Neuron, Vol. 40, 1013–1022, December 4, 2003, Copyright ©2003 by Cell Press

Auditory-Evoked Spike Firing in the Lateral Amygdala and Pavlovian Fear Conditioning: Mnemonic Code or Fear Bias?

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Summary

Amygdala neuroplasticity has emerged as a candidate substrate for Pavlovian fear memory. By this view, conditional stimulus (CS)-evoked activity represents a mnemonic code that initiates the expression of fear behaviors. However, a fear state may nonassociatively enhance sensory processing, biasing CS-evoked activity in amygdala neurons. Here we describe experiments that dissociate auditory CS-evoked spike firing in the lateral amygdala (LA) and both conditional fear behavior and LA excitability in rats. We found that the expression of conditional freezing and increased LA excitability was neither necessary nor sufficient for the expression of conditional increases in CS-evoked spike firing. Rather, conditioning-related changes in CS-evoked spike firing were solely determined by the associative history of the CS. Thus, our data support a model in which associative activity in the LA encodes fear memory and contributes to the expression of learned fear behaviors.

Introduction

Pavlovian fear conditioning is a versatile model paradigm used to probe the neural substrates of fear memory in many species. In a typical experiment, an initially neutral conditional stimulus (CS), such as a tone, is paired with an aversive unconditional stimulus (US) that evokes a host of fear behaviors. After as little as one pairing, subsequent presentation of the CS alone is sufficient to initiate the expression of fear behaviors. A vast body of literature suggests that the amygdala makes a particularly important contribution to fear conditioning (Buchel and Dolan, 2000; Davis, 1992; Fanselow and LeDoux, 1999; Maren, 2001; Paré, 2002), but its precise role is hotly debated (Cahill et al., 1999; Vazdarjanova, 2000).

Electrophysiological recording has revealed that neurons in multiple amygdaloid nuclei exhibit conditional CS-evoked plasticity (Applegate et al., 1982; Maren et al., 1991; Maren, 2000; Quirk et al., 1995). This long-term conditioning-related plasticity is associative (Collins and Paré, 2000; Rogan et al., 1997), does not appear to mirror plasticity from sensory afferents (Maren et al., 2001; Poremba and Gabriel, 2001), and shares many mechanistic similarities with cellular models of memory (Bauer et al., 2001; Goosens and Maren, 2002). These and other findings strongly suggest that the amygdala encodes a neural representation of the CS-US association during fear conditioning, and this mnemonic code contributes to the expression of learned fear behaviors. In agreement with this, CS-evoked spike firing in the amygdala is highly correlated with the expression of fear behaviors, such that CS-evoked spike firing is maximal when fear behaviors are maximally expressed, and CS-evoked spike firing is minimal when fear behaviors are not expressed (Collins and Paré, 2000; Hobin et al., 2003; Maren, 2000; Maren et al., 1991). However, because many indices of fear learning, including fear behaviors and conditional amygdala plasticity, emerge concurrently and with as little as one fear conditioning trial, it is difficult to firmly establish any causal relationship.

Indeed, a number of nonassociative mechanisms related to the expression of a fear state could enhance CS processing in the amygdala. First, it is possible that neuronal processing in the amygdala is altered by the behavioral state of the organism, such that fear states produce enhanced CS processing relative to exploratory or restful states. Behavior has previously been shown to modulate electrophysiological activity in hippocampus (Weiler et al., 1998; Winson and Abzug, 1978). A state of heightened fear could also enhance CS processing by producing general increases in attention or arousal (Davis and Whalen, 2001), which could increase sensory transmission to the amygdala (Ashe et al., 1976; Kapp et al., 1992). Accordingly, amygdala-based processing in humans is enhanced by the availability of attentional resources (Pessoa et al., 2002). In addition, repeated administration of an aversive stimulus such as foot shock produces increases in the excitability of amygdala neurons (Paré and Collins, 2000; Rosenkranz and Grace, 2002), perhaps reflecting changes in cell excitability in other regions (Gabriel, 1976). These alterations in cell excitability may also contribute to nonspecific enhancement of stimulus processing in the amygdala.

To determine whether these nonassociative factors contribute to conditional increases in CS-evoked spike firing in the lateral nucleus of the amygdala (LA), we conducted two experiments in which we manipulated the expression of conditional fear behaviors after fear conditioning. Our data demonstrate profound dissociations between conditional increases in LA spike firing and both conditional increases in LA excitability and conditional fear behavior. Conditional increases in CS-evoked spike firing in LA were exhibited in response to CSs that were paired with a US, regardless of overt behavioral indices of fear or amygdala excitability, while CSs that were not paired with a US never expressed conditioning-related increases in spike firing. Thus, the expression of conditional CS-elicited spike firing is regulated exclusively by the associative history of a CS and is not modulated by the behavioral or attentional state of an organism nor conditioning-related changes in amygdala excitability.

Results

Single-Unit Firing Properties

A total of 341 units from 26 rats contributed to the data reported here. These units were localized to both the

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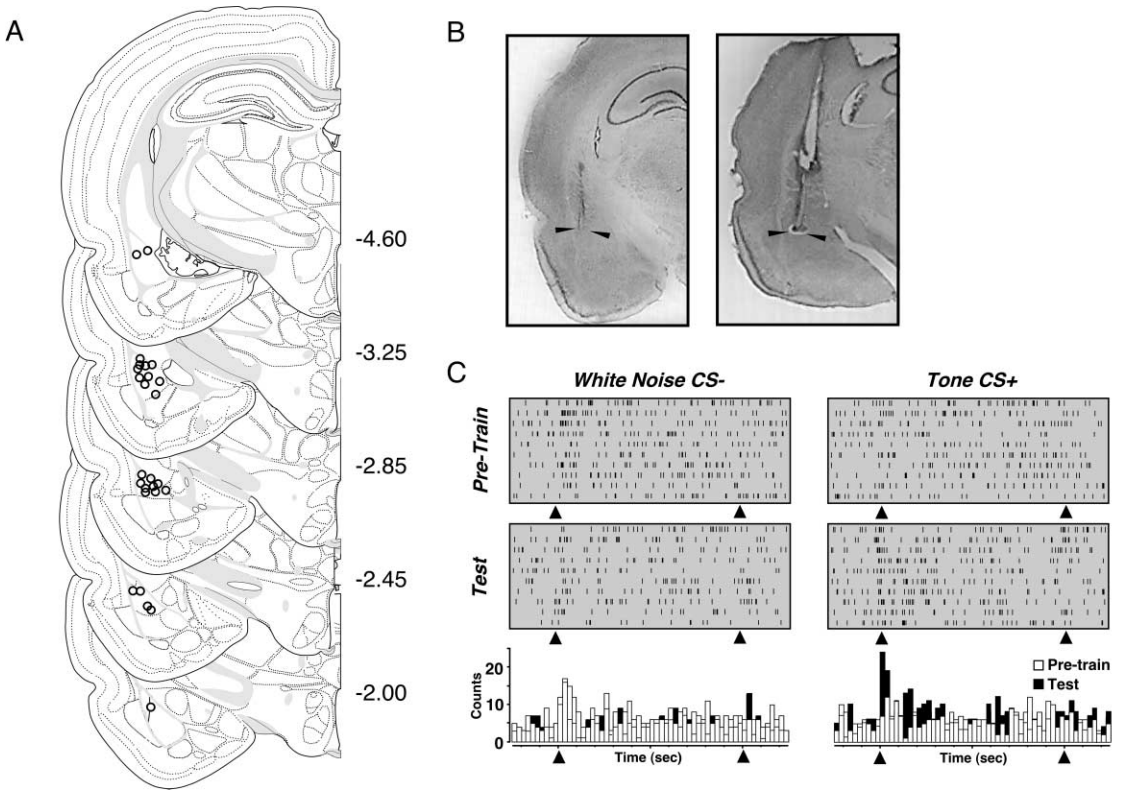

