Brief Communications

Lack of Medial Prefrontal Cortex Activation Underlies the Immediate Extinction Deficit

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We conducted a series of experiments to investigate the neural basis of the immediate extinction deficit, the lack of extinction when the interval between fear memory acquisition and extinction is short. In experiment 1, rats were given extinction training composed of 15 conditioned stimuli (CSs) either 15 min (immediate extinction: I-EXT) or 24 h (delayed extinction: D-EXT) after five tone–shock pairings. In the retention test performed 48 h after conditioning, I-EXT group exhibited significantly higher freezing than D-EXT group. In experiment 2, functional activation in the medial prefrontal cortex (mPFC) was detected using *c-fos* immunoreactivity. The number of Fos-positive neurons in the mPFC was significantly lower in I-EXT group than in D-EXT group. In experiment 3, rats received immediate extinction with microstimulation of the infralimbic region (IL) of the mPFC, either contingently paired or unpaired with the CS. In a subsequent retention test, the paired stimulation group exhibited decreased freezing relative to the unpaired stimulation group. Together, our results suggest that the immediate extinction deficit may be linked to the lack of neuronal activity in the IL.

Introduction

Due to its enduring nature, modification of traumatic fear memories requires lengthy behavioral therapy and pharmacological intervention. To improve therapeutic efficacy, it is essential to understand the neural basis of fear memory regulation. Pavlovian fear conditioning and extinction has proven to be a useful laboratory model for studying neural regulation of fear responses (LeDoux, 2000; Kim and Jung, 2006). In a typical experiment, subjects initially acquire a fear memory by paired presentations of a neutral conditioned stimulus (CS) and an aversive unconditioned stimulus (US). Fear memory is manifested in the form of a conditioned response (CR), which includes autonomic and behavioral reactions. In a subsequent extinction session, the CS is repeatedly presented without the US, resulting in a reduced rate of fear CRs (Pavlov, 1927). Extensive evidence suggests that this reduction is a consequence of the generation of a separate extinction memory rather than unlearning of the conditioned fear response (Bouton, 2004). Unlike the original fear memory, the extinction memory is easily attenuated by changes in temporal and spatial parameters. For example, the extinction memory can be masked by the fear memory if the extinction context changes or if the interval between extinction and test is long (Bouton and Bolles, 1979; Rescorla, 2004).

Manipulations of the interval between fear acquisition and extinction critically influence the strength of the extinction memory. Recent studies have demonstrated that extinction training administered shortly after the acquisition session does not effectively induce long-term suppression of the fear response (Maren and Chang, 2006; Woods and Bouton, 2008; Chang and Maren, 2009) [but see Myers et al. (2006) and Schiller et al. (2008)]. Several possible causes for this "immediate extinction deficit" (IED) have been suggested. Maren and Chang (2006) proposed that elevated fear levels from the acquisition session may be responsible for IED by demonstrating that IED was attenuated when the level of fear during the acquisition session was minimized. Alternatively, Woods and Bouton (2008) proposed, on the basis of their appetitive learning data, that a minimal delay is required for the acquisition memory to be formed and accessible for the next stage of learning. A similar hypothesis was proposed using an associative learning model with multiple state transitions (Wagner and Brandon, 1989). However, none of these studies have examined the basis of IED at the neuronal level nor determined the brain circuits that underlie this phenomenon.

The medial prefrontal cortex (mPFC) has been implicated in the formation and expression of extinction memory. Rats with damage to the mPFC have difficulty recalling extinction (Morgan et al., 1993; Morgan and LeDoux, 1995; Quirk et al., 2000). In addition, the tone responses of infralimbic cortex (IL) neurons are potentiated during retrieval, and their activity correlates with extinction memory. Moreover, local stimulation of the IL facilitated extinction, confirming a causal relationship (Milad and Quirk, 2002). We therefore reasoned that investigating the pattern of mPFC activation at the time of extinction might provide insights into the underling mechanisms of IED. If mPFC is involved in IED, then immediate extinction should lead to under-activation of the mPFC and failure to generate the neuronal changes necessary for extinction memory. Finally, artificial activation of the mPFC should be respected to revive immediate extinction.

