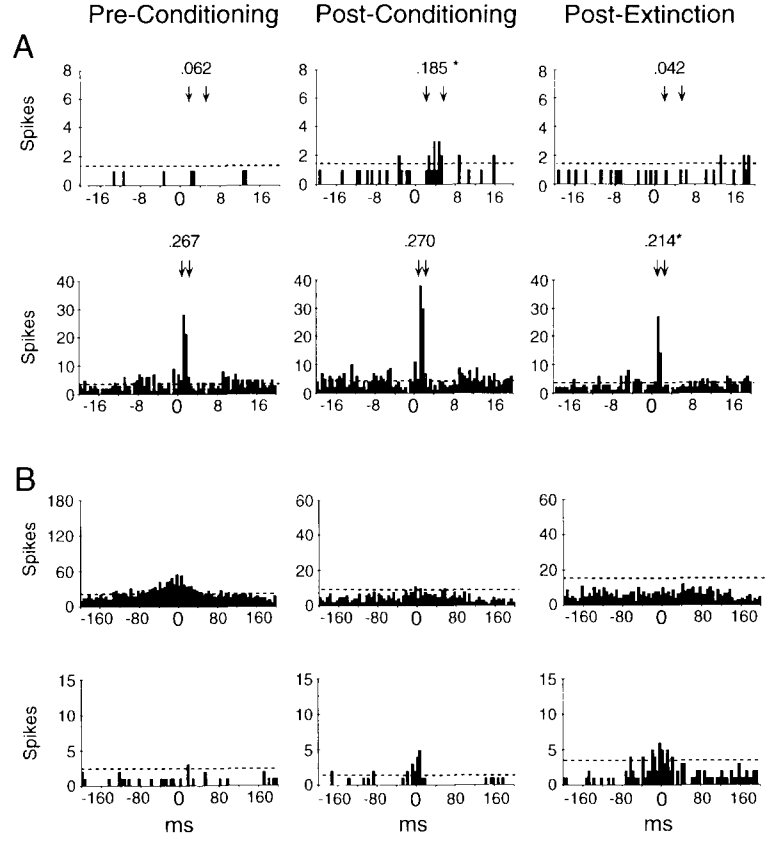

Figure 5. Cross-Correlation Analysis of Spontaneous Activity at Three Points during Training

(A) Two examples of cell pairs showing direct interactions immediately following conditioning (upper) and throughout training (lower). Peaks in the target cell spike distributions were observed at latencies of 4 ms (upper) and 1.5 ms (lower) following reference cell spikes (t_0). Arrows indicate the interval used to generate efficacy values. Training significantly increased the efficacy of the connection between the upper pair of cells. Bin width was 0.5 ms. Dotted lines indicate confidence limits ($p < .01$) for significant interactions, based on a Poisson distribution of interspike intervals (Abeles, 1982).

(B) Two examples of cell pairs showing loose synchrony of cell firing (broad peak at t_0) prior to conditioning (upper) and following conditioning (lower). Conditioning eliminated synchrony in the upper pair and induced synchrony in the lower pair. Note that, unlike changes in direct interactions (see [A]) and tone responses (see Figure 2C), this effect was not reversed with extinction. Bin width was 5 ms.

verse was true for the second pair (Figure 5B, lower), in which a conditioned increase in synchrony occurred. The effects of conditioning on spike synchrony differed from the effects on direct interaction in that the former were not reversed following extinction trials.

In addition to mean rate and cross-correlation analysis, we also analyzed the distribution of interspike intervals of spike trains during spontaneous activity. Interspike interval histograms were generated for 61 LA neurons, 28 of which contained enough spikes to analyze. Of these, 5 showed a significant change in the number of short intervals (<25 ms) 1 hr after training; 4 showed an increase and 1 a decrease in short intervals (χ^2 test, $p < .001$). An increase in short intervals suggests an increase in bursting. An example of a training-induced increase in short intervals is shown in Figure 6. The interspike interval distribution during spontaneous activity (Figure 6A) and tone-evoked responses (Figure 6B) are shown throughout training. Note the increase in short (<10 ms) as well as long (320 ± 100 ms) intervals immediately following conditioning. In the tone-evoked activity, an excitation–inhibition conditioned response developed. A burst shortly after tone onset is followed by an inhibitory period lasting ~300 ms. In this cell, training-induced changes during spontaneous activity mirrored changes in tone-evoked activity. The development of an excitation–inhibition sequence during learning is consistent with a recurrent inhibitory circuit (shown in Figure 6) that is activated by tones as well as in the absence of explicit stimuli. Morphological (MacDonald, 1984; Millhouse and DeOlmos, 1983) evidence exists for such circuits in LA.