Figure 1. Recording Electrode Placements in the Lateral Nucleus of the Amygdala and Discriminative Spike Firing

(A) Recording electrodes were localized to the dorsal and ventral divisions of the lateral nucleus of the amygdala (LA). The spatial localization of the electrodes did not differ across groups of rats, nor did it produce any systematic differences across any of our measures within a group. The brain images are adapted from Swanson (1998), and the position of each section is expressed in mm posterior to bregma.

(B) High-resolution scans of thionin-stained coronal brain sections illustrate representative placements of electrodes in LA from each of the two experiments. Black arrowheads mark the position of wires within LA.

(C) Discriminative auditory fear conditioning was evident within single neurons in the LA. Raster plots indicate trial-by-trial spike firing for each of the 10 test trials during the pretrain and test sessions for the white noise CS^- and tone CS^+ for a representative single unit in LA. The peristimulus time histograms illustrate the sum of spike activity (spike counts) across all CS trials for the pretrain and test sessions (50 ms bins). Arrowheads indicate the onset and offset of the 2 s CS; a 500 ms period preceded and followed the CS. This single unit exhibited robust neuronal discrimination after fear conditioning. Spike firing in the short-latency bins after onset of the CS^+ was greater during the test as compared to the pretrain session and greater than activity elicited by the CS^-.

dorsal and ventral divisions of LA (Figures 1A and 1B). We elected to record from LA because its well-characterized short-latency (<100 ms) responses to auditory CSs exhibit conditional plasticity and this plasticity relates to mnemonic processing (Buchel and Dolan, 2000; Davis, 1992; Fanselow and LeDoux, 1999; Maren, 2001; Paré, 2002). Our population of CS-responsive units exhibited a number of quantitative characteristics that are highly similar to those reported in other studies of single LA neurons. The average preconditioning firing rate of the units was 4.18 Hz (geometric mean, 1.75 ± 0.31 Hz; range, 0.10 to 28.00 Hz). The low spontaneous firing rates and wide spike widths strongly suggest that the majority of the units that we recorded corresponded to single pyramidal neurons rather than interneurons (Paré and Gaudreau, 1996; Washburn and Moises, 1992). 274 of the 341 units (80.4%) were classified as CS responsive. These units exhibited maximal responsivity within 100 ms of CS onset, and conditional changes were manifest within this time frame. Thus, all statistical analyses were confined to the average activity during this time period.

Conditional Fear Is Not Sufficient for Learning-Related Spike Firing in Lateral Amygdala

To determine whether conditional fear behavior, or the enhancement in attentional processing that a fear state engenders, is sufficient to increase CS-evoked spike firing in LA, we compared LA spike firing evoked by a CS^+ and CS^- in rats receiving discriminative auditory fear conditioning to that in rats receiving both discriminative auditory fear conditioning and fear conditioning to the context in which extinction testing was conducted. The administration of contextual fear conditioning was expected to produce equivalent and high expression of multiple behavioral fear responses, as well as facilitated attention or arousal, during extinction sessions in which the CS^+ or CS^- was presented. We measured only conditional freezing to confirm the effectiveness of our manipulation; however, the expression of conditional contextual freezing is highly correlated with the expression of many other conditional fear behaviors (Antoniadis and McDonald, 1999). Examining spike firing to a CS that was never paired with a US under conditions of high conditional fear responses enabled us to deter-

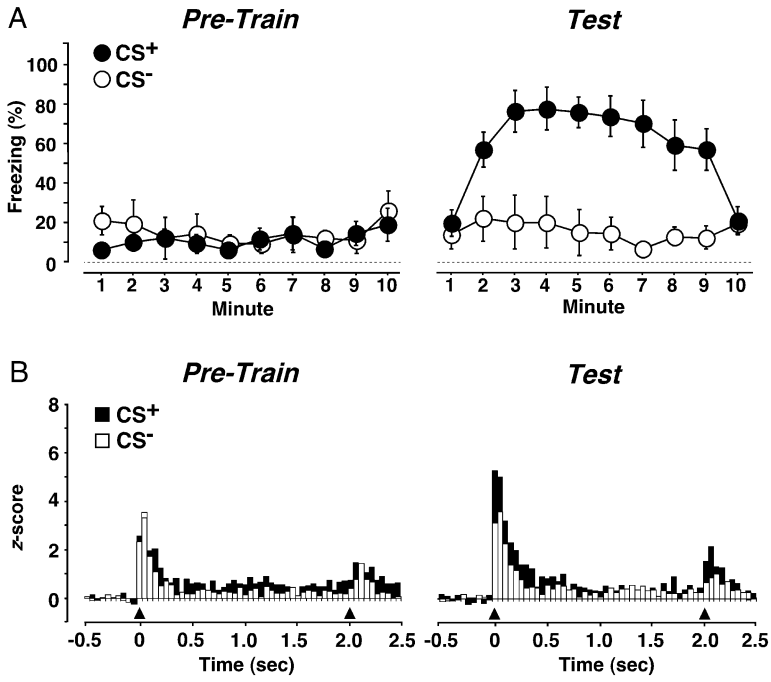

Figure 2. Discriminative Fear Conditioning in the Control Group Produces Behavioral and Neuronal Discrimination to a CS^+ and CS^-

(A) After auditory fear conditioning, conditional freezing increased to a CS previously paired with foot shock (CS^+). In contrast, no change in freezing behavior was observed for a CS that was never paired with foot shock (CS^-). For each test, onset of the first 2 s CS occurred 1 min after the start of the test; CSs were then delivered at 1 min intervals for a total of 10 CS trials. Average freezing during the pre-CS interval (1 min; minute 1) and post-CS intervals (1 min duration; minutes 2–10) are shown.