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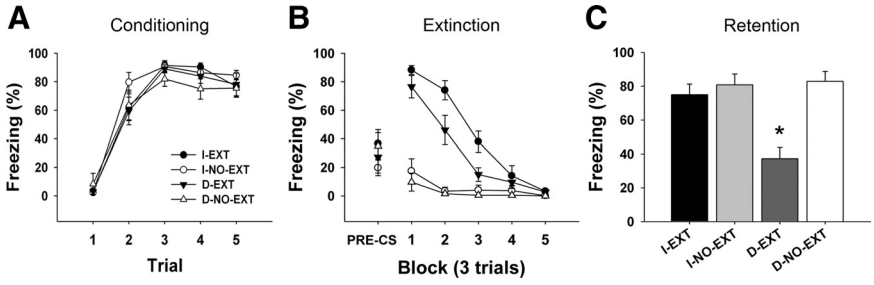

Figure 1. Immediate extinction deficit. ***A***, Percentage of freezing during conditioning. Rats were given five CS–US conditioning trials. All groups exhibited a similar increase in freezing across trials. ***B***, Percentage of freezing during extinction training. The immediate extinction group (I-EXT) and the delayed extinction group (D-EXT) received 15 CS-only trials, 15 min and 24 h after fear conditioning, respectively. Both groups showed similarly attenuated freezing at the end of the extinction training. The immediate no-extinction group (I-NO-EXT) and the delayed no-extinction group (D-NO-EXT) were placed in the extinction context for the same amount of time but without CS presentation at the corresponding time points. All tests were performed in a novel context. ***C***, Averaged percentage of freezing across three test trials. The D-EXT group showed significantly lower freezing relative to the other groups. $*p < 0.05$. All data are presented as means ± SEM.

Materials and Methods

Experiment 1: behavioral data. Rats were subjected to three phases of training: fear conditioning, extinction, and extinction retention test. Rats received five presentations of the CS (2 kHz, 30 s, 80 dB) that coterminated with footshocks (0.5 s, 1 mA) in one context. Either 15 min (I-EXT, $n = 13$) or 24 h (D-EXT, $n = 11$) after conditioning, each group received extinction training in which 15 CSs were presented alone in a different context. Two control groups (I-NO-EXT, $n = 12$ and D-NO-EXT, $n = 10$) were exposed to the extinction context for the same duration of time in the absence of the CS at corresponding time points. All groups were subjected to three tone-alone trials in the extinction context 48 h after conditioning. Details of the fear conditioning procedures are described in the supplemental Methods (available at www.jneurosci.org as supplemental material).

Experiment 2: c-fos immunohistochemistry. Rats were subjected to behavior training procedures as described above, and then killed 90 min after the extinction training. Fos levels were evaluated in I-EXT ($n = 4$) and D-EXT groups ($n = 3$), as well as in four control groups: (1) immediate no extinction, (2) delayed no extinction, (3) immediate unpaired, and (4) delayed unpaired. The unpaired control groups (I-UNPAIRED, $n = 3$ and D-UNPAIRED, $n = 3$) received tones and shocks in an unpaired manner during the conditioning phase and were returned to the extinction context for 15 extinction trials at corresponding time points. The no-extinction groups (I-NO-EXT, $n = 3$ and D-NO-EXT, $n = 4$) were placed in the extinction context for the same amount of time but were not exposed to the CS at corresponding time points.

Fos levels were quantitatively analyzed in the IL, prelimbic cortex (PL), and secondary motor cortex (M2). All procedures for examining Fos expression were similar to those previously described (Jo et al., 2007). Briefly, two images (200×200 μm) per brain region were selected for cell counting. All cell nuclei were identified by 4′,6′-diamidino-2-phenylindole (DAPI) staining (blue), and Fos expression was detected using a rabbit anti-*c-fos* antibody (1:1000; Calbiochem) and a cyanine 3 (CY3) goat anti-rabbit IgG (1:1000; Invitrogen) (red). A neuron with Fos expression in its nucleus was considered a Fos-immunoreactive (Fos-IR) cell. The number of Fos-IR cells in each image was counted blindly, and the ratio of Fos-IR neurons to DAPI-labeled cells per image was calculated.