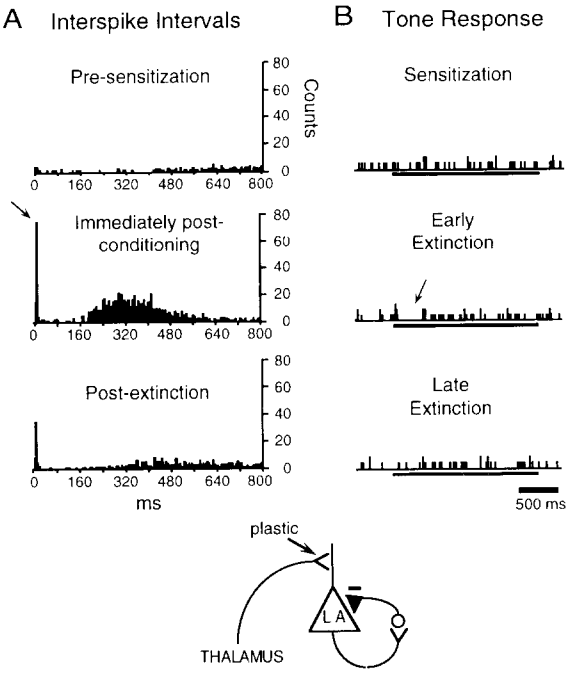

Figure 6. Effects of Training on Interspike Interval Distribution at Three Points during Training

(A) Interspike interval histograms generated from spontaneous activity for a neuron in LA prior to conditioning, immediately following conditioning, and following 30 extinction trials. In this cell, training increased the proportion of short (<4 ms; see arrow) and long (320 ± 100 ms) intervals in the spontaneous spike train. These changes decreased with extinction trials.

(B) Perievent time histograms generated for the same cell shown in (A) at approximately the same points during training. A conditioned tone response, consisting of a small high frequency burst followed by 300 ms of inhibition (most visible during early extinction; see arrow), mirrored the increase in short and long intervals in spontaneous activity (A). This excitation–inhibition sequence suggests a local feedback inhibitory circuit that is active both spontaneously and during tone-evoked trials and is modulated by training. Bin size was 5 ms for the interspike interval histograms (A) and 10 ms for the perievent time histograms (B).

Discussion

A large and growing body of evidence has implicated the amygdala in fear conditioning (Davis, 1992; Kapp et al., 1992; Fanselow, 1994; LeDoux, 1995). Because LA is the region through which sensory inputs enter into amygdala circuitries, and the first opportunity for processing of a conditioned stimulus in the amygdala, we examined whether neurons in LA are modified during fear conditioning. We were particularly careful to distinguish responses obtained from neurons in the dorsal (LAd) and ventral (LAv) subregions, as these have different anatomical connections (LeDoux et al., 1990b; Turner and Herkenham, 1991; Mascagni et al., 1993; Romanski and LeDoux, 1993; Pitkanen et al., 1995) and physiological response properties (Bordi and LeDoux, 1992; Romanski and LeDoux, 1993).

Conditioning of Neuronal Responses in LA

As a result of fear conditioning, the tone-evoked firing rate of cells in LA significantly increased in one-quarter of the cells studied. The magnitude of the change in these neurons was 5-fold. Both the fraction of cells that conditioned and the magnitude of change were similar in LAd and LAv. Changes of this type have previously been observed in the central nucleus (Pascoe and Kapp, 1985), basolateral nucleus (Maren et al., 1991), and LAv (Ben-Ari and Le Gal La Salle, 1972; Segal, 1973). Recent studies by Muramoto et al. (1993) demonstrated conditioned unit activity in the "basolateral nuclear group," which included LAv. In their study, data from the various subnuclei comprising the basolateral group were pooled and are not informative as to changes that occurred in specific subregions. Our results showing that LAd neurons are also modified as a result of conditioning add significantly to the literature by demonstrating that plasticity is present at the earliest stage of amygdala processing.

