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Consolidation of Fear Extinction Requires NMDA Receptor-Dependent Bursting in the Ventromedial Prefrontal Cortex

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SUMMARY

Extinction of conditioned fear is an active learning process requiring N-methyl-D-aspartate receptors (NMDARs), but the timing, location, and neural mechanisms of NMDAR-mediated processing in extinction are a matter of debate. Here we show that infusion of the NMDAR antagonist CPP into the ventromedial prefrontal cortex (vmPFC) prior to, or immediately after, extinction training impaired 24 hr recall of extinction. These findings indicate that consolidation of extinction requires posttraining activation of NMDARs within the vmPFC. Using multichannel unit recording, we observed that CPP selectively reduced burst firing in vmPFC neurons, suggesting that bursting in vmPFC is necessary for consolidation of extinction. In support of this, we found that the degree of bursting in infralimbic vmPFC neurons shortly after extinction predicted subsequent recall of extinction. We suggest that NMDAR-dependent bursting in the infralimbic vmPFC initiates calcium-dependent molecular cascades that stabilize extinction memory, thereby allowing for successful recall of extinction.

INTRODUCTION

One way that organisms learn to predict danger in their environments is through classical fear conditioning, in which cues paired with aversive outcomes come to elicit species-typical fear responses. Conditioned fear learning facilitates detection and avoidance of environmental threats and is dependent on the amygdala (Davis and Whalen, 2001; LeDoux, 2000; Maren and Quirk, 2004; Paré et al., 2004). When conditioned cues no longer predict danger, fear responses to the cues extinguish, permitting behavioral flexibility. Because extinguished fear spontaneously recovers with the passage of time, extinction is thought to involve new learning rather than erasure of fear memories (Bouton et al., 2006; Myers and Davis, 2007; Quirk, 2002). There has been considerable recent interest in the neural mechanisms of extinction learning, given the use of extinction-based therapies for treatment of anxiety disorders (Davis, 2002; Hofmann et al., 2006; Ressler et al., 2004).

Strong support for the "extinction as new learning" hypothesis comes from the dependence of extinction on NMDARs (Baker and Azorlosa, 1996; Falls et al., 1992; Walker and Davis, 2002), which trigger calcium-dependent molecular cascades necessary for plasticity (Bliss and Collingridge, 1993; Riedel et al., 2003). Previous studies have shown that rats given systemic injections of the NMDAR competitive antagonist CPP are able to extinguish normally during an extinction training session, but are unable to recall extinction the following day (Santini et al., 2001; Suzuki et al., 2004). One interpretation of these findings is that NMDARs are required for extinction consolidation. This implies that posttraining blockade of NMDARs would also impair extinction consolidation, though this hypothesis has yet to be formally tested for extinction of conditioned fear.

The site of NMDAR-dependent processes in consolidation of extinction is also a matter of debate. Previous studies showed that blockade of NMDARs in the basolateral amygdala (BLA) interferes with extinction (Falls et al., 1992; Lin et al., 2003), but these studies were unable to distinguish between acquisition and consolidation processes. Studies employing lesion, inactivation, and microinfusion techniques have suggested that the ventromedial prefrontal cortex (vmPFC) is a site of extinction consolidation (Lebron et al., 2004; Morgan et al., 1993; Quirk et al., 2000; Santini et al., 2004; Sierra-Mercado et al., 2006). We therefore examined the possible involvement of prefrontal NMDARs in consolidation of extinction by infusing CPP into vmPFC at various time points before and after extinction of auditory fear conditioning and assessed the ability of rats to recall extinction 24 hr after training.

How NMDARs might facilitate consolidation of extinction is also unclear. Previous studies have shown that

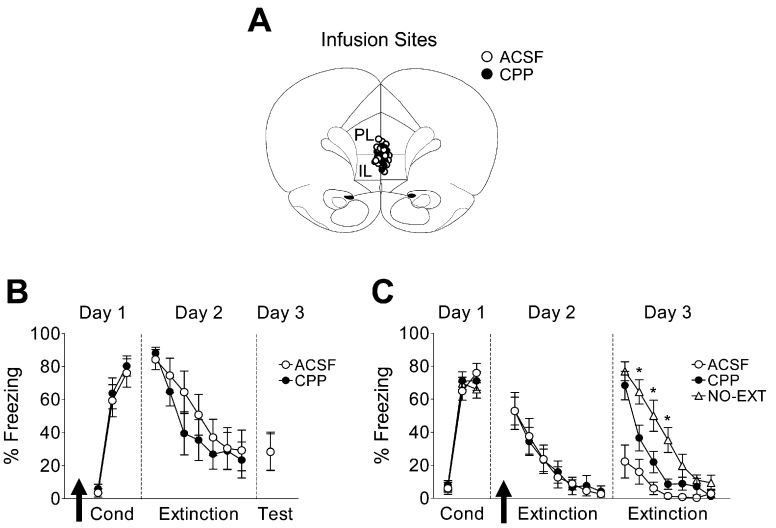

Figure 1. NMDAR Activity in the vmPFC Is Required for Extinction, but Not Acquisition, of Conditioned Fear

(A) Coronal drawing showing location of injector tips aimed at the vmPFC (bregma + 3.20 mm; adapted from Paxinos and Watson, 1998). Rats received an infusion of either artificial cerebrospinal fluid (ACSF) or 60 ng of the NMDAR antagonist CPP.

(B) Rats were infused with CPP (n = 10) or ACSF (n = 10) prior to fear conditioning. CPP-infused rats showed normal acquisition and long-term retention of conditioned freezing. Subsequent extinction was also unaffected by CPP.