(B) Discriminative auditory fear conditioning also produced increases in CS-evoked activity for a CS^+ but not a CS^-. Peristimulus time histograms illustrate the average z scores for all LA units (50 ms bins) during the CS^+ and CS^- sessions before (pretrain) and after (test) fear conditioning. Arrowheads indicate the onset and offset of the 2 s CS; a 500 ms period preceded and followed the CS. Note the increase in CS^+-evoked activity from the pretrain session to the test session, and the greater level of spike firing to the CS^+ compared to the CS^- during the test session.

mine whether behavioral or attentional state modulated CS-evoked LA spike firing.

Initially, we sought to establish strong behavioral and neuronal discrimination between the CS^+ and CS^- in our discriminative auditory fear conditioning paradigm. A total of 83 CS-responsive units from 8 rats (control group) were included in the analyses. As shown in Figure 1C, discriminative fear conditioning produced neuronal discrimination in single LA neurons, which was indicated by greater spike firing to the CS^+ than to the CS^-. 56 of 83 neurons (67.5%) exhibited greater short-latency spike firing (0–100 ms post-CS onset) to the CS^+ than to the CS^- after fear conditioning. Importantly, our fear conditioning procedure also yielded a behavioral discrimination (Figure 2A; stimulus, $F[1,7] = 12.71$, $p < .01$; stimulus X session, $F[1,7] = 26.15$, $p < .01$), such that conditional freezing was significantly greater after fear conditioning only for the CS^+ ($p < .05$, Fisher's PLSD test). Neuronal discrimination in CS-evoked spike firing was also evident in the population average of LA spike firing (Figure 2B; stimulus, $F[1,82] = 8.16$, $p < .01$; stimulus X session, $F[1,82] = 13.39$, $p < .001$): only the CS^+ evoked greater spike firing (0–100 ms) in LA after fear conditioning ($p < .05$, Fisher's PLSD test). Thus, discriminative fear conditioning yielded strong behavioral and neuronal discriminations to the CS^+ and the CS^-.

To determine whether the expression of fear contributes to CS-evoked spike firing, a second group of rats ($n = 9$; 94 CS-responsive units recorded; experimental group) was subjected to both discriminative fear conditioning and contextual fear conditioning to the context in which extinction was conducted. Our manipulation was extremely effective in producing a high level of fear across the extinction sessions to a CS^+ and CS^-: rats exhibited significant increases in conditional freezing after fear conditioning during both the CS^+ and CS^- testing sessions (Figure 3A; session, $F[1,8] = 163.34$, $p < .0001$), and these increases were statistically comparable (stimulus, $F[1,8] = 1.01$, $p = ns$). Despite similar levels of conditional freezing to the CS^+ and CS^-, CS-evoked spike firing was elevated relative to the preconditioning baseline only for the CS^+ (Figure 3B; stimulus, $F[1,93] = 12.80$, $p < .001$; stimulus X session, $F[1,93] = 7.87$, $p < .01$). That is, short-latency (0–100 ms) spike firing evoked by the CS^+ increased significantly after fear conditioning ($p < .05$, Fisher's PLSD test), while spike firing evoked by the CS^- remained at the preconditioning baseline levels ($p = ns$, Fisher's PLSD test). The neuronal discrimination evident in the population average (Figure 3B) was also apparent for single units; 63 of 94 units (67%) exhibited greater short-latency spike firing to the CS^+ compared to the CS^-. Thus, although neuronal and behavioral discrimination were similar in control rats (Figure 4A), neuronal discrimination was present despite the absence of a behavioral discrimination in experimental rats (Figure 4B). This suggests that neither elevated arousal or attentional levels, nor the expression of fear behavior itself, is sufficient to increase CS-evoked spike firing after fear conditioning. This claim is also supported by the observation that spike firing evoked by the CS^- in the experimental group was not modulated by freezing behavior; spike firing evoked by the CS^- remained at preconditioning baseline levels despite high levels of conditional freezing exhibited after fear conditioning. Thus, even if the asymptotic, or "ceiling," levels of conditional freezing observed after fear conditioning masked differences in fear levels between the CS^+ and CS^- testing sessions, our results are still incompatible with claims that the expression of fear behaviors and any correlated increases in attention modulate CS-evoked spike firing.

In addition to examining whether attention or behavior modulates LA activity, we also sought to determine

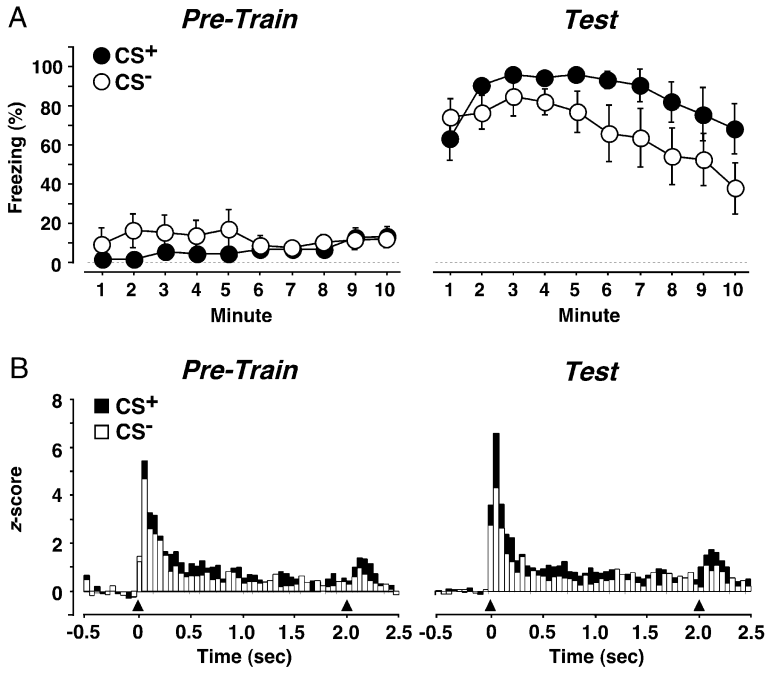

Figure 3. The Expression of Conditional Fear Behavior Is Not Sufficient to Increase Amygdala Spike Firing to a CS

(A) Contextual fear conditioning to the extinction context in the experimental group produced massive and equivalent levels of conditional freezing during extinction testing (test) to a CS^+ and CS^-. For each test, onset of the first 2 s CS occurred 1 min after the start of the test; CSs were then delivered at 1 min intervals for a total of 10 CS trials. Average freezing during the pre-CS interval (1 min; minute 1) and post-CS intervals (1 min duration; minutes 2–10) are shown.