Our conclusion that neurons in LA are modified during learning is at odds with the results of a recent study by Uwano et al. (1995). These investigators found a higher percentage of sensory-responsive cells in the amygdala (as a whole) in conditioned rats compared with naive rats. However, they argue that neurons in LA are not likely to be plastic since they found few examples of cells responding to both auditory and somatosensory stimulation. The majority of multimodal neuronal responses were found in the basolateral and central nuclei, and they conclude that these structures are the earliest sites of sensory

convergence (and plasticity) in the amygdala. However, other studies have found that cells in LAd are responsive to both somatosensory and auditory stimulation (Romanski et al., 1993).

Two possible reasons exist for why Uwano et al. (1995) failed to find evidence for convergence in LA. First, previous studies reporting extensive convergence found it in LAd but not LAv (Romanski et al., 1993). Uwano et al. (1995) may have sampled from LAv to a greater extent than LAd. The latencies that they report for LA (45 ms) are in fact more consistent with LAv than LAd (see below). Second, they used an air puff stimulus to test for convergence but used foot shock for conditioning. The study that found auditory and somatosensory convergence in LAd cells used foot shock (Romanski et al., 1993), which is the typical kind of somatosensory stimulus used in numerous conditioning experiments, including the present study. Also Uwano et al. (1995) did not directly examine whether individual neurons conditioned, but simply compared the proportion of tone-responsive cells in conditioned and nonconditioned animals. They used an increase in the number of tone-responsive cells after conditioning as a measure of plasticity.

In summary, neurons in LA clearly exhibit conditioning-induced changes in auditory responses. While similar changes occur in other amygdala regions, the fact that they occur in LA (particularly in LAd) is notable, as this is the site at which sensory information enters the amygdala during conditioning and the first possible locus where plasticity could occur in the amygdala. Plasticity of neuronal tone responses of the type described here was predicted in a recent network model of amygdala units during fear conditioning (Armony et al., 1995) and by studies showing that auditory responses in LAd are enhanced by induction of long-term potentiation in the pathway that transmits auditory inputs to LA (Rogan and LeDoux, 1995).

Latency of Conditioned Responses

Given that sensory inputs enter the amygdala in LAd and that LAv receives at least some information about sensory stimuli from LAd (Pitkanen et al., 1995), it is reasonable to expect that conditioning-induced changes following tone onset would occur earlier in LAd than in LAv. This is what we found. The average latency of conditioned increases in the tone response of LAd neurons was 21 ms, with over half occurring prior to 15 ms. The latency of conditioned responses in LAv was considerably longer (mean 42 ms). Also, unlike LAv, the majority of LAd cells learned in the first block of training. These data are consistent with the fact that LAv, especially its medial part, receives projections from LAd, and sensory (and conditioned) responses in LAv are probably dependent on the flow of sensory information from LAd (Pitkanen et al., 1995). However, the long delay between conditioning in LAd and LAv (21 ms) is also consistent with other sites as possible sources of conditioned input to LAv, such as the auditory cortex or perirhinal cortex (Romanski and LeDoux, 1993).