(C) Rats were infused with either CPP (n = 12) or ACSF (n = 11) prior to extinction training. No-extinction controls (NO-EXT, n = 12) were infused with ACSF, but were not extinguished on day 2. CPP-infused rats extinguished normally, but showed high freezing on day 3, similar to NO-EXT controls. However, CPP-infused rats showed faster re-extinction than NO-EXT rats. *All p values < 0.05. In this and subsequent figures, behavioral data are plotted in blocks of two trials.

PL, prelimbic area; IL, infralimbic area. Error bars indicate SEM in this and subsequent figures.

NMDARs are necessary for bursting in prefrontal neurons (Jackson et al., 2004; Shi and Zhang, 2003), and it has been suggested that bursting generates synchrony necessary for synaptic plasticity (Buzsaki et al., 2002). However, a direct link between NMDAR-mediated bursting and extinction consolidation has yet to be examined. We therefore used multichannel unit recording to directly assess the effects of both NMDARs (using CPP injections) and extinction training on bursting in vmPFC neurons.

RESULTS

NMDARs in vmPFC Are Required for Extinction, but Not for Acquisition of Conditioned Fear

Rats were chronically implanted with midline cannulae aimed at the vmPFC as previously described (Santini et al., 2004). Figure 1A shows infusion cannula placements in vmPFC. We first examined whether NMDAR-dependent plasticity in the vmPFC is necessary for acquisition of fear conditioning. Rats were infused with CPP or artificial cerebrospinal fluid (ACSF) prior to conditioning. As shown in Figure 1B, CPP had no effect on acquisition of conditioned freezing. Freezing levels in the last trial block of conditioning were 80% and 76% for CPP and ACSF groups, respectively ($t_{(18)}$ = 0.41, p = 0.69). Twenty-four-hour retention of conditioning was also normal in CPP-infused rats. Freezing in the first trial block of extinction was 88% and 84% for CPP and ACSF groups ($t_{(18)}$ = 0.30, p = 0.59). Thus, consistent with lesion and inactivation studies (Corcoran and Quirk, 2007; Lebron et al., 2004; Quirk et al., 2000; Sierra-Mercado et al., 2006), NMDAR-mediated plasticity in the vmPFC is not necessary for acquisition of auditory fear conditioning.

We next examined whether NMDAR-dependent plasticity in the vmPFC is required for extinction. Rats were conditioned on day 1, drug-free. On day 2, they were infused with CPP prior to 15 extinction tones. On day 3, additional tones were given to test for recall of extinction. Three experimental groups were examined: rats infused with CPP, rats infused with ACSF, and rats infused with ACSF but not given extinction training on day 2 (NO-EXT control). As previously observed (Santini et al., 2004), intra-vmPFC infusions reduced the expression of conditioned fear somewhat throughout extinction training; however, CPP did not impair the expression of conditioned fear relative to ACSF. CPP also did not impair within-session extinction. The following day, however, CPP-infused rats showed high levels of freezing similar to NO-EXT controls, indicating poor recall of extinction memory (see Figure 1C). Freezing levels in the first trial block of day 3 were 68%, 22%, and 77% for CPP, ACSF, and NO-EXT groups, respectively. One-way ANOVA of freezing during the first trial block revealed an effect of group ($F_{(2,32)}$ = 12.43, p < 0.001). Post hoc analysis confirmed that CPP-infused rats exhibited significantly higher freezing than ACSF-infused rats (p = 0.001) and were not significantly different from NO-EXT controls (p = 0.73). We also measured spontaneous recovery of freezing on day 3 to facilitate comparisons across groups (for calculation of spontaneous recovery, see Experimental Procedures). Spontaneous recovery values in the first trial block were 92%, 32%, and 130% for CPP, ACSF, and NO-EXT groups, respectively. One-way ANOVA revealed an effect of group ($F_{(2,32)}$ = 15.05, p < 0.001), and post hoc

analysis confirmed that CPP-infused rats exhibited significantly higher recovery than ACSF-infused rats ($p = 0.006$) and were not significantly different from NO-EXT controls ($p = 0.09$). Thus, similar to systemic CPP (Santini et al., 2001), infusion of CPP into the vmPFC impaired recall of extinction without affecting the initial acquisition of extinction.

Despite impaired recall of extinction, CPP-infused rats showed rapid re-extinction (see Figure 1C). A repeated-measures ANOVA of the CPP and NO-EXT groups on day 3 revealed main effects of group ($F_{(1,22)} = 5.72$, $p = 0.026$), trial block ($F_{(6,132)} = 67.84$, $p < 0.001$), and a group × block interaction ($F_{(6,132)} = 3.21$, $p = 0.006$). Post hoc analysis confirmed that CPP-infused rats exhibited significantly less freezing than NO-EXT controls on trial blocks 2, 3, and 4 (all p values < 0.05). Rapid re-extinction suggests that CPP-infused rats had a latent memory of extinction that they were unable to recall at the start of the session.