(B) Despite the absence of behavioral discrimination, LA neurons exhibited conditioning-related increases in CS-evoked spike firing only for a CS previously paired with foot shock (CS^+). CS-evoked spike firing did not increase for a CS that was never paired with foot shock (CS^-). Thus, discriminative LA spike firing persisted in the absence of a behavioral discrimination between a CS^+ and CS^-. Peristimulus time histograms illustrate the average z scores for all LA units (50 ms bins) during the CS^+ and CS^- sessions before (pretrain) and after (test) fear conditioning. Arrowheads indicate the onset and offset of the 2 s CS; a 500 ms period preceded and followed the CS. Note the increase in CS^+-evoked activity from the pretrain session to the test session and the greater level of spike firing to the CS^+ compared to the CS^- during the test session.

whether the postconditioning increases in LA excitability that we (K.A.G. and S.M., unpublished observations) and others (Paré and Collins, 2000; Rosenkranz and Grace, 2002) have observed contribute to CS-evoked spike firing. If changes in LA excitability (as manifested by changes in the spontaneous firing rate of LA neurons) enhance CS-evoked spike firing, it follows that LA excitability should correlate with levels of CS-evoked spike firing. That is, LA neurons should only exhibit increases in spontaneous firing rates during CS^+ extinction sessions. To examine this, the average spontaneous firing rates during the pre-CS periods were calculated for each recording session for each unit. The pattern of changes in spontaneous firing rates across sessions was not affected by whether or not rats had received context conditioning in addition to discriminative fear conditioning (stimulus X session X group, $F[1,175] = 0.67$, $p = ns$). Thus, spontaneous firing rates were averaged across all rats ($n = 17$) for each session. In addition, because preconditioning firing rates did not differ between the pretraining baseline recording sessions for each CS (stimulus, $F[1,176] = 0.95$, $p = ns$), these values were collapsed into a single average value (pretrain). Our data reveal that fear conditioning produces robust increases in the spontaneous firing rates of LA neurons (Figure 5; session, $F[2,352] = 22.55$, $p < .0001$), such that postconditioning spontaneous firing rates (test) were significantly higher than preconditioning firing rates (pretrain) regardless of the stimulus tested (CS^+ or CS^-; $p < .05$, Fisher's PLSD test). These data indicate that LA neurons exhibit generalized increases in excitability after fear conditioning. Nevertheless, this increased excitability was not sufficient to increase spike firing evoked by a CS^-; LA neurons reliably discriminated between the two CSs despite comparable increases in spontaneous firing rates (Figure 5; averaged CS-evoked firing in the control and experimental rats is shown for comparison). Thus, it is unlikely that increases in LA excitability contribute to conditioning-related increases in spike firing to a CS^+.

Conditional Fear Is Not Necessary for Learning-Related Spike Firing in Lateral Amygdala

To determine whether fear behavior or enhanced attention is *necessary* for the expression of conditional increases in CS-evoked spike firing, we performed a second experiment in which we examined CS-evoked LA spike firing after inhibition of freezing behavior by pharmacological inactivation of the central nucleus of the amygdala (CEA). For this experiment, fear conditioning consisted of repeated pairings of one auditory stimulus with foot shock, and unlike the previous experiment, discriminative conditioning was not utilized and no CS^- was presented. After fear conditioning, CS-evoked spike firing in the LA and conditional freezing were assessed after temporary pharmacological inactivation of the CEA and, subsequently, during a drug-free extinction test. We used one of two drugs to inactivate the CEA: D,L-2-amino-5-phosphonovalerate (APV), an NMDA receptor antagonist that may have inactivated only a subset of CEA neurons, or lidocaine (LIDO), a potent sodium channel blocker that likely produced robust inactivation of all CEA neurons. Activity in the CEA is thought to translate activity from the afferent LA into the expression of multiple conditional fear behaviors (Fendt and Fanselow,

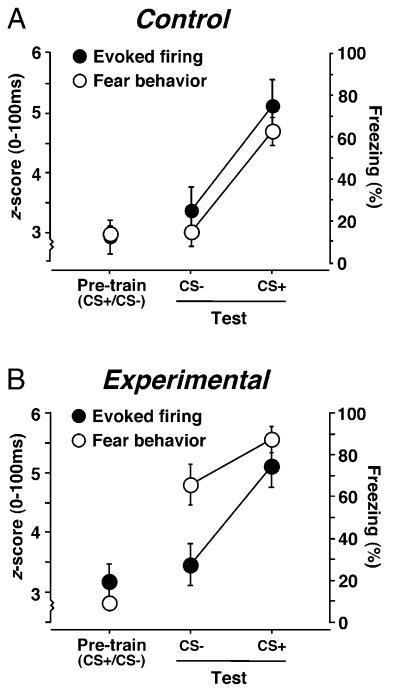

Figure 4. Comparison of Behavioral and Neuronal Discrimination in Rats from the Control and Experimental Groups

Double *y* plots illustrate CS-evoked spike firing ("evoked firing," z scores averaged for each unit for the 100 ms period after CS onset) and conditional freezing ("fear behavior," averaged across 10 CS trials) for the pretrain session (CS^+ and CS^- data pooled) and the CS^+ and CS^- test sessions. Robust neuronal discrimination between the CS^+ and CS^- was evident in both the control (A) and experimental (B) groups, even though behavioral discrimination was prevented in the experimental group (B). The presence of neuronal discrimination in the experimental group, despite high levels of conditional freezing to both auditory CSs, reveals that conditional freezing behavior alone is not sufficient to increase CS-evoked spike firing in LA.

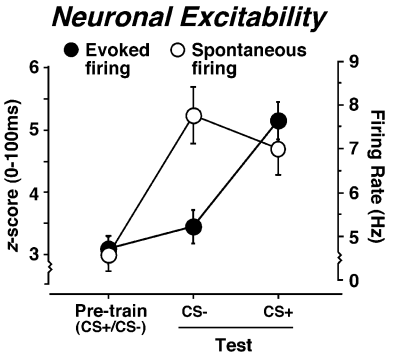

Figure 5. Conditional Increases in the Excitability of Amygdala Neurons Are Not Sufficient to Increase CS-Evoked Spike Firing

Double *y* plots illustrate CS-evoked spike firing ("evoked firing," z scores averaged for each unit for the 100 ms period after CS onset) and spontaneous spike firing during the 500 ms pre-CS periods ("spontaneous firing," firing rate averaged across 10 CS trials for the pretrain session (CS^+ and CS^- data pooled) and the CS^+ and CS^- test sessions in control and experimental rats (the data from the two groups are pooled). Equivalent conditioning-related increases in pre-CS spontaneous firing rate were exhibited by neurons in LA during extinction testing to the CS^+ and CS^-, yet conditioning-related increases in CS-evoked spike firing were observed only for the CS^+.