The conditioning latencies we found in LAd are considerably shorter than those previously reported in the amygdala. Conditioning was observed at latencies of 30–50 ms in the central nucleus (Pascoe and Kapp, 1985) and 49 ms (Muramoto et al., 1993) and 70 ms (Maren et al., 1991) in the basolateral nucleus. Conditioning latencies as early as 15 ms are consistent with the facilitation of thalamic as opposed to cortical projections to LAd. Auditory and somatic response latencies in LAd are as short as 12 and 17 ms, respectively (Bordi et al., 1993; Romanski et al., 1993). The latency of the earliest auditory responses in the auditory cortex is around 10 ms (Sally and Kelly, 1988; Maho et al., 1995). The earliest conditioned increases occur between 10 and 20 ms, but the majority occur between 20 and 40 ms (Kraus and Disterhoft, 1982; Maho et al., 1995). Recent findings from our laboratory show that stimulation of auditory cortex activates LAd neurons with a minimum latency of 10 ms in anesthetized rats (X. F. Li and J. E. L., unpublished data). This suggests that the minimum latency for cortical activation of the amygdala should be greater than 20 ms. Thus, it is likely that more than half of the conditioned responses we observed are too early to be mediated via a cortical relay and more likely reflect the enhancement of thalamic inputs. These data support the hypothesis that direct transmission of auditory stimuli from the auditory thalamus to the amygdala contributes importantly to behavioral responses to threatening acoustic events (LeDoux, 1995) and challenge the notion that only cortico–amygdala pathways are used (Campeau and Davis, 1995).

What Are the Sites of Plasticity in Fear Conditioning?

The loci of plasticity in auditory fear conditioning are a matter of continual debate. In addition to the amygdala, conditioned unit responses have been observed in the medial division of the medial geniculate nucleus (MGm) (Disterhoft and Stuart, 1976; Gabriel et al., 1976; Ryugo and Weinberger, 1978; Edeline and Weinberger, 1992) and auditory cortex (Disterhoft and Stuart, 1976; Kraus and Disterhoft, 1982; Weinberger et al., 1984; Bakin and Weinberger, 1990; Maho et al., 1995). The 15 ms conditioned response latency we observed is consistent with the hypothesis that LA and/or its thalamic input structure are sites of plasticity. Further evidence arguing against the cortex as the sole or even the main initiator of plasticity in subcortical structures is that conditioned responses in auditory cortex require more training trials than those in MGm (Disterhoft and Stuart, 1976).

Anatomical studies show that the major source of thalamic input to LA is from the posterior intralaminar nucleus (PIN), while the adjacent MGm projects mainly to the caudate/putamen (LeDoux et al., 1990a). PIN neurons have not been recorded during conditioning; however, brain stimulation in the PIN served as an effective US for auditory fear conditioning in guinea pigs (Cruikshank et al., 1992). This suggests that convergence of auditory and somatosensory information occurring in the PIN and/or structures to which the PIN projects (LA) are capable of sustaining conditioning. Interestingly, in the same study, stimulation of MGm was ineffective as a US. Both MGm and PIN receive auditory and somatosensory information (Bordi and LeDoux, 1994), but as described above, the PIN projects more heavily to LA than does MGm. In addi-

tion, the degree of sensory convergence may be greater in LA than in the PIN. In the PIN, only 38% of the tone-responsive cells that project to LA also responded to foot shock (Bordi and LeDoux, 1994), while in LA 88% of the tone-responsive cells responded to foot shock (Romanski et al., 1993). Therefore, LA may be the first opportunity in the auditory processing stream for massive sensory convergence capable of sustaining plasticity. In our study, over half of the LA neurons that were tone responsive also conditioned. Thus, the contribution of processes in the PIN to early plasticity in LA may be small compared with the contribution of processes within the LA itself. Other evidence of plasticity in LA is the robust long-term potentiation observed following high frequency stimulation of MGm/PIN (Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995).

Local plasticity in LA, according to most cellular learning theories (Hebb, 1949; Konorski, 1948; Kandel and Spencer, 1968), requires coactivation of LA neurons by the CS and US. We have emphasized the earliest components of tone responses in LA, but the tone presentation lasted 1500 ms before the onset of the shock, inviting speculation as to the underlying mechanisms of CS–US integration, especially mechanisms that might bridge the delay between the early tone responses and the response to the US. In fact, we found that 4 of the 10 LAd cells that conditioned were significantly tone responsive during the last 500 ms of the stimulus prior to conditioning, thus fulfilling the temporal requirement for CS–US pairing. Furthermore, we suspect that LAd neurons may also be activated throughout the entire tone, but at subthreshold levels. Subthreshold depolarization is sufficient to induce synaptic plasticity (Kelso et al., 1986; Gustafsson et al., 1987). All neurons with sustained responses had a stronger initial burst at tone onset. It may be that, early in training, sustained responses (whether supra- or subthreshold) are sufficient to allow CS–US pairing throughout the tone.