Posttraining Activation of vmPFC NMDARs Is Required for Consolidation of Extinction

To determine whether NMDAR activity *after* extinction is necessary for consolidation of extinction, rats were conditioned and extinguished drug-free, and then infused with CPP or ACSF either 0, 2, or 4 hr after extinction training (see experimental design in Figure 2B). Twenty-four hours after each infusion, rats were given additional extinction tones to test for recall of extinction, and the percent spontaneous recovery of freezing was assessed. Rats infused with CPP immediately after extinction training (but not 2 or 4 hr after) showed impaired recall of extinction at test (see Figure 2C and Figure S1 in the Supplemental Data available online). Freezing levels in the first trial block were 46% and 9% for CPP and ACSF groups, respectively ($t_{(15)} = 2.90$, $p = 0.011$), corresponding to spontaneous recovery values of 64% and 14%, respectively. Analysis of recovery values at all time points revealed a significant effect of CPP infusions given pretraining ($t_{(21)} = 3.56$, $p = 0.002$) and immediately posttraining ($t_{(15)} = 3.01$, $p = 0.009$), but not at 2 hr ($t_{(15)} = 0.80$, $p = 0.44$) or 4 hr ($t_{(9)} = 1.11$, $p = 0.29$) posttraining. Note that the effect of immediate posttraining infusions was not as large as that seen with pretraining infusions. Thus, NMDAR-dependent processes necessary for consolidation of extinction likely initiate during the extinction session and continue for up to 2 hr after training.

Blockade of NMDARs Reduces Bursting in the vmPFC without Altering Firing Rate

Given that the effects of systemic CPP on extinction (Santini et al., 2004; Suzuki et al., 2004) were replicated with intra-vmPFC infusions of CPP in the posttraining period, we hypothesized that systemic CPP might alter the spontaneous firing properties of vmPFC neurons. To investigate this possibility, rats were chronically implanted with movable microelectrodes in the medial prefrontal cortex to monitor the activity of single neurons. Unit recording

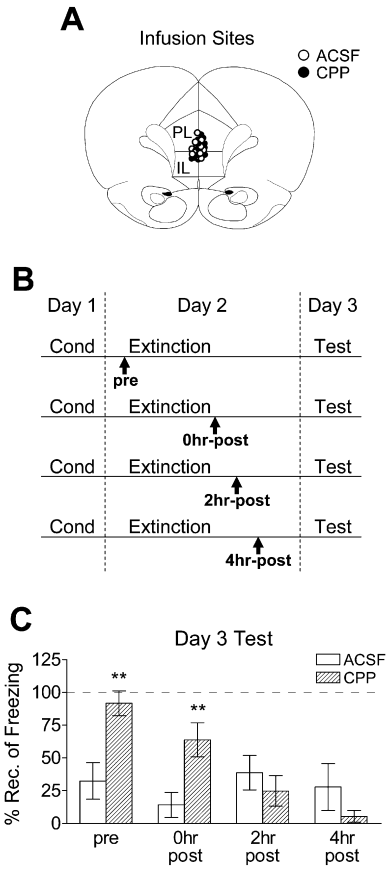

Figure 2. Consolidation of Extinction Requires Posttraining NMDAR Activity in the vmPFC

(A) Coronal drawing showing infusion sites.

(B) Summary of experimental procedures. Rats were infused prior to extinction (pre; see Figure 1) or at one of the following postextinction time points: immediately after (0hr-post, ACSF: n = 8, CPP: n = 9), 2 hr after (2hr-post, ACSF: n = 8, CPP: n = 9), or 4 hr after (4hr-post, ACSF: n = 6, CPP: n = 5).

(C) Exactly 24 hr after infusion, rats were tested for extinction memory by assessing the percent recovery of freezing. Rats infused with CPP prior to, or immediately after, extinction training showed significantly higher spontaneous recovery of freezing than ACSF-infused rats, indicating poor recall of extinction. **All p values < 0.01. CPP infusions at 2 or 4 hr after extinction training had no effect. Dashed line at 100% indicates return of conditioned freezing to pre-extinction levels.

sites are shown in Figure 3A. Following recovery from surgery, rats were injected with saline or 10 mg/kg of CPP, the same dose that impaired recall of extinction in this task (Santini et al., 2001). Spontaneous activity of single neurons was collected prior to and 60 min after injection of both saline and CPP.

A total of 96 neurons from 9 rats were maintained across all four recording sessions (pre-sal, post-sal, pre-CPP, and post-CPP). Average firing rates were similar before and after treatment with CPP (see Figure 3B). Mean rates were 3.3 Hz and 3.2 Hz for pre-CPP and post-CPP sessions, respectively. Nonparametric analysis revealed no

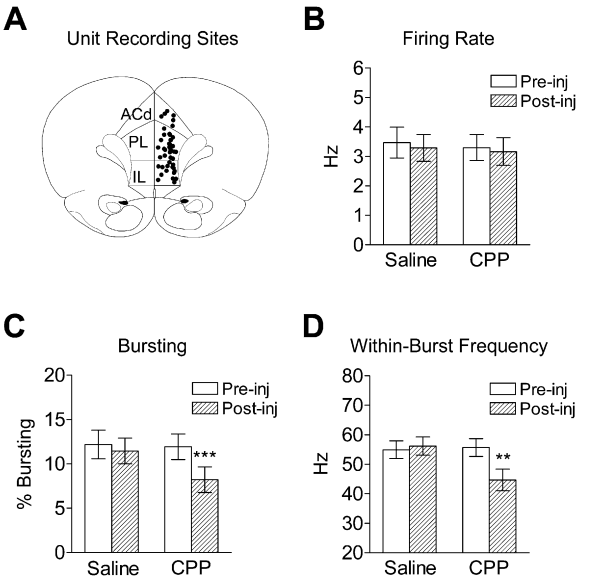

Figure 3. Blockade of NMDARs Reduces Bursting in vmPFC, with No Change in Firing Rate

(A) Coronal drawing showing recording sites. Spontaneous activity from single prefrontal neurons (n = 96) was recorded prior to, and 1 hr after, an i.p. injection of saline or 10 mg/kg of CPP, in freely moving rats.

