Two different lateral amygdala cell populations contribute to the initiation and storage of memory

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Single-cell activity was recorded in the dorsal subnucleus of the lateral amygdala (LAd) of freely behaving rats during Pavlovian fear conditioning, to determine the relationship between neuronal activity and behavioral learning. Neuronal responses elicited by the conditioned stimulus typically increased before behavioral fear was evident, supporting the hypothesis that neural changes in LAd account for the conditioning of behavior. Furthermore, two types of these rapidly modified cells were found. Some, located in the dorsal tip of LAd, exhibited short-latency responses (<20 ms) that were only transiently changed. A second class of cells, most commonly found in ventral regions of LAd, had longer latency responses, but maintained enhanced responding throughout training and even through extinction. These anatomically distinct cells in LAd may be differentially involved in the initiation of learning and long-term memory storage.

Pavlovian fear conditioning has been used extensively to study how the brain learns about stimuli associated with danger. In fear conditioning, an initially neutral conditioned stimulus (CS), after being paired with an aversive unconditioned stimulus (US), begins to evoke defensive fear responses. Evidence indicates that the amygdala is central in fear conditioning[1–4], but its precise contributions have been debated[5,6].

Anatomical tracing, lesion and electrophysiological recording studies suggest that the lateral nucleus of the amygdala (LA), and especially the dorsal subnucleus (LAd), is the sensory gateway to amygdala circuits[1], and that the processing of the CS by LA neurons is enhanced by the co-occurrence of the US[7–11]. Whereas these and other findings[12,13] strongly suggest that LAd might be an important site of plasticity, several questions remain unanswered about the nature of the physiological changes observed in LAd during fear conditioning.

First, no study to date has examined in detail the relationship between the acquisition rates of neuronal changes in the LAd and behavioral learning. Whether the neural changes in LAd actually account for the conditioning of fear behavior is therefore not known. In previous electrophysiological studies, the emphasis was on the recording of unit activity rather than on behavior[7,9,11]. This is partly because subjects exhibit behavioral fear reactions such as freezing after the first US presentation, making it difficult to accurately assess freezing conditioned to the CS. Here we bypassed this technical problem by using a procedure in which fear conditioning was superimposed on an operant bar-pressing task[14,15]. Although rats freeze in the presence of the CS once it is associated with the US, they press a bar during the periods when the CS is not presented. As a result, this task allows a sensitive measurement of CS-elicited behavioral fear and can be used to assess the rate of learning of behavioral fear responses in relation to the acquisition of cellular plasticity by amygdala neurons.

A second unresolved issue is whether the amygdala is a site of permanent storage of fear memories[5,6]. Previous studies in rats found that amygdala activity, after initial increases early in training, 'resets' toward baseline levels during later training[16]. A similar finding was obtained in imaging studies in humans undergoing fear conditioning[17,18]. These data have been interpreted as evidence that the amygdala is not a site of storage[6]. However, these studies have typically not used concurrent measurements of amygdala activity and behavior to evaluate their relationship. In addition, relatively few cells were recorded in the previous rat study. Therefore, as the finding that amygdala changes are transient has important implications for the way amygdala contributions to fear learning should be viewed, we re-evaluated neural activity in LAd using the procedures described above.

RESULTS
Behavior
Fear conditioning was assessed using two measures, freezing and suppression of bar-pressing. Although both sensitively assess fear conditioning[15], they reflect dissociable aspects of behavioral fear mediated by different neural systems[19]. The use of the bar-pressing procedure makes it possible to assess both CS-elicited freezing and suppression, as the motivation to press the lever for food overcomes the tendency to remain immobile after shock presentation.

The 13 rats receiving paired CS–US trials exhibited increased fear levels, as measured by both conditioned suppression and freezing, during conditioning trials and early extinction trials (**Fig. 1a** and **b**). In contrast, the nine rats receiving unpaired CS and US presentations showed no evidence of CS-elicited fear at any point.

Unit activity
A total of 170 LAd cells from 22 rats were included in the analyses. Of these, 100 cells were from 13 rats in the paired group,
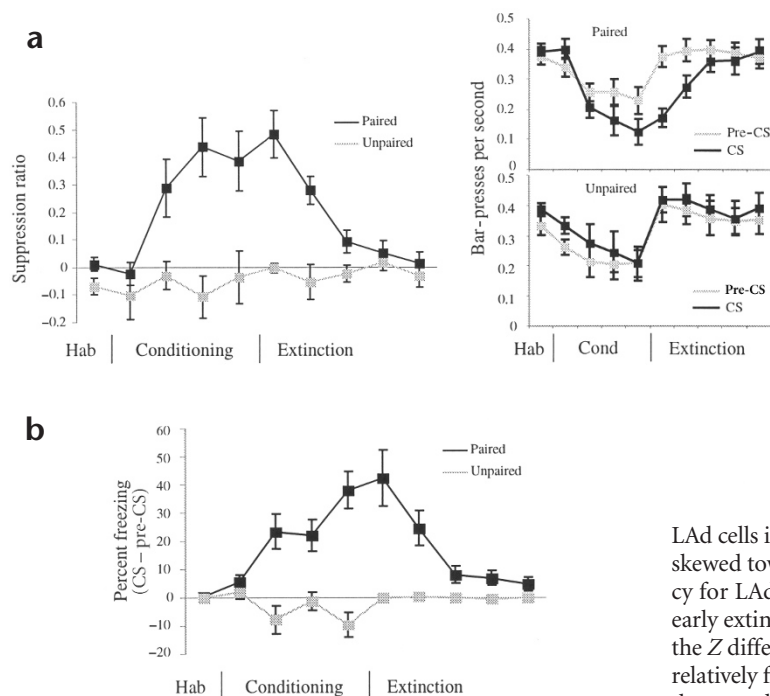
a