Functional Connectivity during Spontaneous Activity

We did not observe a general effect of conditioning on spontaneous firing rate in LA; thus, conditioning does not appear to "turn on" or "turn off" LA as a whole. However, we did observe effects of training on the interspike interval distribution of some LA neurons, as did Ben-Ari and Le Gal La Salle (1972). More importantly, we observed alterations in the functional connectivity between LA cell pairs as a result of training. Training-induced synchrony and desynchrony of firing suggest that LA neurons are part of a network that encodes the training event. The increase in short-latency (1–4 ms) direct coupling of LA cells further suggests that synaptic strength in the network undergoes long-term changes with training, and that LA is in fact a site of associative plasticity. Although the percentage of cell pairs that showed direct interactions was relatively low (7%), it agrees with estimates from visual cortex (Hata et al., 1991) and the dentate gyrus (Sakurai, 1993). A number of recent studies have shown changes in functional connectivity between pairs of neurons induced by behavioral training (Ahissar et al., 1992; Sakurai, 1993; Wilson and McNaughton, 1993; Vaadia et al., 1995). Our data are also consistent with the observation by Ben-Ari (1972) that spontaneous activity may include traces of the latent processes that explain the changes observed in the responses during training.

Extinction

It is well known that, following the extinction of behavioral responses, persistent memory for conditioning can be demonstrated by a variety of manipulations (LeDoux et al., 1989; Bouton, 1994). This has led to the notion that responses are inhibited during extinction without erasure of the memory for conditioning. The question arises as to where this persistent memory trace is stored. The conditioned increases in tone responses of most LA neurons returned to preconditioning levels following extinction. The same was true for the short-latency functional interactions between cell pairs. Thus, it appears that extinction resets some aspects of LA physiology to the preconditioning state. A striking exception to this involved the changes in the synchrony of spontaneous firing between LA cell pairs, which did not revert to the original state following 30 extinction trials. This suggests that, in addition to their role in encoding the training experience, LA cells may also participate in the permanent (nonextinguishable) storage of conditioned fear.

In conclusion, we have shown that fear conditioning enhances the earliest components of tone responses in LA neurons. Such early plasticity is important because it enables the LA to signal danger as quickly as possible and initiate defensive behaviors. Early increases in tone responses in LA neurons are transmitted through intra-amygdala circuits to the central nucleus, which then directly controls autonomic and behavioral responses via projections to the brain stem (see Davis, 1992; Kapp et al., 1992; LeDoux, 1995) without necessarily requiring cortical processing (LeDoux, 1995). The changes we observed in functional connectivity between LA neurons suggest that, once learned, stimulus–fear associations may be stored in the amygdala, where they are in a position to continually modulate the sensory input stream.

Experimental Procedures

Surgery

Studies were performed on male Sprague–Dawley rats weighing 300–350 g at the time of surgery. The methods used were similar to those of previous studies (Bordi et al., 1993). Rats were pretreated with atropine sulfate (0.24 mg/kg, intraperitoneally) and anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Body temperature was controlled with a heating pad. Burr holes were drilled above the amygdala (6.0 mm anterior, 5.3 mm lateral to earbar-zero) and above frontal cortex and cerebellum for insertion of self-tapping set screws.

A movable electrode assembly (modified from Kubie, 1984) was stereotaxically implanted in the left neostriatum just dorsal to LA (2.3 mm dorsal to earbar-zero). The assembly contained a bundle of 20 insulated nichrome wires (22.5 μm diameter) within a stainless steel tube (0.45 mm diameter). The diameter of the bundle was ~0.5 mm. The wires were cut with a sharp pair of scissors so that they were equal in length and extended ~2 mm beyond the end of the tube. Tip impedance of individual wires was 1–2 MΩ at 1000 Hz. In some rats, the tube containing the bundle was passed through a larger guide

tube (0.81 mm diameter) to improve placement accuracy. Each microwire was fixed to one pin of a ten pin connector (Augat) with silver paint. Two connectors were used. The connectors were molded in a triangular base of made of dental acrylic. Three screws protruded from the base to form a tripod. The screws were threaded into nylon cuffs that were cemented to the skull with Grip Cement (Dentsply, Maumee, OH). Each quarter turn of the screws advanced the microdrive 112 μm. After cementing the drive in place, the wound was closed with suture, and antibiotic ointment was topically applied. Rats were allowed 1 week of recovery prior to testing.