(B) Average firing rate was unchanged by CPP.

(C) The percentage of spikes occurring within bursts was significantly reduced by CPP. ***$p < 0.001$.

(D) CPP also significantly reduced the within-burst frequency. **$p < 0.01$.

ACd, dorsal anterior cingulate area.

significant difference between pre-CPP and post-CPP firing rates (Wilcoxon matched-pairs test: $Z = 1.15$, $p = 0.25$). To investigate bursting, we used a definition of bursting based on previously reported firing properties of prefrontal neurons (Shi and Zhang, 2003): three or more spikes exhibiting interspike intervals (ISIs) less than 25 ms (first interval) or 50 ms (subsequent intervals). CPP significantly reduced the percentage of spikes occurring within bursts (see Figure 3C). Bursting values were 11.9%, and 8.2% for pre-CPP and post-CPP sessions, respectively. The difference between pre-CPP and CPP was highly significant ($t_{(94)} = 4.10$, $p < 0.001$, paired t test, Bonferroni corrected). The within-burst frequency was also reduced significantly by CPP (see Figure 3D; pre-CPP versus post-CPP, $t_{(94)} = 3.31$, $p < 0.01$, Bonferroni corrected).

A reduction in bursting, with no change in average firing rate, suggests that CPP selectively reduced the occurrence of short, but not long, ISIs in medial prefrontal cortex neurons. The selective effect of CPP on short ISIs is apparent in Figure 4A, which shows representative 6 s epochs of spike trains recorded before and after administration of CPP. An ISI histogram for this cell (see Figure 4B) confirmed that there was a reduction in short (<40 ms) but not long (e.g., 100 ms) intervals. A group ISI histogram shown in Figure 4C confirmed the loss of short intervals, with significant reductions in bins 10–20 ms ($t_{(94)} = 5.63$, $p < 0.001$) and 20–30 ms ($t_{(94)} = 3.67$, $p < 0.01$) (paired t test, Bonferroni corrected). The responses of individual neurons to injections of saline and CPP are shown in scatter plots in Figures 4D and 4E, respectively. For CPP, the majority of cells fell below the 45° line, indicating a reduction in short intervals by CPP ($p = 0.009$; Fisher exact test comparing saline versus CPP). Reductions in short ISIs following CPP occurred in all three subregions of the medial prefrontal cortex (ACd, dorsal anterior cingulate; PL, prelimbic; and IL, infralimbic; see Figure S2 for examples).

To determine if the effects of CPP on extinction and bursting were correlated, we compared the dose of CPP used above (10 mg/kg) to a lower dose (5 mg/kg) shown to impair hippocampal long-term potentiation (Wayner et al., 2000) and water-maze learning (Gureviciene et al., 2003). Replicating our previous observation (Santini et al., 2001), injection of 10 mg/kg of CPP prior to extinction training significantly impaired recall of extinction the following day (see Figure 5A). Spontaneous recovery levels were 90% for 10 mg/kg CPP and 32% for saline rats ($t_{(17)} = 2.32$, $p = 0.033$). In contrast, 5 mg/kg of CPP had little effect on recall of extinction. Spontaneous recovery of freezing was 51% for 5 mg/kg CPP and 37% for saline-treated rats. This difference was not significant ($t_{(27)} = 0.72$, $p = 0.48$). Paralleling these behavioral findings, 5 mg/kg of CPP had no significant effect on bursting in vmPFC neurons (pre-CPP, 11.9%; post-CPP, 10.4%; $t_{(42)} = 1.81$, $p = 0.08$) (see Figure 5B). Within-burst frequency also showed no significant differences (pre-CPP, 40 Hz; post-CPP, 36 Hz; $t_{(42)} = 1.00$, $p = 0.32$). Thus, the effects of CPP on extinction and bursting were correlated across two CPP doses, consistent with the hypothesis that NMDAR-mediated bursting in vmPFC is necessary for consolidation of extinction.

Postextinction Bursting in the Infralimbic vmPFC Predicts Subsequent Recall of Extinction

Our findings thus far indicate that NMDARs in the vmPFC are necessary for both burst firing and consolidation of extinction. But are the two linked? To address this, rats implanted with unit-recording electrodes in the vmPFC were fear conditioned and extinguished (see Figure 6B1). Twenty-four hours later, when rats were tested for recall of extinction, there was a bimodal distribution of freezing (see Figure 6B2). Of 15 rats tested, 9 rats showed good recall of extinction (Low Fear group, <40% freezing) and 6 rats showed poor recall of extinction (High Fear group, >60% freezing) ($F_{(1,13)} = 63.48$, $p < 0.001$, repeated-measures ANOVA). This replicates prior studies in which untreated rats show a large variability in extinction recall (Herry and Garcia, 2002; Milad and Quirk, 2002).