Experiment 3: artificial mPFC activation. To stimulate neuronal activity of the IL, a bipolar stimulating electrode (Plastics One) was implanted in the IL (0.125 mm; impedance, 15–30 kΩ) (see supplemental Methods, available at www.jneurosci.org). Rats were allowed to recover for 7 d and were then subjected to fear conditioning as described above. After conditioning, rats received electrical stimulation of the IL during immediate extinction training. The stimulation consisted of a 300 ms train of square pulses (0.2 ms pulse width, 100 Hz) produced by a pulse generator (MASTER-8, A.M.P.I), and was delivered through a stimulus isolator (A360R-C, World Precision Instruments) to maintain the current at 100 μA. The paired-stimulation group (I-PAIRED; $n = 21$) received IL stimulation 100 ms after CS onset. In addition to I-PAIRED group, six control groups were included; unpaired-stimulation groups (immediate or delayed), no-stimulation groups (immediate or delayed), stimulation-only group (immediate), and M2-stimulation group (immediate). The unpaired-stimulation groups randomly received explicitly unpaired tones and IL stimulation during the extinction training either 15 min (I-UNPAIRED; $n = 16$) or 24 h (D-UNPAIRED; $n = 11$) after conditioning. The no-stimulation groups (I-NO-STIM, $n = 14$ and D-NO-STIM, $n = 17$) were subjected to extinction training at the corresponding time points but did not receive IL stimulation, while the stimulation-only group (I-STIM; $n = 9$) received IL stimulation without CS presentation during immediate extinction training. As an anatomical control, the M2-stimulation group (M2-STIM; $n = 12$) received M2 stimulation paired with the CS during immediate extinction training.

Results

Immediate extinction deficit

To compare the effects of immediate and delayed extinction on long-term extinction memory without interference from baseline fear, we employed an ABB design using one context for acquisition and the other for extinction and extinction retention. All groups exhibited robust freezing at the last conditioning trial ($F_{(3,42)} = 0.401$, $p = 0.753$) (Fig. 1*A*). For extinction training, all rats showed similarly low freezing during the 5 min baseline period ($F_{(3,42)} = 0.750$, $p = 0.529$). At the beginning of extinction training, the I-EXT and D-EXT groups showed robust freezing which declined gradually, whereas the I-NO-EXT and D-NO-EXT groups maintained low freezing throughout the session (Fig. 1*B*). ANOVA revealed that there were main effects for group ($F_{(3,42)} = 46.312$, $p < 0.01$) and trial block ($F_{(4,168)} = 59.934$, $p < 0.01$), as well as a group × trial block interaction ($F_{(12,168)} = 13.032$, $p < 0.01$). *Post hoc* analysis confirmed that extinction groups froze significantly more than the no-extinction groups (all $p < 0.01$). In addition, the I-EXT group showed higher freezing than the D-EXT group ($p < 0.01$). A direct comparison between I-EXT and D-EXT groups revealed that the I-EXT group froze significantly more than the D-EXT group in the second and third trial block (all $p < 0.05$). However, rats in both groups showed equally attenuated freezing by the end of the extinction training ($p = 0.703$), indicating that loss of freezing within the extinction session was equivalent in the two groups regardless of the postacquisition interval.

Despite an equivalent reduction in freezing during the extinction session, the two extinction groups differed in the retention test. ANOVA showed a main effect for group ($F_{(3,45)} = 10.805$, $p < 0.01$), and *post hoc* analysis confirmed that the D-EXT group froze significantly less than the other groups (all $p < 0.01$) (Fig. 1*C*). However, the I-EXT group showed a high freezing level, which was similar to the no-extinction groups (all $p > 0.05$).