1999; LeDoux et al., 1988; Maren, 2001) as well as producing increased arousal and attention (Gallagher, 2000; Kapp et al., 1992). By inactivating the CEA, we planned to abolish the expression of conditional fear behaviors and eliminate fear-related increases in attention. After several hours, when the CEA was functional, a second session of extinction testing was conducted. We expected that the expression of conditional fear behaviors would accompany the restoration of function to the CEA. By comparing CS-evoked spike firing across the two extinction sessions, we were able to ascertain whether conditional fear behavior (indexed by the expression of conditional freezing) was necessary for the expression of conditional increases in LA spike firing.

A total of 97 CS-responsive units from 9 rats (APV, $n = 4$; LIDO, $n = 5$) were included in the analyses. Guide cannulae in these rats were localized to the medial division of CEA (Figure 6), the subregion of CEA that projects to the brainstem areas controlling fear behaviors (Jolkkönen and Pitkänen, 1998; Petrovich and Swanson, 1997). Data from these analyses reveal that inactivation of the CEA successfully eliminated the expression of conditional increases in freezing behavior (Figure 7A; session, $F[2,12] = 12.58$, $p < .01$). Inactivation of the CEA (test [drug]) produced levels of conditional freezing that were comparable to those exhibited prior to fear conditioning (pretrain [no drug]) and significantly lower than those shown in a later extinction session in which the CEA was active (test [no drug]; $p < .05$, Fisher's PLSD test). This effect was equally robust regardless of the drug used to produce inactivation (drug, $F[1,6] = 0.12$, $p =$ ns; drug X session, $F[2,12] = 0.45$, $p =$ ns). A comparison of CS-evoked spike firing under conditions of low and high expression of conditional fear behaviors reveals that the expression of conditional increases in spike firing did not covary with levels of conditional fear (Figure 7B). Specifically, significant and equivalent conditional increases in CS-evoked spike firing were exhibited during extinction sessions in which the CEA was either active or inactive (session, $F[2,192] = 33.62$, $p < .0001$; $p =$ ns, Fisher's PLSD test). Thus, conditional increases in CS-evoked amygdala spike firing are maintained in the absence of both conditional fear behaviors and the elevations in attention and arousal that accompany a fear state (Figure 8A).

Interestingly, although inactivation of CEA did not affect CS-evoked spike firing in LA, it did prevent the expression of conditioning-induced increases in the spontaneous firing rate of LA neurons (Figure 8B). The mechanism by which this occurs is not clear. Because the CEA does not directly regulate activity in other amygdaloid nuclei (Jolkkönen and Pitkänen, 1998), inactivation of the CEA may have inhibited the LA via an undetermined multisynaptic pathway. Alternatively, low concentrations of either APV or LIDO may have diffused into LA, thereby reducing synaptic transmission in LA (Maren and Fanselow, 1995), although apparently not reducing transmission enough to attenuate CS-evoked spike firing in LA. Nonetheless, these data reveal a disso-

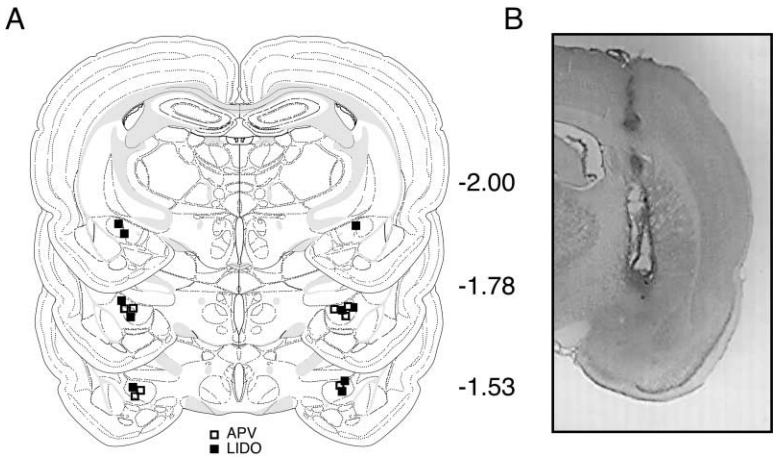

Figure 6. Cannula Placements in the Central Nucleus of the Amygdala

(A) Guide cannulae were localized primarily to the medial division of the central nucleus of the amygdala (CEA). The brain images are adapted from Swanson (1998), and the position of each section is expressed in mm posterior to bregma.

(B) A high-resolution scan of a thionin-stained coronal brain section depicts a representative cannula placement in CEA. Although not shown in the scan, cannula placements in CEA were bilateral.

ciation between the excitability of neurons in LA and CS-evoked spike firing, indicating that conditioning-related increases in the excitability of LA neurons are not necessary for the expression of conditional CS-evoked spike firing in LA.

Discussion

Fear conditioning gives rise to a number of behavioral and neuronal changes that could enhance or modulate CS processing in the amygdala. We sought to determine whether these changes contribute to conditioning-related increases in CS-evoked spike firing. Here we report strong, bidirectional dissociations between performance-related factors and conditional spike firing in the amygdala. Under special conditions, we observed that high expression of conditional fear behaviors and amygdala hyperexcitability did not increase spike firing to a CS never paired with foot shock. Following pharmacological inactivation of CEA, we observed conditional