A total of 70 neurons in the vmPFC were recorded at four time points: immediately prior to extinction and 30 min, 1 hr, and 2 hr following extinction. There were no significant differences between low and high fear groups in the rate of bursting at any of these time points ($F_{(1,68)} = 2.58$, $p = 0.11$). However, separating infralimbic (IL) and prelimbic (PL) subregions of vmPFC revealed

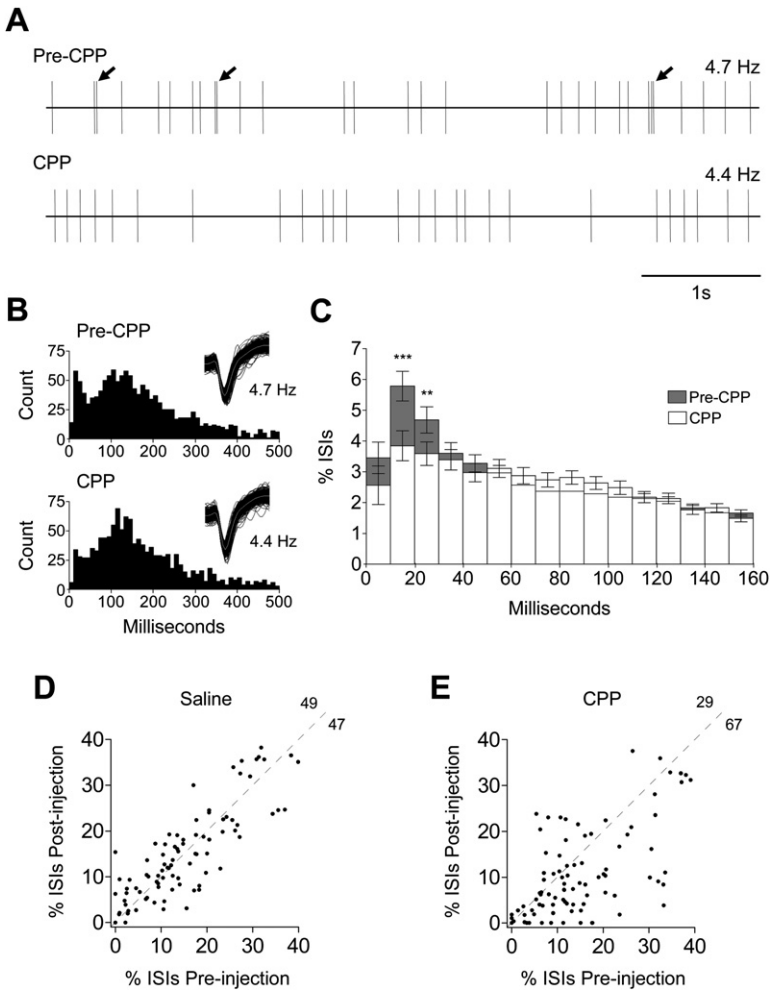

Figure 4. Blockade of NMDARs Selectively Reduces the Incidence of Short Interspike Intervals in vmPFC

(A) Sample 6 s epochs of spike trains of a representative vmPFC neuron before and after injection of 10 mg/kg of CPP. Note the absence of short intervals (arrows) with CPP.

(B) Interspike interval (ISI) distributions and waveforms of the neuron shown in (A). CPP reduced the early peak consisting of the shortest intervals (<40 ms; bin width = 10 ms).

(C) Normalized population ISI histograms show significant reduction in short intervals by CPP. ***p < 0.001, **p < 0.01, t test, Bonferroni corrected.

(D and E) Scatter plots show the percent short ISIs (<40 ms) for each cell before and after injections. The proportion of cells that decreased short ISIs after injection was significantly higher with CPP than with saline (Fisher exact, p = 0.009).

significant differences (see Figures 6C and 6D). In IL, low fear rats showed significantly more bursting than high fear rats at each of the three postextinction time points. Repeated-measures ANOVA of the IL data showed a significant effect of group ($F_{(1,28)} = 4.40$, $p = 0.045$) with post hoc tests confirming significant differences at all postextinction time points (all p values < 0.01). In contrast, bursting in PL neurons did not differ significantly between groups ($F_{(1,38)} = 0.25$, $p = 0.62$). The same pattern of results was evident in average firing rate data. The low fear group had higher average firing rate in IL than the high fear group at postextinction time points 30 min and 2 hr (Kruskal-Wallis ANOVA: all p values < 0.05), but no significant differences prior to extinction (p = 0.15). No significant differences were observed in PL firing rates. Thus, complementing the CPP findings above, rats showing impaired bursting in the IL-vmPFC were unable to consolidate extinction learning.

DISCUSSION

We used infusion and unit-recording techniques to determine the role of prefrontal NMDARs in consolidation of fear extinction. We showed that (1) pretraining blockade of NMDARs in vmPFC impaired 24 hr recall of extinction, leaving the initial acquisition of extinction intact; (2) posttraining blockade of NMDARs in vmPFC also impaired recall of extinction, implicating prefrontal NMDARs in consolidation of extinction; (3) systemic blockade of NMDARs selectively reduced high-frequency bursting in vmPFC neurons; and (4) the degree of bursting in infralimbic vmPFC neurons after extinction training predicted subsequent recall of extinction.

Previous studies using systemically administered CPP showed that NMDARs are not necessary for within-session extinction, but are necessary for long-term retention of extinction (Santini et al., 2001; Suzuki et al., 2004). These studies, however, did not determine the location of NMDAR-dependent consolidation processes. Our findings indicate that the vmPFC is a critical site of NMDAR-dependent consolidation of extinction. CPP infused into vmPFC prior to, or immediately after, extinction training impaired 24 hr recall of extinction. Infusions made 2 or 4 hr after training had no effect. Thus, 2 hr of postextinction NMDAR-dependent processing in the vmPFC is sufficient to consolidate extinction. NMDAR-mediated