It is possible that the higher level of freezing in the I-EXT group was due to slower extinction during the preceding session. To exclude this possibility, we matched the freezing levels between the two extinction groups (I-EXT = 13, D-EXT = 7) by selecting a subset of rats (freezing level of 30% or greater during the second block of the extinction session). ANOVA revealed that there was no difference in freezing levels during extinction train-

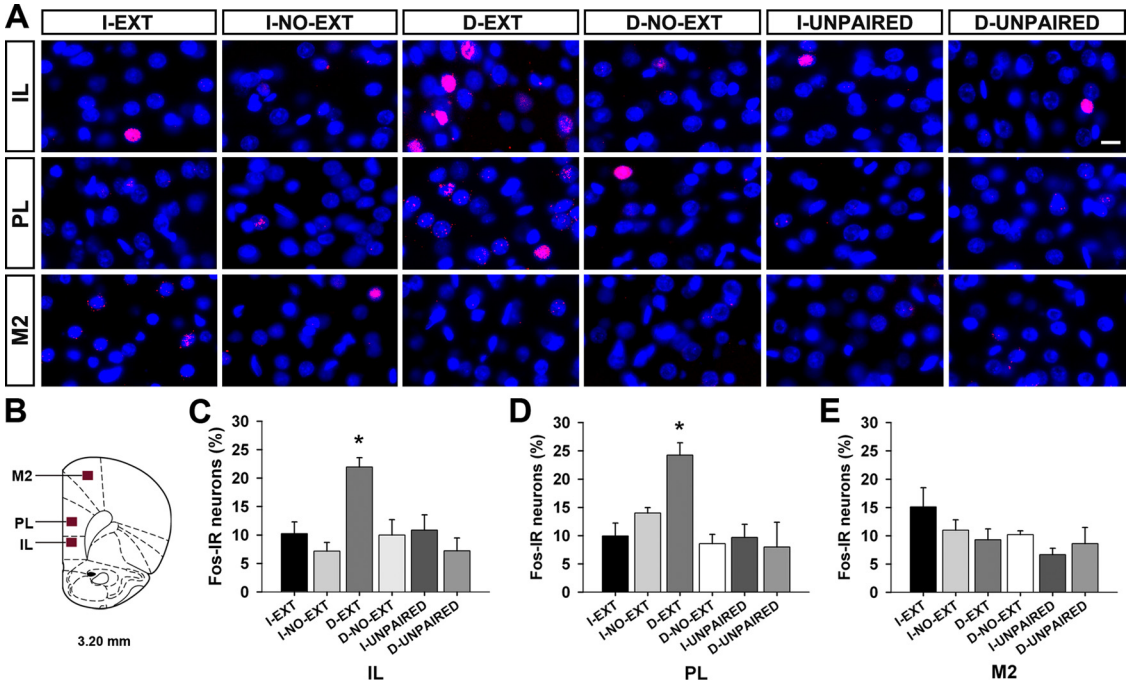

Figure 2. Fos expression in the mPFC following immediate extinction. ***A***, Representative photomicrographs showing Fos expression in the IL, PL, and M2. Fos expression was identified using CY3 fluorescence (red), and nuclei were identified by DAPI staining (blue). Scale bar, 10 μm. ***B***, Schematic diagram illustrating the IL, PL, and M2. ***C–E***, Percentages of Fos-IR neurons in the IL, PL, and M2. Fos-IR neurons in both the IL and PL were found in significantly greater numbers in the D-EXT group than in other groups. Numbers of Fos-IR neurons in the I-EXT group were not different from the controls. D-EXT, Delayed extinction group; I-EXT, immediate extinction group; I-NO-EXT, immediate no extinction group; D-NO-EXT, delayed no extinction group; I-UNPAIRED, immediate unpaired conditioning group; D-UNPAIRED, delayed unpaired conditioning group. $^*p < 0.05$. All data are presented as means ± SEM.

ing (group × trial block, $p = 0.223$) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In addition, the I-EXT group still showed a higher level of freezing than the D-EXT group ($t_{(18)} = 3.277$; $p < 0.01$). Thus, our data indicate that extinction training conducted shortly after conditioning does not lead to long-term extinction memory, which is consistent with a previous report (Maren and Chang, 2006).