Unit Recording and Spike Sorting

Signals from the electrodes were passed through unity gain field effect transistors (#U401, Marshall Electronics, El Monte, CA) in source-follower configuration. The output of the transistors was connected via a cable and slip-ring commutator to multichannel preamplifiers (AM Systems, Everett, WA) with passive filters set at 300 Hz (high pass) and 5 kHz (low pass). No additional filters were used. The amplified signals were monitored with digital oscilloscopes (Hitachi VC6025) and an audio amplifier. Signals were also digitized at 16 kHz and written to the disk of a PC microcomputer (Gateway 2000, model 486 DX2/50E, N. Sioux City, SD) using DataWave Technologies (Longmont, CO) hardware and DataWave Discovery software. Signals exceeding a voltage trigger level were captured in 2 ms epochs.

Voltage and time parameters of the stored waveforms were analyzed off-line to identify clusters of waveforms indicative of separate neurons. On average, it was possible to identify 1–4 clusters on each active wire. The most useful waveform parameters were: peak voltage, valley voltage, time of peak, spike height, and the voltage at several experimenter-determined times. After setting the cluster boundaries, spikes were replayed in storage mode to verify the cluster assignment. Small units that could not be reliably separated from background noise during all phases of the training were discarded. Following determination of single unit clusters, files containing only the time-stamped unit events were created and used for subsequent data analysis.

Preconditioning Screening of Unit Activity

The electrodes were advanced in 50–100 μm steps until spontaneous activity was encountered simultaneously on several wires. The units were then tested for tone responses. Auditory stimuli were generated with a Spirit-30 digital signal processing board (Sonitech International) controlled by a DataWave Discovery clocked sequence that was running on the same PC as the data acquisition system. After digital to analog conversion, stimuli were amplified (Denon POA 2400) and amplitude attenuated by the clocked sequence. The auditory stimuli were delivered through a speaker placed in the ceiling of a soundproof chamber (Industrial Acoustics, Bronx, NY) that contained the test box. The test box was 25 × 30 × 25 cm and fitted with an electrifiable floor (Coulbourn Instruments). The top of the box was open. The amplitude of the sound was measured to be 80 dB (± 5 dB) at all points in the test box at all frequencies used. To test for tone responsivity, tone pips (50 ms duration) of varying frequencies as well as white noise (also 50 ms) were given. Because each animal could only be conditioned once, the electrode was moved until multiple tone-responsive cells were encountered. When 2 or more wires exhibited tone-responsive activity at a depth corresponding to LA, conditioning commenced.

Conditioning

The CS consisted of a 5 kHz pure tone (80 dB, 2 s). This frequency gave fairly good responses in most neurons prior to training but is lower than the best frequency (typically >12 kHz) reported for most LA neurons (Bordi et al., 1993). A frequency giving submaximal responses prior to training was selected so that robust increases might be observed as a result of conditioning. The US was a foot shock (0.5 mA, 0.5 s) delivered through the floor gratings. These parameters are similar to those used in behavioral studies of fear conditioning. Following the protocol of Weinberger and associates (e.g., Weinberger and Diamond, 1987), training was divided into three phases: sensitization, conditioning, and extinction. During sensitization, tones and shocks were explicitly unpaired. The tones were separated by a varying interval of 1–3 min, and shocks occurred randomly between the tones. Ten sensitization trials were followed immediately by conditioning. During conditioning, tones occurred on the same schedule as sensitization, except the shock always occurred during the last 500 ms of the tone. Twenty conditioning trials were given, followed by a 1 hr rest period in the home cage, after which the rat was returned to the test chamber for extinction trials. During extinction, 10–30 CS tones were delivered with the same schedule as in sensitization and conditioning, but without any shocks. The entire training sequence took ~3 hr. Comparison of responses during the early extinction trials with the sensitization trials gives a measure of the effects of paired as opposed to random presentation of the tone and shock (Weinberger and Diamond, 1987).