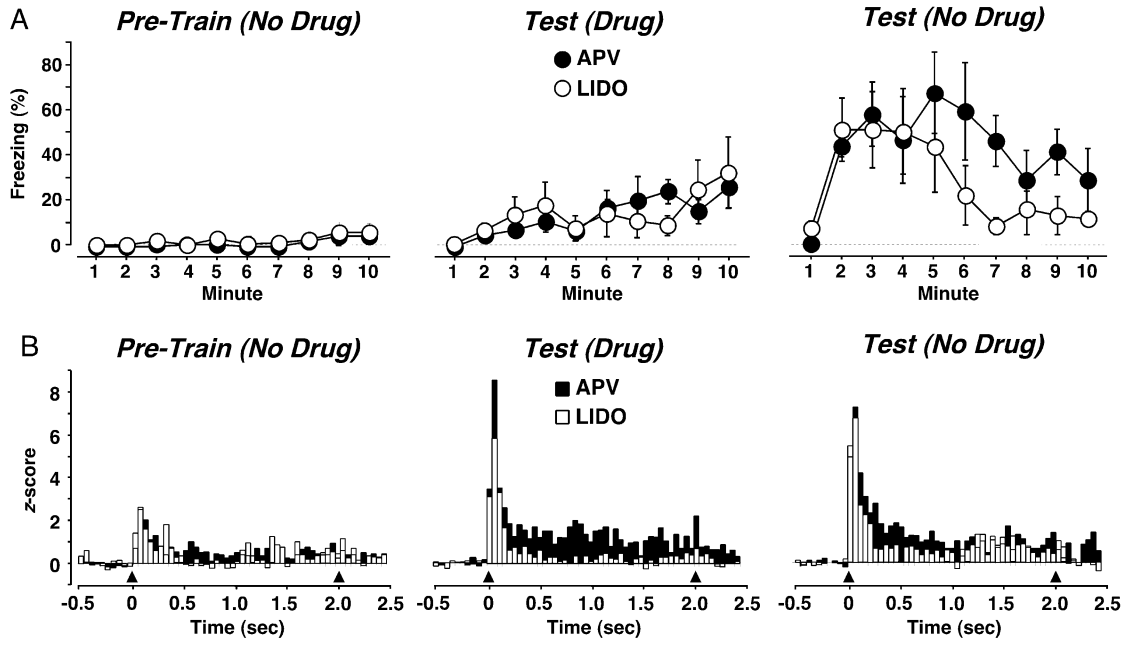

Figure 7. Conditional Fear Behavior Is Not Necessary for the Expression of Conditional Increases in CS-Evoked Spike Firing in the Amygdala

(A) Conditional freezing was expressed at low levels prior to auditory fear conditioning, and similarly low levels of conditional freezing were exhibited after fear conditioning when either APV or LIDO was used to render the CEA inactive during extinction testing (test [drug]). Robust conditional freezing was later exhibited by these same rats when given additional extinction testing after intra-CEA infusion of vehicle (test [no drug]). For each test, onset of the first 2 s CS occurred 1 min after the start of the test; CSs were then delivered at 1 min intervals for a total of 10 CS trials. Average freezing during the pre-CS interval (1 min; minute 1) and post-CS intervals (1 min duration; minutes 2–10) are shown. Thus, inactivation of the CEA temporarily blocked the expression of conditional fear behaviors. A different pattern was observed for CS-evoked amygdala spike firing (B). Regardless of whether the CEA was inactive (test [drug]) or active (test [no drug]), conditional increases in CS-evoked spike firing were observed. Peristimulus time histograms illustrate the average z scores for all LA units (50 ms bins) during the CS^+ and CS^- sessions before (pretrain [no drug]) and after (test [drug], test [no drug]) fear conditioning. Arrowheads indicate the onset and offset of the 2 s CS; a 500 ms period preceded and followed the CS. Note the increase in CS-evoked activity from the pretrain session to the test (drug) and test (no drug) sessions, despite the different levels of conditional freezing during these test sessions. Conditional increases in CS-evoked spike firing in LA neurons persisted in the absence of overt indices of fear.

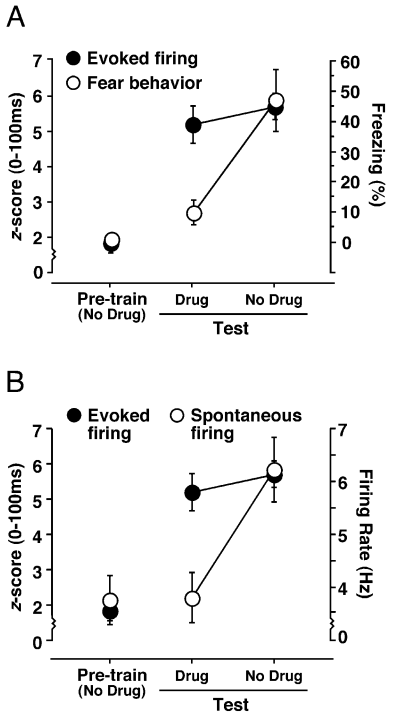

Figure 8. Conditional Increases in Freezing Behavior and in the Excitability of LA Neurons Are Not Necessary for the Expression of Conditional Increases in CS-Evoked Spike Firing

Double *y* plots illustrate CS-evoked spike firing ("evoked firing"; z scores averaged for each unit for the 100 ms period after CS onset; A and B), conditional freezing ("fear behavior," averaged across 10 CS trials; A), and spontaneous spike firing during the 500 ms pre-CS periods ("spontaneous firing," firing rate averaged across 10 CS trials; B) for the pretrain session (no drug) and the test (drug) and test (no drug) sessions. Note that the same CS-evoked spike firing data is plotted in both (A) and (B). Although inactivation of the CEA prevented the expression of conditional freezing (A) and conditional increases in the spontaneous firing rates of LA neurons (B), these neurons exhibited conditional increases in CS-evoked spike firing that were comparable to those exhibited in a later session, when the CEA was active.

increases in LA spike firing despite the absence of both fear behavior and hyperexcitability of amygdala neurons. Thus, the expression of conditional freezing and conditioning-induced increases in amygdala excitability were neither necessary nor sufficient to produce increases in CS-evoked spike firing in LA.

These observations indicate that conditioning-related changes in CS-evoked activity are not attributable to facilitated CS processing accompanying fear-related increases in attention or arousal. In addition, these data suggest that a state of immobility does not enhance passive acoustic properties, enabling sound waves to produce larger responses in the cochlea or its efferents. Also, it is clear that the behavioral state of an organism does not modulate CS-evoked spike firing. By breaking the tight correlation between rapidly expressed conditional plasticity and other changes that accompany fear learning, our data provide particularly compelling evidence that conditional plasticity in LA is reflective of a mnemonic code, rather than a bias imparted by the expression of fear behavior. In agreement with this, the only factor that predicted the amount of spike firing generated by a CS was its associative history with a US.