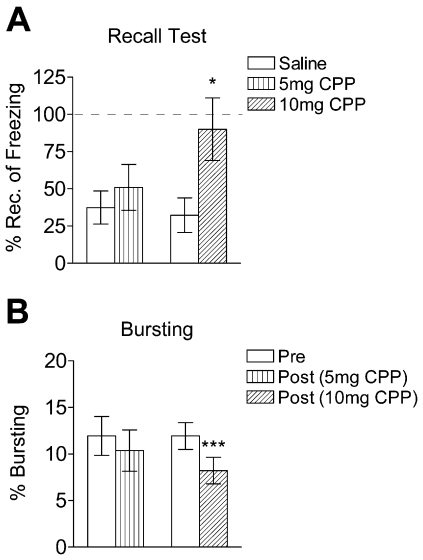

Figure 5. The Effects of CPP on Extinction and vmPFC Bursting Are Correlated across Doses

(A) Rats were injected i.p. with either saline or CPP (5 or 10 mg/kg) prior to extinction training and tested for extinction memory the next day by assessing the percent recovery of freezing. Rats injected with 10 mg/kg of CPP showed significantly higher spontaneous recovery of freezing than saline-injected rats, indicating poor recall of extinction (CPP, n = 10; Saline, n = 9). *p = 0.033. In contrast, rats injected with 5 mg/kg of CPP showed low levels of spontaneous recovery, indicating good recall of extinction (CPP, n = 15; Saline, n = 14).

(B) Spontaneous activity from 43 vmPFC neurons was recorded before and after rats received 5 mg/kg of CPP. As with extinction behavior, 5 mg/kg of CPP did not reduce the percentage of spikes occurring within bursts. (10 mg/kg bursting data from Figure 3C are plotted for comparison purposes.)

calcium influx is thought to trigger molecular cascades, such as the mitogen-activated protein kinase (MAPK) signaling pathway, to stabilize recently acquired memory (Bliss and Collingridge, 1993; Elgersma and Silva, 1999; Lamprecht and LeDoux, 2004). Consistent with this, blockade of MAPK (Hugues et al., 2004) or protein synthesis (Santini et al., 2004) in the vmPFC prevents consolidation of extinction.

Despite impaired consolidation of extinction, CPP-infused rats showed savings in their rate of re-extinction, suggesting that structures other than the vmPFC also learn extinction. Indeed, previous studies demonstrated that plasticity in the basolateral amygdala (BLA) is necessary for extinction. Blockade of NMDARs, protein kinases, and protein synthesis in the BLA impairs extinction (Falls et al., 1992; Lin et al., 2003); however, these studies did not distinguish between extinction acquisition and consolidation. More recent investigation revealed that the BLA mediates the initial acquisition of extinction, via NMDARs and MAPK (Herry et al., 2006; Sotres-Bayon et al., 2007). In contrast to BLA, plasticity in vmPFC is necessary for the recall of previously learned extinction. Lesions, inactivation, or inhibition of protein synthesis in the vmPFC do not affect extinction within a session, but prevent recall of extinction the following day (Lebron et al., 2004; Morgan et al., 1993; Quirk et al., 2000; Santini et al., 2004; Sierra-Mercado et al., 2006). Thus, as recently suggested, the vmPFC may work together with the BLA and other structures to consolidate and later express extinction memory (Barrett et al., 2003; Berlau and McGaugh, 2006; Sotres-Bayon et al., 2006).

We observed that NMDAR blockade with CPP selectively reduced bursting in vmPFC neurons. Because CPP was administered systemically, it is possible that reduced bursting in vmPFC was due to an effect on structures afferent to vmPFC. Nonetheless, we favor the idea that CPP acted directly on vmPFC neurons because CPP infused into vmPFC impaired consolidation of extinction, and because previous in vitro studies using prefrontal slices reported that NMDAR agonists and antagonists increased and decreased bursting, respectively (Shi and Zhang, 2003; Zhang and Shi, 1999).

How might NMDAR-mediated bursting in vmPFC neurons facilitate extinction-related plasticity? Bursting is believed to promote synaptic plasticity by providing synchronized activity of inputs (Buzsaki et al., 2002). It has been suggested that strong inputs generate bursts that can back-propagate through the dendritic compartment, triggering calcium entry through NMDARs and voltage-gated calcium channels (Harris et al., 2001; Paulsen and Sejnowski, 2000). Burst-related calcium currents generated by strong inputs are thought to sum with smaller calcium currents triggered by weak inputs (Blair et al., 2001; Gold and Bear, 1994). In this way, strong "teaching" inputs could cause Hebbian strengthening in weak inputs with which they are coactive (Magee and Johnston, 1997; Pike et al., 1999). Such strengthening could account for extinction-related plasticity that has been previously observed in the vmPFC (Barrett et al., 2003; Herry and Garcia, 2002; Milad and Quirk, 2002).

Several lines of evidence suggest that the hippocampus might supply a teaching input to vmPFC. Recall of extinction is context dependent (Bouton et al., 2006) and requires hippocampal processing during extinction training (Corcoran et al., 2005). Stimulation of the hippocampus induces bursting in vmPFC (Tierney et al., 2004) and modulates the response of vmPFC neurons to BLA inputs (Ishikawa and Nakamura, 2003). In the present study, successful recall of extinction was correlated with the degree of IL bursting following extinction training, and a recent report showed potentiation of hippocampally evoked potentials in IL during the same postextinction period (Hugues et al., 2006). Consolidation of extinction, therefore, may involve hippocampus-dependent potentiation in the IL-vmPFC. Experiments are underway to determine if postextinction bursting in IL-vmPFC requires hippocampal input.