b

Fig. 1. Behavioral measures of conditioned fear. (**a**) Mean (± s.e.m.) suppression ratio for both the paired and unpaired groups throughout the three phases of the experiment, habituation, conditioning and extinction. Positive suppression ratios indicate bar-press suppression during the CS, whereas a suppression ratio of 0 indicates no change in press rates during the CS. The suppression ratios are derived from the raw bar-press rates during the pre-CS period (60 s) and during the CS (20 s), which are depicted on the right for the paired (top) and unpaired (bottom) groups (rates are given in bar-presses per second). (**b**) Mean (± s.e.m.) CS-elicited freezing levels (freezing during the 20-s CS minus freezing during the previous 20 s) for both the paired and unpaired groups throughout the three phases of the experiment. For (**a**) and (**b**), data points are four trial blocks averaged together, except the first point, which is the average of the final 6 trials of habituation.

and 70 were from 9 rats in the unpaired group (example of recording site, **Fig. 2**).

Consistent with previous studies[7], the spontaneous firing rates of LAd neurons were low. The average firing rate was 2.7 Hz; however, 61% of the cells had rates less than 1 Hz, and the geometric mean (± s.e.m.) was 0.4 ± 1.2 Hz. The preponderance of low rate and wide spike-width cells suggests that, like in the hippocampus[20], many of the cells in our sample were pyramidal-type projection cells as opposed to interneurons[21]. Conditioning did not have any systematic effect on spontaneous firing rates ($p > 0.25$, repeated-measures analysis of variance), measured at various time points throughout the experiment (see Methods).

Seventy-three of the 100 cells in the conditioned group were classified as CS-responsive. This was determined by combining peri-event time histograms (PETHs) for all phases of the experiment (see Methods). The average response latency, defined as the first 10-ms bin following stimulus onset that had significantly greater firing than pre-CS levels, was 42 ± 6 ms; this average was skewed by 7 cells with response latencies that exceeded 100 ms. Of the 66 cells with latencies under 100 ms, the average was 28 ± 2 ms. Nineteen cells responded with latencies under 20 ms.

Conditioned responding

CS-evoked activity an hour after conditioning (during early extinction trials) was compared to pre-conditioning (habituation) levels to determine if training-induced changes in neuronal responsivity were evident an hour after training for the entire neuronal population. For each cell, the CS-evoked activity from 0–200 ms following CS-onset was quantified as an average Z-score (see Methods) during extinction and habituation. The difference between these two Z-scores provided a measure of the change in a cell's responsivity, and is depicted for each of the 100

LAd cells in the paired group in Fig. 3 (left). The population was skewed toward positive Z difference scores, indicating a tendency for LAd cells to exhibit greater CS-elicited responses during early extinction trials than during habituation trials. In contrast, the Z difference scores in the unpaired group (**Fig. 3**, right) were relatively flat, and centered on a difference of zero, suggesting little to no change from pre-training levels. In support of this observation, the mean Z difference score for the paired group was 0.66 ± 0.15, significantly greater than the unpaired group's mean of 0.05 ± 0.06 ($p < 0.001$, one-tailed t-test), which did not differ significantly from zero.

To test for changes in neuronal responsivity occurring during the conditioning phase of the experiment, the above analysis was also applied to data recorded during conditioning. For this analysis, the 16 conditioning trials were divided into four 4-trial blocks, and the Z difference score was calculated for each block relative to habituation response levels. To capture any increased responsivity that may have been transient in some cells, the maximum Z difference score of the four conditioning blocks was determined for each cell (**Fig. 4**). This analysis revealed that the paired group (mean ± s.e.m., 0.90 ± 0.11) had significantly greater Z difference scores than the unpaired group (0.48 ± 0.10; $p < 0.01$).

To further explore the acquisition of conditioned responding relative to behavior on a cell-by-cell basis in the paired group, individual cells showing plasticity at various time points during training were identified as follows: for each cell, neuronal CS-elicited firing during conditioning trials was compared, in 4-trial blocks, to habituation levels ($p < 0.05$, t-test). Twenty-four cells from this paired group passed this criterion for cell-by-cell plasticity during at least one of the conditioning blocks. With respect to CS-onset times, increases in firing were seen as early as 10–20 ms after the onset of the stimulus (**Fig. 5a**). The change
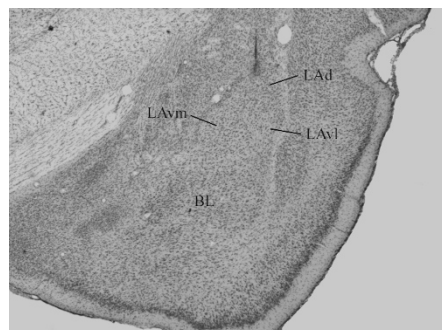
Fig. 2. Photomicrograph of a thionin-stained brain section from a representative rat, showing electrode tract and lesion site in LAd. LAd, dorsal subnucleus of the LA; LAvm, medial division of the ventral LA; LAvl, lateral division of the ventral LA; BL, basolateral amygdala.