One concern with the present study is that we only measured one fear response (freezing) and that other fear responses either failed to reach a ceiling after unsignaled shock in the first experiment or survived CEA inactivation in the second experiment. Nonetheless, it is likely that fear responses other than freezing were similarly altered by our behavioral and pharmacological manipulations. In support of this, contextual fear conditioning has been shown to increase the expression of multiple fear behaviors, including heart rate changes, ultrasonic vocalizations, and body temperature changes (Antoniadis and McDonald, 1999, 2000). Fear conditioning is also thought to bring about a state of nonspecific arousal or attention (Kapp et al., 1992). Thus, when we examined whether high levels of conditional fear were sufficient to increase spike firing in LA to a CS that was never paired with a US, it is likely that the expression of many fear behaviors were near asymptotic, as was conditional freezing, and that the animals were in a highly aroused and attentive state. Similarly, when we examined whether abolishing conditional fear alters CS-evoked spike firing in LA, inactivation of the CEA likely reduced the collective expression of several behaviors, as well as reducing attentional processing (Holland and Gallagher, 1999; Holland et al., 2000). Indeed, the CEA is believed to be the final common output pathway for various conditional fear behaviors (Maren and Fanselow, 1996), insofar as it sends efferents to multiple fear response systems including the lateral hypothalamus (controlling changes in blood pressure; LeDoux et al., 1988), the periaqueductal gray (controlling freezing; LeDoux et al., 1988), and the medulla (controlling changes in respiration; Kapp et al., 1982). Because our manipulations strongly modulated conditional freezing (either producing very high levels or extremely low levels of conditional freezing), we assume that other fear behaviors were also strongly expressed or inhibited. However, it is possible that fear behaviors not measured in this study exerted modulatory effects on CS-evoked spike firing in LA.

An unexpected outcome of the present study was the discovery that fear conditioning induced increases in the spontaneous firing rate of LA neurons that were independent of both CS-evoked spike firing and ongoing behavior. Indeed, spontaneous firing rates among LA neurons in the control group were elevated during both the CS^+ and CS^- test sessions, in which freezing behavior was highly discriminative. Although we did not examine naive (nonshocked) rats, this pattern of results suggests that foot shock exposure alone may have produced a nonassociative sensitization of neuronal excitability in LA. Interestingly, this sensitization effect did not manifest itself in behavior insofar as the elevated spontaneous activity of LA neurons during the CS^- test session in control rats was not accompanied by conditional freezing. However, inactivation of CEA markedly reduced the conditioning-related increase in spontaneous activity (and conditional freezing) without affecting CS-evoked spike firing. This is interesting in light of studies implicating the CEA and LA in nonassociative sensitization of fear (Bellgowan and Helmstetter, 1996; Hitchcock et al., 1989; Sananes and Davis, 1992). Nevertheless, the presumed shock sensitization of neuronal excitability in LA we observed was dissociable from conditioning-related increases in CS-evoked spike fir-

ing, suggesting that they represent parallel but distinct learning processes in the amygdaloid circuitry.

The present report confirms and extends results from previous examinations of the relationship between amygdala neuronal activity and measures of fear learning. We have previously shown that conditioning-related changes in LA spike firing parallel conditional freezing, even after extensive overtraining (Maren, 2000). Other studies have reported dissociations between conditional amygdala spike firing and fear behavior. In these cases, conditional amygdala plasticity has been reported to persist after behavioral fear responses have been extinguished (McEchron et al., 1995; Quirk et al., 1997; Tang et al., 2001), suggesting that amygdala activity is important for the initiation of fear behaviors rather than reflecting the performance of fear behaviors. However, fear responses likely extinguish at very different rates within an extinction session. For example, elevated levels of stress hormones may remain even after conditional freezing is extinguished. Because these studies measured only a single fear behavior, they were unable to rule out the possibility that elevated levels of amygdala plasticity reflected other nonextinguished conditional fear responses.

An alternative approach has been to utilize a paradigm in which conditional fear is more gradually acquired (by reducing US intensity, for example) and determine whether conditional plasticity or conditional fear behavior emerges first (McEchron et al., 1995; Repa et al., 2001). For example, Repa et al. (2001) reported that some amygdala neurons exhibited significant conditional increases in CS-evoked spike firing prior to behavioral learning, thereby supporting the claim that amygdala neurons exhibit activity changes that predict behavior. However, because additional cells exhibited conditional plasticity at the same time or after behavioral learning, these data also support the claim that behavior predicts changes in neuronal activity in LA. Hence, the results of the present study are particularly compelling insofar as they represent a strong dissociation between conditional spike firing and other measures of fear learning. To our knowledge, we are the first to demonstrate in the same study that high levels of conditional fear are insufficient to increase CS-evoked spike firing and that the expression of conditional fear is not necessary for the expression of associative plasticity in the LA.

In combination with extant data, our findings lend support to a model in which local plasticity in LA represents fear memories and contributes to the initiation of conditional fear responses. By this view, the establishment of Pavlovian fear memories depends on synaptic plasticity mechanisms in the LA that register CS-US contingencies during fear conditioning (Bauer et al., 2001; Blair et al., 2001). Long-term potentiation (LTP) in auditory afferents to the LA, which has been demonstrated to occur during auditory fear conditioning (Rogan et al., 1997), may support conditioning-related increases in auditory CS-evoked spike firing in LA neurons (Maren, 1999). And although the importance of LA synaptic plasticity in fear conditioning is well established (Goosens and Maren, 2002), further studies are required to understand whether these plasticity mechanisms support the development of conditioning-related increases in CS-evoked spike firing in the amygdala.

In conclusion, the present results reveal that CS-evoked spike firing in the LA reflects an associative memory, not a bias imparted by the fear state the CS engenders. Because neuronal responses in the amygdala are dissociable from fear behaviors, our results provide strong evidence that CS-evoked spike trains in LA represent a mnemonic code that is a cause, not a consequence, of conditional fear responses. Accordingly, these data lend further support to the view that the amygdala, and the LA in particular, plays a critical role in associative processes governing the encoding, storage, and retrieval of Pavlovian fear memories. And while associative factors regulate the expression of CS-evoked single-unit activity in the LA, further work is required to understand whether such activity is itself necessary and sufficient for fear memory.

Experimental Procedures

Subjects

Studies were performed on male Long-Evans rats (400–600 g; Harlan Sprague-Dawley, Indianapolis, IN). Procedures were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Surgery

Under sodium pentobarbital anesthesia (65 mg/kg i.p.), small burr holes were drilled for placement of a multichannel recording probe aimed at the dorsal division of the lateral amygdala (LA) using standard stereotaxic methods. Some rats also received bilateral stainless steel guide cannulae (23 gauge; 20 mm) aimed at the central nucleus of the amygdala (CEA) using standard methods. Dental acrylic and jeweler's screws were applied to the skull to hold the implants in place, and 2–3 days were permitted for recovery.

Fear Conditioning

Fear conditioning and extinction testing were conducted in modified observation chambers (30 × 24 × 40 cm; MED-Associates Inc., Burlington, VT) located in sound-attenuating cabinets. Foot shock unconditional stimuli (USs; 0.5 s, 1.0 mA) were delivered through the grid floor. Auditory conditional stimuli (CSs; 2 s; 80 dB) were delivered via a speaker mounted to one wall of each chamber. Conditioning and extinction were conducted in context A or context B, which differed in visual, odor, and tactile cues.