Fos expression in the mPFC following immediate extinction

We next investigated the possibility that IED may be caused by a lack of neuronal activity in the mPFC. Behavioral training during the conditioning and extinction sessions was performed as described above (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Different levels of Fos expression in the IL and PL were observed among groups (Fig. 2*A*). ANOVA revealed a main effect of group on the number of Fos-IR neurons in the IL ($F_{(5,19)} = 5.079$, $p < 0.05$) and the PL ($F_{(5,19)} = 5.654$, $p < 0.05$), but not in the M2 ($F_{(5,19)} = 1.573$, $p = 0.231$) (Fig. 2*C–E*). *Post hoc* analysis confirmed that the fraction of Fos-IR neurons in both the IL (all $p < 0.05$) and PL (all $p < 0.05$) was significantly greater in the D-EXT group than in the other groups. All the other groups, including the I-EXT group, displayed an equally low level of Fos expression in the IL (all $p > 0.05$) and PL (all $p > 0.05$) in comparison to D-EXT group. Therefore, our data suggest that IED results from a lack of neuronal activity in the mPFC.

The effects of IL stimulation during immediate extinction on long-term extinction memory

We next examined the possibility that IL stimulation during the immediate extinction could remedy IED. Only rats in which the electrode was precisely positioned in the IL were included in the analysis (Fig. 3*B*). All groups showed robust freezing at the last conditioning trial ($F_{(6,93)} = 1.851$, $p > 0.05$) (Fig. 3*C*). During extinction training, all groups showed a similar pattern of reduction in freezing, except for the I-STIM group. ANOVA revealed that there were main effects for group ($F_{(6,93)} = 3.076$, $p < 0.01$) and trial block ($F_{(4,372)} = 64.585$, $p < 0.01$), as well as a group × trial block interaction ($F_{(24,372)} = 2.499$, $p < 0.01$). *Post hoc* analysis confirmed that the I-EXT group froze significantly less than the other groups in the first trial block ($p < 0.01$) (Fig. 3*D*). These results indicate that electrical stimulation of the IL had no effect on the level of freezing during the extinction session. The retention test was conducted 48 h after fear acquisition in the absence of IL stimulation. A one-way ANOVA revealed a significant effect for group ($F_{(6,93)} = 6.732$, $p < 0.01$). *Post hoc* analysis confirmed that the I-PAIRED group froze significantly less than other groups (all $p < 0.05$), but did not differ from the D-NO-STIM ($p > 0.05$) or D-UNPAIRED groups ($p > 0.05$) (Fig. 3*E*). Thus, our data suggest that IL stimulation paired with the CS mitigates IED.

Discussion

We conducted a series of experiments to investigate the neural mechanism that underlies the loss of long-term extinction memory following immediate extinction training (IED). We first confirmed that extinction training administered immediately after fear conditioning failed to induce long-term suppression of fear memory. Using *c-fos* immunoreactivity, we found that Fos expression in the mPFC was significantly lower in the immediate extinction group than in the delayed extinction group. Finally, we were able to mitigate IED by electrical stimulation of the IL paired with the presentation of the CS. Together, these findings provide compelling evidence that IL activation is necessary for the long-term suppression of fear CRs and that IED is caused by a lack of neuronal activity in the IL.