Fig. 3. Neuronal plasticity during early extinction, for all cells. The distribution of Z difference scores is depicted, showing the average CS-elicited response from 0–200 ms after stimulus onset, expressed as a Z score, during early extinction minus habituation levels, for each of the 100 LAd cells in the paired group and 70 LAd cells in the unpaired group. Values are organized along the abscissa in rank order, from the smallest to largest Z difference score. Each bar represents exactly one cell. Note the shift of the paired population toward positive Z difference scores, indicating a tendency for these LAd cells to exhibit greater CS-elicited responses during early extinction trials than during habituation trials.
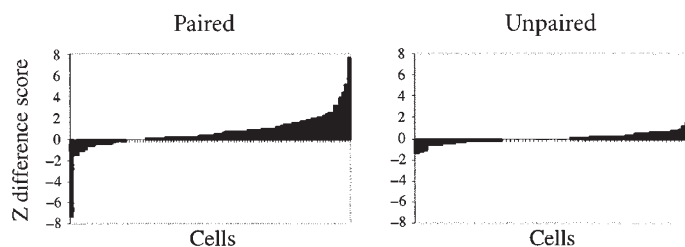
in neural response approached its maximum level during the first two blocks of conditioning trials (**Fig. 5b**). In contrast, conditioned fear behavior was more gradually acquired, reaching maximal levels toward the end of conditioning (**Fig. 1**).

To better determine whether the changes in neural response preceded changes in behavioral response, a single-trial analysis was performed on the plastic cells. For each of these cells, the first conditioning trial during which the CS response significantly exceeded habituation levels ($p < 0.05$, one-tailed) was compared to the first trial on which behavioral evidence of fear conditioning was observed. To compare neural changes to the earliest possible evidence of behavioral learning in our protocol, the first trial of behavioral learning was defined as the first trial on which either significant freezing or suppression occurred ($p < 0.05$, one-tailed), whichever came first. For a significant majority of the cells (17 of 24; $p < 0.05$, binomial probability test), the changes in the neural response occurred earlier or on the same trial as the changes in the behavior (**Fig. 6a** and **b**). This observation—enhanced LAd neural activity preceding fear behavior—is also evident in the group data, as shown in Fig. 6c, where the average CS response is plotted on a trial-by-trial basis relative to the trial on which behavior was learned. Note that the averaged neural response began to increase before fear behavior was evident (that is, it increased from trials –5 to –1), and, in fact, peaked at the trial at which fear behavior was first detected (trial 0).

Even for the seven cells that first showed evidence of increased neural responsivity on the same trial as behavioral learning (**Fig. 6a** and **b**), the increased firing of these cells likely preceded the behavioral response. That is, the mean latency of the increased firing of these 7 cells was within 20–30 ms of the onset (the first auditory pip) of the CS on which behavioral fear was first evident. However, the shortest latency behavioral response elicited by a CS itself is electromyographic (EMG) neck muscle activity, which is not expressed until 150–200 ms following CS onset[22]. In general, behavioral reaction times in response to a warning stimulus, even for practiced human subjects, are of a similar latency[23]. Therefore, neural responses occurring 20–30 ms after CS onset very likely precede behavioral conditioned fear responses such as freezing.
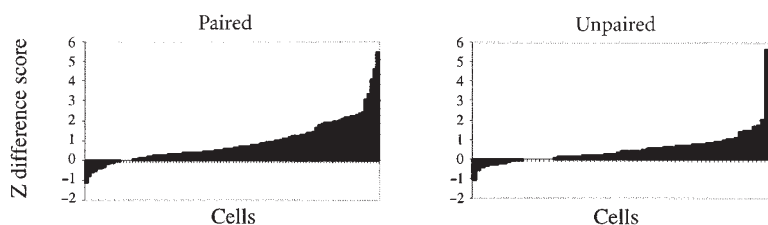
Persistence of conditioned response

The averaged neural response of plastic cells reached maximum levels during the first two 4-trial blocks of conditioning, and then tended to diminish during later conditioning trials (**Fig. 5b**). To determine how individual cells contributed to this pattern, a 'persistence' value was quantified for each cell by dividing the increase in CS responses (over habituation levels) during late conditioning (final 8 trials) by the increased CS response during early conditioning (first 8 trials). Therefore, a persistence value of zero indicates that responses returned to baseline (pre-conditioning) levels, whereas values of one or greater indicate that responses remained elevated in late conditioning. The distribution of persistence values suggests that there are two types of cells (**Fig. 7a**). One group has persistence values clustered around 0, indicating that their increased responses returned to baseline levels, whereas a second group of cells has values clustered above 1.0, indicating that their increased responses were maintained throughout later conditioning trials. Therefore, a cutoff persistence level of 0.75, which bisects this dual distribution, was used to classify these cells as either 'transiently plastic' (12 cells) or 'long-lasting plastic' (12 cells). As expected based on their classification, the response of transiently plastic cells fell back to pre-training levels during later conditioning trials, whereas long-lasting plastic cells maintained their elevated firing levels throughout conditioning (**Fig. 7b**). Both cell types (9 of 12 transiently plastic and 8 of 12 long-lasting plastic cells) tended to exhibit conditioned changes before behavioral fear was evident, though the long-lasting plastic cells took longer to reach their maximal firing rates (**Fig. 7b**).