An additional group of rats was trained as above, except tones and shocks were never paired at any point during training. Thus, they received 10 unpaired presentations during sensitization and an additional 20 during what would have been conditioning. These served as an additional sensitization control for nonassociative conditioning (Rescorla, 1967).

Data Analysis

Tone-Evoked Activity

Perievent time histograms of the tone responses were constructed off-line on a Power Macintosh computer with software (BRAHMS) written specifically for this purpose and provided by Dr. Norman Weinberger. For most analyses, the bin width for the histograms was 10 ms. Neurons were judged tone responsive if any of the first 7 bins following tone onset (70 ms total) significantly exceeded the average firing rate during the 500 ms pretone period. Owing to the unusually low spontaneous firing rates of LA neurons, we tested only for significant firing rate increases ($p < .05$, one tailed t test with Bonferroni correction). If a bin with only 1 spike passed this criterion, it was thrown out as not significant unless immediately followed by a bin with another spike. If a neuron passed this criterion for tone responsivity, each of the seven 10 ms bins was compared with the pre-rate to determine the onset latency of the tone response ($p < .05$, one-tailed t test). Ten tone trials were summed together for tone–response data analysis.

To test tone responses for conditioned changes, blocks of 3 trials during sensitization were compared with blocks of 3 trials during early extinction. To control for changes in mean firing rate, the tone response was defined as the rate in a bin minus the rate in the pretone period. We confined our search to only conditioning-induced increases in tone responses to avoid the potential problem of counting as conditioned decreases cells that were lost during training. Neurons were judged to have significantly increased their tone response with training if the tone response in 1 or more bins was significantly greater during the first 2 blocks of extinction relative to the last 3 blocks of sensitization ($p < .05$, one-tailed t test with Bonferroni correction). Bins with 1 spike were never counted as significant. Cells that significantly conditioned were then analyzed on a bin by bin basis to determine latency of conditioning ($p < .05$, one-tailed t test with Bonferroni correction).

Spontaneous Activity

Periods of spontaneous activity (5 min) were collected at four points during the experiment: immediately prior to sensitization, immediately following conditioning, and immediately prior to and following extinction. The effect of training on spontaneous activity was examined with three measures: mean firing rate, interspike interval distribution, and cross-correlation of spike trains of simultaneously recorded pairs of cells. All three analyses were performed off-line with DataWave software. A mean and SD were calculated for spontaneous rates by averaging the rate for five segments of equal duration (usually 1 min). The effect of training on mean firing rate was tested with an ANOVA (repeated measures, $p < .05$). Interspike interval distributions were examined for evidence of bursting (short intervals). The fraction of intervals <25 ms in duration was compared at different points during training with the χ^2 test.

Cross-correlograms were examined for coincident firing (peak at t_0) and short-latency interactions (peak within 4 ms of t_0). Interactions were judged significant if they deviated significantly ($p < .01$) from that predicted by two Poisson spike trains (Abeles, 1982). For correlograms showing short-latency interactions, bins within 4 ms of t_0 containing greater than expected spikes based on the 40 bins preceding t_0 ($p < .05$, two-tailed) were identified. After identifying this window for one

phase of the experiment, the same window was applied to the other phases. The method of Levick et al. (1972) was used to determine the efficacy of functional connectivity between 2 cells. The method was modified so that efficacy was defined as the number of spikes *above expected* in the window divided by the number of spikes of the reference cell. This method controls for changes in the spontaneous rate of either the reference or target cell. The effect of training on efficacy was tested with the χ^2 test.

Histological Analysis

Following extinction trials, rats were given an overdose of pentobarbital (100 mg/kg), and anodal current (40 μA, 8 s) was passed through one of the active recording wires. The rat was then transcardially perfused and the brain removed and placed in buffered formalin with 30% sucrose and 2% potassium ferrocyanide. Frozen sections were mounted on slides and stained with thionin. Electrode location was determined by the Prussian blue reaction formed by the iron deposit of the lesioned wire.

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