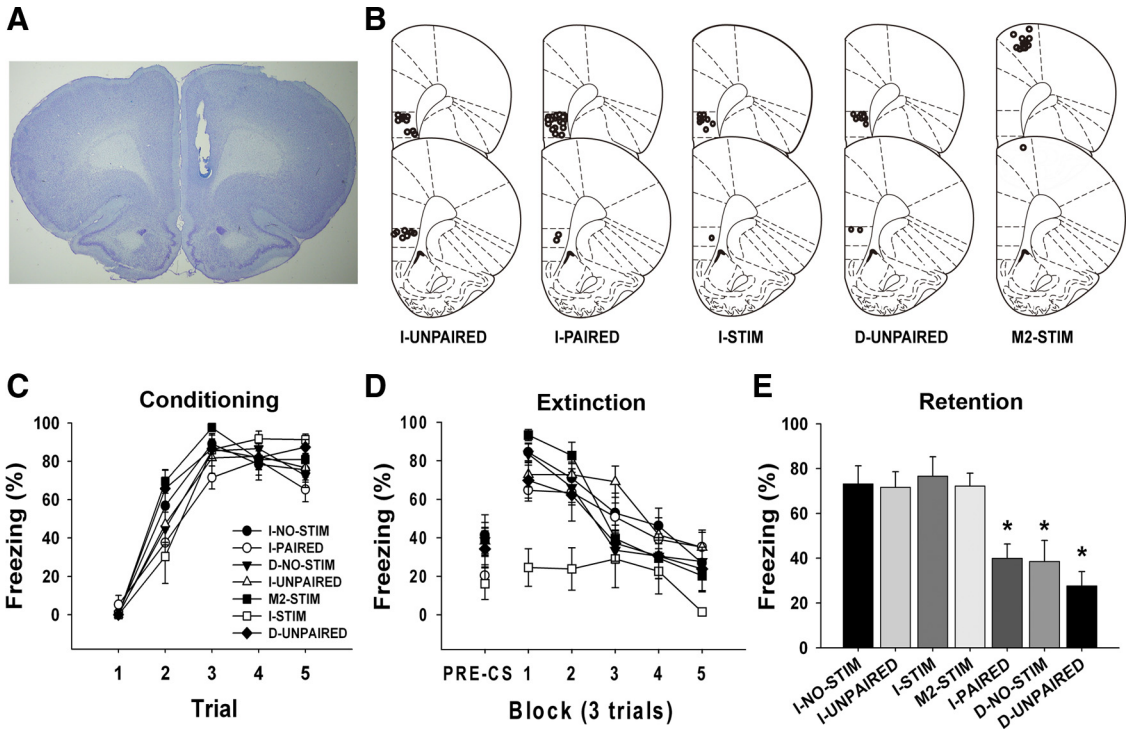

Figure 3. Effects of IL stimulation during immediate extinction training on long-term extinction memory. ***A***, A representative image showing a coronal section from a rat with a stimulating electrode in the IL. ***B***, Location of stimulation sites in the IL for the immediate unpaired-stimulation group, immediate paired-stimulation group, immediate stimulation-only group, delayed unpaired-stimulation group, and immediate M2-stimulation group [modified from Paxinos and Watson (1998)]. ***C***, Percentage of freezing during conditioning. Rats were given five CS–US conditioning trials. All groups showed robust freezing in the last conditioning trial. ***D***, Percentage of freezing during extinction training. The immediate paired-stimulation group (I-PAIRED) and M2-stimulation group (M2-STIM) received IL or M2 stimulation at 100 ms after CS onset during immediate extinction training, respectively. The immediate unpaired-stimulation group (I-UNPAIRED) and delayed unpaired-stimulation groups (D-UNPAIRED) received explicitly unpaired IL stimulation and tones at corresponding time points. The immediate no-stimulation and delayed no-stimulation groups received extinction training at 15 min (I-NO-STIM) or 24 h (D-NO-STIM) after conditioning, but never received IL stimulation. In addition, the stimulation-only group (I-STIM) received IL stimulation in the absence of CS presentation. All groups showed a similar pattern of reduced freezing, except for the I-STIM group. ***E***, Averaged percentage of freezing across three test trials. The I-PAIRED group froze significantly less than other groups, but did not differ from the D-NO-STIM or D-UNPAIRED groups. $*p < 0.05$. All data are presented as means ± SEM.

The results of the current study strongly support a role for mPFC activation in extinction, consistent with several previous [for review, see Myers and Davis (2002) and Sotres-Bayon et al. (2004)]. For example, extinction training induced *c-fos* immunoreactivity in the mPFC (Herry and Mons, 2004; Santini et al., 2004) and increased the excitability of IL neurons (Santini et al., 2008). In addition, IL stimulation paired with the CS facilitated extinction memory (Milad and Quirk, 2002; Milad et al., 2004). In the current experiment, delayed extinction also increased Fos expression in the mPFC. Following immediate extinction, however, few Fos-positive neurons were found, indicating that the mPFC was not fully active. The mPFC hypothesis was confirmed in the final experiment: artificial inflation of IL activity counteracted IED.