These two classes of cells could also be distinguished based on other characteristics. First, the long-lasting plastic cells had a smaller CS response before training, during habituation (for example, Figs. **7b**, and **8a** and **b**). In addition, auditory response latencies of 20 ms or less, which suggest direct activation from thalamic efferents[24], were only seen in the transiently plastic group (**Fig. 8a** and **b**). Furthermore, plasticity was greatest at these short latencies in the transiently plastic cells, whereas increased firing in very long latency bins (over 100 ms) was much more common in the long-lasting plastic cells. Finally, the transiently plastic cells were exclusively found near the dorsal tip of LAd, whereas the long-

Fig. 4. Neuronal plasticity during conditioning, for all cells. The distribution of Z difference scores is depicted, showing the average CS-elicited response from 0–200 ms after stimulus onset, expressed as the maximum Z-score of four conditioning 4-trial blocks minus habituation levels (see text for details), for each of the 100 LAd cells in the paired group and 70 LAd cells in the unpaired group. Values are organized along the abscissa in rank order, from the smallest to largest Z difference score, and each bar represents exactly one cell.
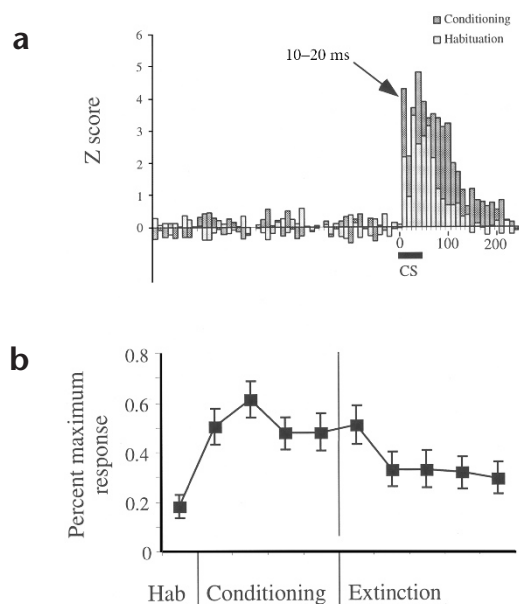
Fig. 5. Latency and time course of neuronal plasticity. (**a**) Mean PETHs (spikes normalized to the pre-CS baseline; 10-ms bins) for two phases of the experiment (habituation trials 1–8 and average of conditioning trials 1–8 and 9–16) for all conditioned cells from the paired group (*n* = 24 cells). (**b**) Time course of the average CS response of plastic cells. Mean (± s.e.m.) CS response for all conditioned cells from the paired group (*n* = 24 cells) throughout the three phases of the experiment. Data points are four trial blocks averaged together, except the first point, which is the average of the final 6 trials of habituation.

lasting plastic cells were found throughout LAd, and were most prevalent in the ventral half of LAd (**Fig. 8c**).

Extinction of conditioned responding
Both transiently plastic and long-lasting plastic cells showed enhanced CS-evoked activity an hour after the conditioning phase, that is, during early extinction trials (**Fig. 7b**). The transiently plastic cells, after returning to pre-training response levels during late conditioning, again exhibited increased responses during early extinction trials ($p < 0.05$, one-tailed *t*-test), only to fall back to baseline levels late in extinction. Long-lasting plastic cells, on the other hand, maintain their increased CS-evoked firing rates not only in late conditioning trials, but also in both early and late extinction trials (all such time points are significantly elevated from habituation levels, $p < 0.02$).

DISCUSSION
We recorded from LAd neurons while simultaneously monitoring behavior during fear conditioning, primarily to address two previously unresolved issues regarding learning-induced neural activity. Specifically, do the changes in LAd neural activity precede behavioral changes during learning, and do the neural changes persist?

Anatomical considerations
Recordings were made in LA because this region has been implicated in fear conditioning using a variety of different experimental approaches[1,4,5]. However, LA is composed of several subdivisions. We focused on LAd because tract tracing and physiological studies show that this region is the primary target of pathways that transmit the auditory CS to the amygdala[25–27] and is a site of CS and US convergence[28], and because previous studies have shown short-latency conditioned changes in neural activity in this region[7,9,11].

Distinct neural responses were found in the dorsal versus the ventral portions of LAd—dorsal LAd cells were transiently plastic whereas plasticity in ventral LAd cells persisted even through extinction. This anatomical distinction is supported by the results of tract tracing studies[25,26] as well as studies that have mapped the localization of certain molecular changes in LA during fear conditioning[29]. These findings pinpoint the locus of plasticity

during fear conditioning to relatively small populations of neurons that may differentially account for the initial learning and subsequent maintenance of the long-term memory of the training experience, as discussed in more detail below.

LAd neural plasticity is associative
One hour after conditioning, only cells from animals receiving paired CS–US presentations showed evidence of enhanced responding to the CS. Cells from animals receiving explicitly unpaired CS–US presentations (which resulted in no observable behavioral fear response to the CS itself) exhibited little to no change in responsivity to the CS. Similarly, during the conditioning phase, neuronal CS-elicited firing in the paired group showed greater increases than in the unpaired group. Therefore, changes in the paired group are likely due to the coding of the CS–US association rather than to sensitization or other non-associative effects caused by exposure to the US.

LAd neural changes predict behavior
A previous study of LAd plasticity during fear conditioning found that the changes in neural activity occur within the first three or four training trials[16]. However, that study did not have a concurrent measure of behavioral learning. Because fear conditioning can be learned in as little as one trial[30], the possibility remains that behavioral changes may in fact have preceded the changes noted in LAd activity, which would suggest that changes in LAd processing are not critical to the generation of behavioral fear responses.