Freezing behavior was used as a behavioral index of conditional fear. Conditioning chambers rested on load-cell platforms that recorded chamber displacement in response to the rats' motor activity. The output of the load-cell of each chamber was set to a gain that was optimized to detect freezing behavior. The load-cell amplifier output from the chamber was digitized at 5 Hz and was continuously acquired online using DataWave software (DataWave Technologies, Longmont, CO). Freezing was quantified by calculating the number of observations at or below a freezing threshold (load-cell activity of 5). To avoid counting momentary inactivity as freezing, an observation was scored as freezing only if it fell within a contiguous group of at least five observations that were all less than the freezing threshold. Thus, freezing was scored if a rat was immobile for at least 1 s. Within each session, the freezing observations were converted to a percentage of total observations for each minute. This method has been shown to yield greater than 95% concordance with visual scoring of freezing behavior.

Discriminative fear conditioning used two distinct auditory CSs (either a 2 kHz tone or a white noise burst; 2 s duration; 80 dB amplitude; 10 ms rise time), only one of which was paired with foot shock (the CS^+). The CS that was never paired with foot shock was termed the CS^-. The stimulus used as the CS^+ was counterbalanced across rats. Prior to discriminative fear conditioning, rats were placed in context B for two baseline recording sessions (pretrain). Each of the CSs (10 trials; 60 s ISI) was presented in a separate session. Discriminative conditioning later occurred in context A over two sessions. The first session consisted of ten CS^- presentations;

the second session consisted of ten coterminating CS^+/foot shock pairings. Some rats were returned to their home cages and received no further training. These rats served as a control group to confirm that this fear-conditioning paradigm produced robust behavioral and neuronal discrimination between a CS^+ and CS^-. Other rats (experimental group) received an additional training session after the CS^+ training session. These rats were returned to the baseline recording context (context B) and received 10 unsignaled foot shocks (60 s ISI) to generate fear of the context in which they would later be tested. Approximately 16 hr after the last conditioning session, all rats were placed in context B for two extinction sessions (test). In each session, one of the CSs (either CS^+ or CS^-) was presented (10 trials; 60 s ITI), and the testing order for CSs was counterbalanced across rats. Importantly, both auditory CSs (white noise or tone) produced equivalent patterns of results when used as the CS^+, and the order in which CS extinction tests (CS^+ or CS^-) were conducted also had no effect on the results (data not shown).

In a second experiment, reversible inactivation of the CEA was used to assess the necessity of conditional freezing for LA single-unit activity. This experiment used a standard (single CS) auditory fear-conditioning paradigm. Rats were infused with VEH (0.3 μl/side; 0.1 μl/min) and placed in a conditioning chamber for a pretraining session in which ten auditory CSs (white noise burst) were presented (60 s ISI; first CS presented 1 min after placement in the chamber). Rats were later returned to the conditioning chambers for fear conditioning consisting of 10 CS-US pairings. Approximately 18 hr later, the rats were infused with either APV (0.3 μl/side) or LIDO (0.5 μl/side) and immediately placed in the conditioning chamber for extinction testing (test [drug]). 12 hr later, rats were infused with VEH (0.3 μl or 0.5 μl/side) and immediately placed in the conditioning chamber for a second extinction test (test [no drug]). Both extinction sessions consisted of 10 CS presentations (60 s ISI; first CS presented 1 min after placement in the chamber), and all conditioning and extinction was conducted in context A.

Electrophysiological Recordings

Single-unit recording probes consisted of a bundle of eight tungsten wires (25 μm diameter; 150–250 kΩ) inserted into a 28-gauge stainless steel cannula. Wires were cut to extend approximately 1 mm beyond the tip of the cannula. Differential recordings were made via a recording cable containing an eight-channel operational-amplifier headstage (source-follower configuration) passing neuronal signals from the electrode to a computer via a commutator. Neuronal data were amplified (10,000×) and filtered (0.6–6 kHz) (Neuralynx, Tucson, AZ) prior to acquisition with commercial software (DataWave Technologies, Longmont, CO). These data were acquired in 3 s epochs for each CS trial (0.5 s before, 2 s during, and 0.5 s after each CS presentation).

Drug Infusion

Rats were placed in plastic buckets containing pine shavings. Injection cannulae were inserted into the guide cannulae, and infusions of either vehicle (VEH; 0.1 M PBS [pH 7.4]), D,L-2-amino-5-phosphonovalerate (APV; 10 μg/μl, 0.3 μl/side; Sigma Chemical, St. Louis, MO) or 2% lidocaine (LIDO; 0.5 μl/side; Sigma), were administered using standard methods. Infusions were delivered at a rate of 0.1 μl/min, and 2 min were allowed for diffusion before the injectors were removed. Rats were then immediately transported to the conditioning chambers.

Data Analysis

Single-unit data were analyzed offline using Experimenter's Workbench and Autocut software (DataWave Technologies, Longmont, CO) and NeuroExplorer software (NEX Technologies, Littleton, MA). Single units were extracted from each channel using window discriminators and cluster analysis. Auto- and cross-correlograms were generated to insure that clusters corresponded to distinct single units. For each unit, data were binned (50 ms) and each bin was normalized to the 500 ms pre-CS average for that session, producing a z score for each bin. For each recording session, unit data were summed across trials, generating a peristimulus time histogram (PSTH) for each cell, and the average PSTH of all cells was calculated for each group during each recording session. Cells were excluded if they failed to show CS responsivity (unit activity of at least 3 standard deviations above baseline in either of the first two 50 ms bins after CS onset) in at least one session, or if the CS responsivity exceeded 30 standard deviations. The spontaneous firing rate of each unit was calculated by measuring the number of times the unit fired in the 500 ms period immediately preceding each CS presentation and calculating a single average rate across the 10 trials for each session. We have previously determined that spontaneous firing rates do not vary with successive CS presentations during extinction sessions (K.A.G. and S.M., unpublished observations).

Histology

After the last experimental session, rats were overdosed with sodium pentobarbital, and a weak anodal current (80 μA, 10 s) was passed through the electrode wires to aid in the identification of electrode placements. Rats were then transcardially perfused with 0.9% saline and 10% formalin. The brains were postfixed in 10% formalin/30% sucrose for at least 48 hr. Coronal brain sections (55 μm) were cut on a cryostat, and sections were stained with thionin to visualize cell bodies, electrode tracks, and cannula tracks.

Acknowledgments

This research was supported by grants from the NIMH (R29MH57865; R01MH065961) to S.M. and a Howard Hughes predoctoral fellowship to K.A.G.

Received: June 10, 2003
Revised: August 28, 2003
Accepted: October 17, 2003
Published: December 3, 2003

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