Understanding the neural mechanisms of fear extinction could lead to improved methods for treatment of pathological forms of fear such as phobias and posttraumatic stress disorder, which are treated with extinction-based exposure therapies (Foa, 2000; Rothbaum and

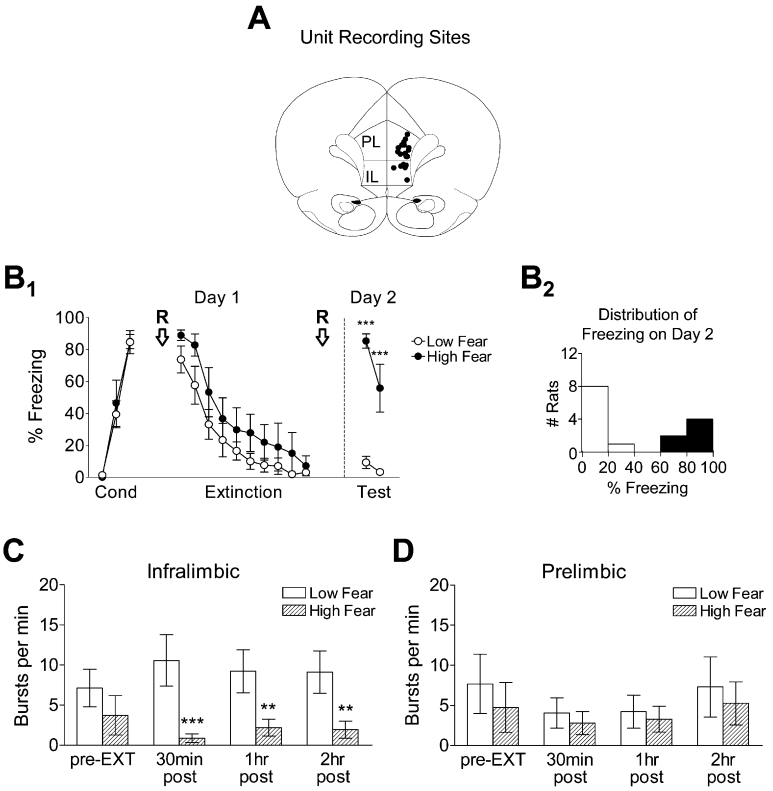

Figure 6. Bursting in the Infralimbic vmPFC Predicts Subsequent Recall of Extinction

(A) Coronal drawing showing placement of unit-recording electrodes in infralimbic (IL) and prelimbic (PL) subregions of vmPFC.

(B) Rats were conditioned and extinguished and tested for extinction recall the following day. Rats were divided into two groups based on the degree of extinction recall (B_2): those showing good extinction recall (Low Fear, <40% freezing, n = 9) and those showing poor extinction recall (High Fear, >60% freezing, n = 6). ***$p < 0.001$.

(C) IL neurons exhibited significantly more bursting in low fear rats compared to high fear rats when recorded ("R") at each of three postextinction time points (30 min, 1 hr, and 2 hr) (Low Fear, n = 18; High Fear, n = 12). ***$p < 0.001$, **$p < 0.01$.

(D) Bursting in PL neurons did not differ between groups (Low Fear, n = 25; High Fear, n = 15).

Davis, 2003). Consistent with the NMDAR dependence of extinction, the NMDAR partial agonist d-cycloserine (DCS) has been recently used as an adjunct to exposure therapy for acrophobia (Ressler et al., 2004) and social anxiety (Hofmann et al., 2006). The posttraining involvement of NMDARs in consolidation of extinction observed in the present study supports the idea that DCS could be given immediately following exposure sessions and still be effective (Richardson et al., 2004). Though DCS is thought to act in the amygdala (Davis et al., 2006), our findings suggest that DCS may also act in the vmPFC to facilitate consolidation of extinction. Recent functional imaging studies in humans implicate the vmPFC in recall of extinction (Kalisch et al., 2006; Milad et al., 2006; Phelps et al., 2004). A testable prediction from our study is that DCS given shortly after extinction-based therapy would activate vmPFC, thereby improving the patients' ability to consolidate and recall extinction.

EXPERIMENTAL PROCEDURES

Subjects

All procedures were approved by the Institutional Animal Care and Use Committee of the Ponce School of Medicine in compliance with the NIH guidelines for the care and use of laboratory animals (Publication number DHHS NIH 86-23). Male Sprague-Dawley rats weighing 270–330 g were housed and handled as described previously (Quirk et al., 2000). Food was restricted to 18 g/d of standard laboratory rat chow until rats reached 85% of their free-feeding weight. Rats were then trained to press a bar for food on a variable interval schedule of reinforcement (VI-60) in order to maintain a constant level of activity against which freezing can be reliably measured (Quirk et al., 2000).

Surgery

After bar-press training, rats were implanted with a single 26 gauge stainless-steel guide cannula (Plastics One, Roanoke, VA) in the vmPFC as described previously (Santini et al., 2004). Stereotaxic coordinates were 2.8 mm anterior, 1.0 mm lateral, and 4.1 mm ventral from bregma, with the cannula angled 11° toward the midline in the coronal plane. Another set of rats was implanted with movable arrays of 16 microwires (22 μm; Stablohm 650; California Fine Wire). Before implantation, the tip of each wire was plated with gold by passing a cathodal current of 1 μA while cables were submerged in a gold solution. This procedure reduces impedance of the wires to a range of 250–350 kΩ. Electrodes consisted of two or three wire bundles of different length in order to record simultaneously from the infralimbic (IL), prelimbic (PL), and dorsal anterior cingulate (ACd) subregions of the medial prefrontal cortex. Stereotaxic coordinates were 2.9 mm anterior and 0.6 mm lateral, and the ventral coordinate was 5.0, 4.0, and 2.0 mm ventral from bregma for IL, PL, and ACd, respectively. Rats were allowed 7 days to recover from surgery.