In the present study, three factors allowed the comparison of neural and behavioral conditioning rates on a trial-by-trial basis. First, we used a relatively low shock intensity (0.4 mA) for the US to slow down the rate of behavioral learning. Second, greater statistical reliability of neuronal data was achieved by presenting 20 separate auditory presentations as a single CS during each trial[8]. Third, the use of the food-motivated bar-pressing task ensured that the animals would be active rather than immobile in the intertrial period, and thus allowed a sensitive measurement of CS-elicited behavioral fear responses on each trial. With this approach, we demonstrated that the neural changes preceded the behavioral changes for most of the LAd cells that showed evidence of plasticity in their CS-evoked activity during the conditioning phase. These results support the hypothesis that increased LAd neural activity leads to the initiation of behavioral fear responses.

One possible caveat is that learning may normally take place earlier than it was observed via freezing or suppression, as these behaviors may have been masked early in training by the conflicting drive to bar-press for food rewards. However, it cannot be argued that the increased neural responsivity in the LAd is merely a consequence of the behavioral conditioned responses observed here, as the neural changes are observed before the onset of behavioral changes. Furthermore, the strong tie between LA neural activity and behavioral fear conditioning is supported both by the present data (the neural activity peaks on the same

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Fig. 6. Acquisition of neuronal versus behavioral measures of learning. (**a**) First significant trial for conditioned responding of neuronal units (*y*-axis) plotted versus the first significant trial for conditioned responding of fear behavior (*x*-axis) as determined by bar-press suppression and freezing, whichever changed first. Each conditioned cell from the paired group is plotted once (*n* = 24 cells). The outlined 45-degree area indicates cells that first showed neuronal conditioning on the same trial on which behavioral conditioning was first detected. The circled region represents cells for which the neuronal changes were detected on the same or earlier trials than behavioral changes. (**b**) Data summarized from (**a**), showing the difference for each cell, in trials, between the first significant neuronal trial and the first significant behavioral trial. Negative numbers indicate cells for which neuronal unit changes were detected before behavioral changes, whereas zero indicates cells for which unit and behavioral changes were detected on the same trial. Dark gray bars represent all cells for which unit changes were detected on the same or earlier trials than behavioral changes, as summarized in the right panel of (**b**). (**c**) Mean CS-response for all conditioned cells from the paired group (*n* = 24 cells) throughout habituation and conditioning. Conditioning trials are aligned based on the trial of behavioral learning (trial 0).


trial as the onset of the behavior), and by many previous studies that implicate the LA as critical for fear conditioning (discussed in more detail below)[5]. Thus, the most parsimonious interpretation of the present results is that the increased LAd activity contributes to the generation of the learned fear behavior.

Persistence of LAd plasticity

Half the LAd cells that increased their CS-evoked firing rates during conditioning maintained those increased response levels throughout the later trials of conditioning. Because behavioral fear levels, as measured by both suppression and freezing, were also elevated throughout the later conditioning trials, these elevated LAd response levels may reflect aspects of fear memory stored within the LAd.

At first glance, the current results are at odds with some previous studies of amygdala physiology in both rats and humans. In rats, cells that increased their responses early during training showed a general trend toward diminished responses late in training[16]. In human fMRI studies, amygdala activation decreased with additional training trials[17,18], though this decrease was not significant in one of the studies[18].

A number of differences between these previous studies and the present design may help explain the conflicting results. First,
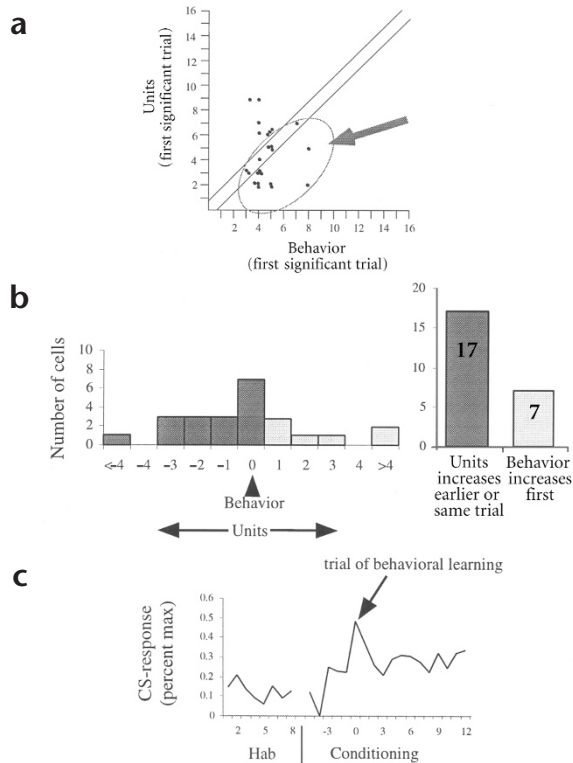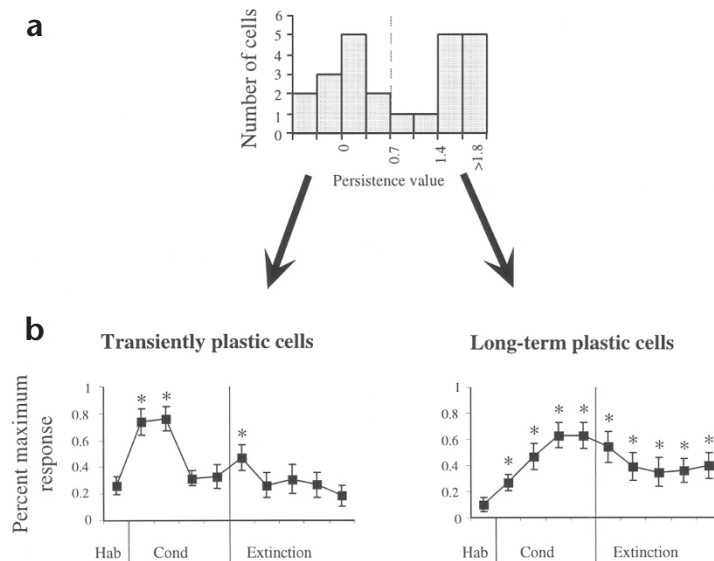
the conclusions from the study in rats[16] were based on a smaller sample of cells showing conditioned responding, which may have hampered detection of the long-lasting plastic cells. Second, the analyses in that study focused on only the first 50 ms of the CS-elicited responses. Because in the present study the shortest latency responses were found in the transiently plastic cells, which were the main cells reported in the previous study[16], the analytic or recording methods used in the previous study may have biased the sample. In contrast, in the present study, the entire envelope of the CS response was analyzed, up to 250 ms after the CS onset.