A key question that emerges from this study is why immediate extinction training was unable to activate the mPFC. Considering the reciprocal projections and dynamic interaction between the amygdala and the mPFC (Cassell et al., 1989; McDonald, 1991; McDonald et al., 1996; Vertes, 2006), it is possible that the amygdala suppressed the mPFC. This hypothesis is consistent with the observations that fear conditioning inhibited the excitability of IL neurons (Garcia et al., 1999; Ishikawa and Nakamura, 2003; Santini et al., 2008), and that stimulation of the basolateral amygdala decreased the responsiveness of mPFC neurons (Pérez-Jaranay and Vives, 1991). In favor of this hypothesis, a high level of fear before the extinction session disrupted long-term extinction memory (Maren and Chang, 2006). However, in our study extinction training was performed in a different context to minimize the initial fear level. As a result, little freezing was observed during the baseline period, indicating that the fear levels were low before the CS was presented. Thus, the loss of long-term extinction memory following immediate extinction training was not due to fear levels per se in our study.

Alternatively, inhibitory modulation of the mPFC might be mediated by stress response systems. Stress can be induced by intense physical stimulation or strong negative emotion (Selye and Horava, 1953). Once initiated, the effects of stress can linger on, even after the source of immediate stress is removed. Given that electric footshock elevated plasma levels of corticosterone for 90 min (Weinstock et al., 1998), stress levels may be higher in the immediate extinction condition than during delayed extinction, despite the lack of a measurable behavior (i.e., freezing) in both conditions. Activation of stress responses by the conditioning session could suppress the mPFC preventing it from becoming activated by extinction training. In support of this model, acute stress rapidly blocked the induction of long-term potentiation (LTP) in both amygdala–mPFC (Maroun and Richter-Levin, 2003) and hippocampus–mPFC (Rocher et al., 2004) pathways. Furthermore, acute stress induced dendritic retraction of pyramidal neurons in the IL, thereby causing an impairment in extinction (Izquierdo et al., 2006). From these results, it is reasonable to assume that the suppressed neuronal activity in the mPFC is the result of the temporally adjacent acquisition session, which involved physical and emotional stress.

Yet another hypothesis is that the associative learning memory must be encoded in the mPFC before a subsequent extinction

memory can be processed, suggesting that a short postacquisition interval may not allow sufficient time for critical changes in the mPFC to occur. Retention of long- but not short-term memory in certain types of associative learning has indeed been shown to require delayed synaptic changes in the mPFC (Takehara-Nishiuchi et al., 2006; Takehara-Nishiuchi and McNaughton, 2008). This hypothesis is also consistent with the role of the mPFC in other studies, which show that mPFC is not required for primary learning but becomes essential when experimental conditions, such as CS–US contingencies, are reversed in a subsequent phase (Schoenbaum et al., 2000). Similarly, one learning theory proposed a temporal memory model in which a "ready state" for extinction learning is not achieved until the acquisition phase has been stabilized (Wagner and Brandon, 1989). This "consolidation delay" hypothesis offers an explanation for the dynamic nature of extinction memory following appetitive as well as aversive learning (Woods and Bouton, 2008). Together, empirical data and theoretical work support that mPFC may play a role in prerequisite first stage learning, providing essential contribution to the second stage learning. However, further studies are needed to specifically test the time course of neural events in the mPFC following initial learning.

Although our data strongly support the theory that a short posttraining interval renders extinction training immensely ineffective, the optimal timing of early intervention following a traumatic event is still controversial. Some have reported that early intervention is effective in alleviating the severity of posttraumatic stress disorder (PTSD) symptoms (Campfield and Hills, 2001), whereas others have demonstrated that early intervention exacerbates trauma-related fear due to increased stress (Bisson et al., 1997; Gray and Litz, 2005). Results from laboratory experiments are equally confusing, perhaps due to differences in experimental protocols and parametric variations. For example, Myers et al. (2006) show that immediate extinction effectively eliminated spontaneous recovery, renewal, and reinstatement, implying the fear memory was permanently removed. On the other hand, Schiller et al. (2008) reported normal spontaneous recovery and reinstatement following immediate extinction in both humans and rats. It is difficult to directly compare these two studies as they differ in many significant ways, including the type of CR (potentiated startle vs freezing), US intensity, and the number of training trials. Further studies, especially those aimed at exploring different experimental parameters, are needed to elucidate which conditions differently drive the long-term consequences of immediate extinction. In the long run, this and related studies may inform development of optimal therapeutic options for mental disorders related to persistent fear memory.

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