Fear Conditioning

Rats in the behavioral experiments were fear conditioned and extinguished in standard operant chambers (Coulbourn Instruments, Allentown, PA) located inside sound-attenuating boxes (Med Associates, Burlington, VT) in an isolated testing room. Details of the apparatus have been previously described (Quirk et al., 2000). On day 1, rats received five habituation trials (sine wave tones at 4 kHz, 30 s, 75 dB), immediately followed by fear conditioning consisting of seven presentations of the tone that coterminated with footshocks (0.5 s, 0.45 mA). On day 2, rats were returned to the same operant chamber and were given extinction training consisting of tone-alone trials (15 for infusion experiments and 20 for systemic injection experiments). On day 3, rats

were given additional tone-alone trials to test for extinction memory. The interval between successive tones was variable, with an average of 3 min. Food was available on a VI-60 schedule throughout the experiment. Behavior was recorded with digital video cameras (Micro Video Products, Bobcaygeon, Ontario, Canada).

Infusions of CPP

Blockade of NMDARs in the vmPFC was achieved via local infusion of 60 ng in 0.5 μl of the NMDAR antagonist CPP [3-((+/−)2-carboxypiperazin-4yl)propel-1-phosphate; Sigma, St. Louis, MO] dissolved in artificial cerebrospinal fluid (ACSF), and the pH was maintained at 7.40. CPP is particularly well-suited for investigating consolidation processes because of its long-lasting duration of action (Abraham and Mason, 1988). Previous work has shown that the dose of CPP used in the present study, when infused into the vmPFC, does not affect spontaneous locomotion (O'Neill and Liebman, 1987). For the infusions, cannula-dummies were removed from guide cannulas and replaced with 33 gauge stainless-steel injectors, which were connected by polyethylene tubing (PE-20; Small Parts Inc., Miami Lakes, FL) to 10 μl syringes mounted in an infusion pump (Harvard Apparatus, South Natick, MA). Drugs were infused at a rate of 0.1 μl/min for 5 min. Infusions were performed at one of five time points: prior to fear conditioning, prior to extinction training, immediately after extinction, 2 hr after extinction, or 4 hr after extinction training.

Unit Recording

Rats were acclimated to recording procedures, and electrodes were driven in steps of 44 μm until single units were isolated using electrophysiological data acquisition package (Plexon Inc., Dallas, TX). The effect of CPP on the firing properties of neurons from IL, PL, and ACd was assessed by recording spontaneous activity from well-isolated cells. Activity was recorded for 5 min prior to and 1 hr after systemic injection of saline or CPP (5 or 10 mg/kg). After collecting data from this location, electrodes were advanced 176 μm and then advanced in steps of 44 μm to search for additional cells. Each rat was used in up to two experiments (5–7 days between experiments).

The effect of extinction training on the firing properties of PL and IL neurons was assessed in a separate group of rats. In this experiment, rats received fear conditioning (5 tone-shock trials; 0.35 mA footshocks) and extinction (20 tone trials) in a single day (2 hr in between) and were tested for recall of extinction 24 hr later. Spontaneous activity was recorded for 5 min immediately prior to extinction training and at three postextinction time points: 30 min, 1 hr, and 2 hr after training. In both experiments, food was always available in a VI-60 schedule.

Signals exceeding a voltage threshold were digitized at 40 kHz and stored on a PC. Principal component vectors and/or voltages were plotted in three-dimensional space, and clusters formed by single cells were cut using Offline Sorter software (Plexon Inc., Dallas, TX). Data were then imported to NeuroExplorer software (NEX Technologies, Littleton, MA) in which comparisons of interspike interval histograms and cross-correlations were performed to prevent repetition of cells. Firing rate, bursting, burst rate, within-burst frequency, and interspike intervals were analyzed. A burst was defined as three or more consecutive spikes with an interval of less than 25 ms between the first two spikes and less than 50 ms in subsequent spikes.

Histology

At the conclusion of single-unit experiments, a marking lesion was produced at the tip of the wires by passing an anodal current of 25 μA for 18 s. Rats were perfused with 10% buffered formalin, brains were removed, and microlesions were marked with a green reaction (6% ferrocyanide) while fixing the tissue in 30% sucrose/10% buffered formalin. Location of marking lesions and injector tips (from infusion experiments) were reconstructed onto coronal drawings adapted from Paxinos and Watson (1998) from 40 μm Nissl-stained sections.

Data Analysis

Freezing behavior provided a measure of conditional fear. Freezing is defined as the absence of all movements except those related to breathing (Blanchard and Blanchard, 1972) and was quantified during the 30 s tone presentation from digitized videos using commercial software (FreezeScan, Clever Systems, Reston, VA). Data were averaged in blocks of two trials, and spontaneous recovery of freezing at test was calculated as follows: (freezing on first block of trials on day 3)/(freezing on last block of trials on day 1) × 100. Group comparisons were made using either Student's unpaired t tests or repeated-measures analyses of variance (ANOVA) with post hoc Tukey HSD tests (Statistica; StatSoft, Tulsa, OK). These tests were also used to detect extinction-training-induced alterations in the firing properties of vmPFC neurons. Paired t tests with Bonferroni corrections and Fisher exact tests were used to detect CPP-induced alterations in bursting and interspike intervals, and nonparametric Wilcoxon matched-pairs tests and Kruskal-Wallis ANOVAs were used to analyze firing rate data.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/53/6/871/DC1/.

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