With regard to the failure of the human studies to detect increased amygdala activity late in training, one possibility is that amygdala activity during later trials may be restricted to the persistently responding cells that store the trace. Because fewer cells are involved at the end than at the beginning of training, the later activity might go undetected in fMRI studies, especially because learning-induced amygdala activation is near the detection threshold for this technique[17]. A second possibility is that fear levels of human subjects may actually decrease during later conditioning phases, especially in light of the low intensity

Fig. 7. Persistence of neuronal plasticity during conditioning. (**a**) Persistence values (see text for details) for all conditioned cells from the paired group (*n* = 24 cells). Dashed line, cutoff used to distinguish transiently plastic cells from long-term plastic cells. (**b**) Mean (± s.e.m.) CS-response for transiently plastic and long-term plastic cells (*n* = 12 cells per group) throughout the three phases of the experiment. Data points are four trial blocks averaged together, except for the first point, which is the average of the final six trials of habituation. Asterisks indicate trial blocks during which the response is significantly greater than habituation levels (*p* < 0.05).
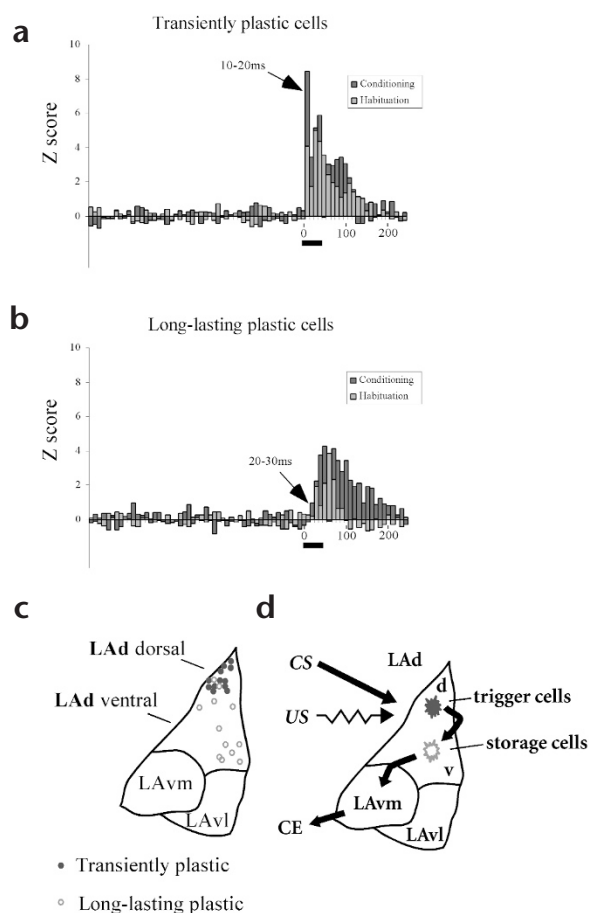
Fig. 8. Breakdown of transiently plastic versus long-lasting plastic cells, by latency of plasticity and anatomical location. (**a**, **b**) Mean PETHs (spikes normalized to the pre-CS baseline; 10-ms bins) for two phases of the experiment (habituation trials 1–8 and average of conditioning trials 1–8 and 9–16) for transiently plastic cells (**a**; n = 12 cells) and long-term plastic cells (**b**; n = 12 cells). (**c**) Anatomical recording locations for transiently plastic cells (gray circles) and long-term plastic cells (white circles). (**d**) Diagram of our hypothesis of how LAd cells encode fear learning. Abbreviations are as in Fig. 2. d and v, dorsal and ventral portions of LAd, respectively; CE, central nucleus of the amygdala.

shock used as a US. Consistent with this possibility, when humans' fear responses were measured using skin conductance changes, they virtually disappeared during later fear conditioning trials[17].

Two different cell populations in LAd

A number of differences between the transiently plastic and long-lasting plastic cells, in addition to the differential persistence levels of their enhanced auditory responding, further suggest that they may represent two different populations of LAd cells. The transiently plastic cells tended to have shorter latency and more robust auditory responses before training. Furthermore, the transiently plastic cells tended to be located more dorsally, near the dorsal tip of the LAd, whereas the long-lasting plastic cells were most prevalent in the ventral regions of this subnucleus. One possibility consistent with these characteristics is that the transiently plastic cells receive more direct projections from the thalamus, whereas the long-lasting plastic cells instead are the targets of intra-amygdala, and/or cortico–amygdala projections. This is suggested by the short latency responses of the transiently plastic cells, as latencies less than 20 ms are possible only through direct thalamic projections[24], whereas the longer latencies of the long-lasting plastic cells are consistent with additional synapses being involved.

Further support that two cell types exist in LAd comes from recent experiments in our lab. For instance, one study of the biochemical mechanisms of fear conditioning found that cells exhibiting activation of mitogen-activated protein kinases after learning are much more prevalent in ventral rather than dorsal LAd, and that such changes are critical for the consolidation of fear conditioned memories that last six hours or longer[29]. In addi-

tion, tract-tracing studies reveal that the regions of LAd containing the two types of cells may receive slightly different afferent information from the auditory thalamus[25,26]. As the functional roles of the thalamic regions in question are poorly understood, the significance of these projections, and whether they contribute to the transient versus long-lasting properties found in LAd cells, remains to be determined.

Given that LAd has approximately 20,000 excitatory neurons (based on unpublished stereological cell counts) and that the dorsal and ventral LA are roughly the same size, plasticity may be triggered and stored within two populations of about 10,000 cells in LAd (**Fig. 8d**). If so, the problem of identifying cell biological correlates of fear conditioning in the mammalian brain would become a tractable pursuit.

The CS responses of the two cell types conform well to the changes in CS processing predicted by a classical learning theory, the Pearce–Hall model[31]. In this theory, attentional processes raise the associability of a CS when the discrepancy between the expected and received US is high on recent trials. This describes the firing behavior of the transiently plastic cells, which respond most to the CS during early conditioning and early extinction trials. Conditioned responding in the model is controlled by the strength of the previous conditioning to the CS, which is fairly well represented by the firing of the long-lasting cells. The finding that these cells maintain an elevated responsivity late into extinction may reflect the maintenance of a memory trace of the training experience, which is known to persist even after behavioral fear has been extinguished[32].

Locus of synaptic plasticity

The present data leave open the possibility that the measured changes in neuronal responsivity may be due to plasticity that occurs outside the LAd, in afferent structures such as the thalamus[33,34] and/or auditory cortex[35,36], both of which contain cells with responses that can be modified by fear conditioning. However, certain aspects of thalamic and cortical plasticity are amygdala-dependent[37,38]. Although further study will help better resolve the contributions of thalamic and cortical plasticity to LAd activity, it is likely that some if not most of the plasticity in LAd is due to local integration of the CS and US. For example, the LAd is a site of massive CS–US convergence[28], more so than afferent areas in the thalamus[39]. Studies of long-term potentiation (LTP) indicate that LA synapses are capable of plasticity[4,12,13], and evidence suggests that fear conditioning induces LTP in LA[8,10,29]. Fear learning is blocked by temporary disruption of amygdala function during training, even when the amygdala is intact during later testing[30,40–43]. Furthermore, fear learning is impeded by local disruption of putative learning mechanisms in the amygdala, including macromolecular synthesis, as well as the intracellular cascades mediated by mitogen-activated protein kinases and cyclic AMP-dependent protein kinase[29,44,45]. Collectively, these and other findings strongly implicate LAd as a site

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of plasticity during fear conditioning[1,4,5]. The present findings suggest it may also be involved in long-term storage.

Summary

The present data add a previously missing foundation to the hypothesis that fear conditioning may in part be subserved by increases in LAd responsivity to the CS after it is paired with the US. The results show that LAd responsivity increases rapidly but incrementally during early training trials until presumably some threshold is reached, at which time behavioral learning is expressed. Furthermore, it seems that the initiation of plasticity and storage of long-term memories may be differentially encoded by increased CS-responsivity of cells in the dorsal versus ventral parts of the LAd.

METHODS

Animals and bar-press training. Studies were done on male Sprague–Dawley rats weighing 300–350 g before behavioral training. All procedures were in accordance with Public Health Service guidelines and were approved by the animal use committee of New York University.

Animals were kept on a restricted diet to maintain them at ~95% body weight. They were then placed in an operant conditioning box (24 × 31 × 35 cm, MED Associates, St. Albans, Vermont) and were trained to press a bar for food rewards (45 mg; Noyes, Lancaster, New Hampshire) until a minimum of 10 responses/min at a 60-s variable-interval (VI60) reinforcement schedule was reached, which typically took about 1 week of training.

Surgery. Surgical procedures were similar to those in previous studies[7]. Once the bar-press response was learned, subjects were pretreated with atropine (0.24 mg/kg, intraperitoneally) and were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Supplemental doses of anesthetic were administered throughout surgery as needed, and body temperature was regulated by a gel heating pad. Burr holes were drilled above the amygdala[46], and above frontal cortex and cerebellum for insertion of self-tapping set screws to anchor the implant to the cranium.

An electrode assembly[47] of 8–10 independently movable bundles of wires was stereotaxically implanted so that the electrode wires were positioned in the neostriatum just dorsal to LA (2.5 mm dorsal to earbar-zero). Each individual wire bundle was sheathed in a protective stainless steel tube (33 G), and consisted of 4 or more individual nichrome wires (25 micron diameter), a tetrode, or a stereotrode[48] made from nickel/chromium alloy wires (13 micron). All wires were insulated except for the cut tip (impedance < 1 MΩ at 1 kHz). The overall configuration of wires had a diameter of ~0.7 mm. At the end of surgery, the drive was cemented in place and rats were allowed five days to recover.

Unit recording. After recovery, animals were given additional bar-pressing sessions to ensure performance was at least at pre-surgery levels. During this time, the electrode was connected to a head stage containing unity-gain operational amplifiers. A cable then passed the signal through a hole in the top of the conditioning chamber to multichannel differential amplifiers (LYNX, Tucson, Arizona) via a slip-ring commutator (Crist Instruments, Damascus, Maryland) that allowed the rat to move freely. Signals were amplified (10,000× gain), passively filtered (600–6,000 Hz), digitized at ~25 kHz/channel, and displayed on digital oscilloscopes and on a computer monitor using Experimenter's Workbench 32 software (DataWave Technologies, Longmont, Colorado). Spike waveforms corresponding to single cells were sorted off-line on the basis of waveform parameters using cluster isolation methods as described previously for single wires[7], stereotrodes and tetrodes[48], using the DataWave software. Only cells that were well isolated throughout the experiment were analyzed.

Each wire bundle was advanced in 40-μm steps until discriminable single units were isolated at depths believed to be in LAd. Units were tested for auditory responses using a number of experimenter-produced stimuli such as claps, taps and vocalizations. The bundles were lowered until multiple auditory-responsive cells were isolated, at which time conditioning began.

Conditioning. Conditioning took place in an operant conditioning box similar to the one where the animal had been trained to bar-press. The operant box was enclosed in a larger sound-attenuating chamber. The conditioning protocol was modified from previous studies in our lab[7,8]. The CS was a 20-s series of acoustic white noise pips (50-ms duration, 5-ms rise/fall, 80 ± 5 dB, open field) delivered at 1 Hz, emitted from a speaker mounted near the ceiling of the operant box. The use of multiple auditory presentations as a single CS allowed greater sampling of neuronal data within each trial[8]. The US was a mild electric footshock (0.4 mA, 0.5 s) delivered through the grid floor of the test box. A constant background pseudo-white noise (55 dB) produced by a ventilator fan was present throughout the sessions.

The experiment consisted of three phases. During habituation, the CS was presented alone for 8 trials. These were immediately followed by 16 conditioning trials in which the CS and US co-terminated. The rat was then placed in its home cage for 1 h, after which it was returned to the test box for 20 extinction trials, during which the CS was again presented alone. Trials were separated by a variable mean interval of 4 min (range, 3–5 min). The entire training session lasted about 5 h. To test for effects of training on spontaneous neural activity, 10 min of spontaneous activity were recorded at four times during the experiment: before habituation, immediately following conditioning, just before extinction and following extinction. The spontaneous activity was recorded while the subject rested in the conditioning box, with a wall preventing access to the bar-pressing lever. In addition to the paired group described above, a separate group (unpaired) received explicitly unpaired CS and US presentations during the conditioning phase of the experiment, to control for non-associative influences of conditioning.

Data analysis. Data were analyzed using a combination of NEX spike train analysis software (Plexon, Dallas, Texas), Matlab and Excel, as described below.

Bar-press suppression[15] was measured using the suppression ratio $(r_{pre} - r_{cs})/(r_{pre} + r_{cs})$, in which r_{pre} and r_{cs} indicate the mean press rates during the 60 s before the CS and during the CS, respectively. This yields a value of 1 for complete suppression, 0 for no suppression, and negative values down to –1 for facilitation elicited by the CS. Freezing was defined as cessation of all movement other than respiratory activity or slight ear twitches to the onset of auditory stimuli, and was measured by a blind observer using a stopwatch, from a videotaped recording of the experiment. Testing for changes from habituation levels, for both behavioral and neural analyses, used the final six habituation trials as baseline.

PETHs of auditory responses were constructed for each cell, using 10-ms bins. As each CS consisted of 21 auditory pip presentations[8], the last of which overlapped with the US during conditioning, shock artifact made recording during the twenty-first pip of conditioning trials impossible. Therefore, analyses of auditory responses were always based on the first 20 stimuli per CS.

To investigate the effects of training on the entire population of LAd cells, PETHs for each cell were summed over all stimuli for the trials being analyzed, and normalized to the 500-ms pre-stimulus baseline using a standard Z-score transformation. That is, each bin of the normalized PETH expressed the number of standard deviations above or below the mean baseline firing rate. The CS response of a given cell in one phase of the experiment, expressed as an average Z-score, was then subtracted from the cell's CS response during another phase. The distribution of the resulting 'Z difference scores' across the population of cells yielded an index of plasticity for the region.

In addition, neurons were analyzed on a cell-by-cell basis to determine whether they showed statistical evidence of training-induced enhanced responding. First, the envelope of the CS-evoked response was determined for each cell by finding the earliest and latest bins that showed elevated CS-evoked activity. Specifically, a PETH was constructed using all trials from all phases of the experiment. All PETH bins following stimulus onset that exceeded the average firing rate during the 500-ms pre-stimulus period by 1.65 standard deviations or more were included, until 2 consecutive bins failed to reach this criteria, or until 25 post-CS bins (250 ms) had been considered. In addition, at least one of the bins was required to be three standard deviations above pre-stimulus levels, or the cell was classified as not CS-responsive. The resulting response time window, calculated separately for each cell, was then analyzed further for increases in CS-elicited firing (the firing rate during the cell's response

time window minus the rate during the 500-ms pre-stimulus period) over the course of the experiment.

Histology. At the end of the experiment, small lesions were made by passing current (4 μA, 8 s) through recording wires from most of the wire bundles from which cells were recorded. Animals were transcardially perfused with buffered formalin. The brains were removed and stored in a formalin-sucrose solution, with 2% nitroferrocyanide added to visualize iron deposits left by the lesioned wires (Prussian blue reaction). Frozen sections (40 μm thick) were cut on a sliding microtome or cryostat, and sections were stained for Nissl bodies. Lesion sites were used to locate the regions of the recorded cells. The known configuration of the electrode wire bundles allowed the reconstruction of all recording sites[47].

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