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Electrical Stimulation of Medial Prefrontal Cortex Reduces Conditioned Fear in a Temporally Specific Manner

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The authors recently showed that extinction of auditory fear conditioning leads to potentiation of tone-evoked activity of neurons in the infralimbic (IL) subregion of the medial prefrontal cortex, suggesting that IL inhibits fear after extinction (M. R. Milad, & G. J. Quirk, 2002). In support of this finding, pairing conditioned tones with brief (300-ms) electrical stimulation of IL reduces conditioned freezing. The present study showed that IL stimulation inhibits freezing if given 0.1 s after tone onset (the latency of tone-evoked responses) but has no effect if given either 1 s before or 1 s after tone onset. This suggests that IL gates the response of downstream structures such as the amygdala to fear stimuli.

Recently, there has been increased interest in the neural mechanisms of fear extinction (Davis & Myers, 2002; Garcia, 2002). During extinction, conditioned fear responses to a tone previously paired with a footshock diminish after repeated presentations of the tone alone. Behavioral data suggest that extinction does not erase conditioning but forms a new memory that inhibits the conditioned response (Bouton, 1993; Pavlov, 1927; Quirk, 2002; Rescorla, 2001). Previous studies have implicated the ventral medial prefrontal cortex (vmPFC) in inhibition of fear after extinction (Barrett, Shumake, Jones, & Gonzalez-Lima, 2003; Herry & Garcia, 2002; Morgan, Romanski, & LeDoux, 1993; Quirk, Russo, Barron, & Lebron, 2000; but see Gewirtz, Falls, & Davis, 1997). Rats with lesions of vmPFC can extinguish fear responses within a session but are unable to recall extinction 24 hr later (Lebron, Milad, & Quirk, 2003; Quirk et al., 2000). Paralleling these lesion findings, we recently reported that tone responses of neurons in the infralimbic (IL) subregion of vmPFC were potentiated 24 hr after extinction training, when rats were recalling extinction (Milad & Quirk, 2002). Moreover, rats with the largest increases in IL tone responses exhibited the least freezing to the tone. Collectively, these findings suggest that extinction potentiates IL activity, which inhibits freezing after extinction.

To test this hypothesis, we paired conditioned tones with brief (300 ms) trains of IL stimulation designed to mimic naturally occurring tone responses of IL neurons (Milad & Quirk, 2002). IL stimulation delivered 0.1 s after tone onset (the same latency as tone-evoked activity) markedly reduced freezing to the tone. In contrast, stimulation delivered 2 min before the tone in an unpaired control group had no effect.

By what mechanism does IL stimulation reduce freezing to tones? One possibility is that it produces a prolonged anxiolytic effect independent of the tone. If this were the case, stimulation administered before the tone should reduce freezing to subsequent tone stimuli. The unpaired control group just described rules out prolonged effects lasting 2 min or more; however, a shorter prolonged effect lasting tens of seconds could still account for our findings. Alternatively, IL stimulation might reduce freezing by gating the response of downstream structures to tone stimuli. In this case, only IL stimulation paired with tones (mimicking tone-evoked activity) should reduce freezing.

In the present study, we attempted to distinguish between these possibilities by varying the timing of IL stimulation with respect to tone onset. We reasoned that if stimulation of IL has a persistent anxiolytic effect, stimulation 1 s before tone onset should be as effective as stimulation at 0.1 s after tone onset. If, however, IL acts by gating the response of downstream structures to tones, then stimulation 1 s before the tone should have no effect. Accordingly, we repeated our original experiment and tested two additional time points: 1.0 s before tone onset and 1.0 s after tone onset. The latter was done to determine whether there was a temporal limit to the effectiveness of IL stimulation. Determining the most effective timing of prefrontal stimulation could be a first step in developing this technique for possible clinical use in patients with phobias and posttraumatic stress disorder (PTSD; Eschweiler et al., 2000). A preliminary report of these data has been presented in abstract form (Vidal-Gonzalez, Milad, & Quirk, 2002).

Method

Subjects

The procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Ponce School of Medicine in compliance with National Institutes of Health guidelines for the care and use of laboratory animals. Male Sprague–Dawley rats weighing approximately 300 g were transferred from the Ponce School of Medicine colony to the laboratory, where they were housed individually in transparent polyethylene cages located in a negative pressure clean room (Colorado Clean Room, Ft. Collins, CO) and maintained on a 12-hr light–dark schedule with free access to water. Food was restricted to 10–15 g of

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standard rat chow until rats reached 85% of their original body weight. They were then trained to press a bar for food on a variable interval schedule of reinforcement (VI-60) to maintain a constant level of activity against which freezing responses could be reliably measured (Quirk et al., 2000; Santini, Muller, & Quirk, 2001). Bar-press training was given in the same chamber used for fear conditioning and extinction (described subsequently).

Surgery

The procedures for implanting stimulating electrodes were similar to those previously described (Milad & Quirk, 2002). A concentric bipolar electrode 0.2 mm in diameter with an exposed tip length of 0.25 mm (Rhodes Medical Instruments, Woodland Hills, CA) was implanted in the right IL. Stereotaxic coordinates were 2.9 mm anterior, 1.0 mm lateral, and 4.9 ventral to bregma, angled 6° toward midline (Paxinos & Watson, 1998). Rats were implanted unilaterally rather than bilaterally to minimize track-related damage to the dorsal mPFC, which has been shown to increase conditioned freezing (Morgan & LeDoux, 1995). At the conclusion of the experiment, the tip of the electrode was marked through passing of a 20-μA current for 20 s. The location of electrodes was reconstructed with standard histological techniques from Nissl-stained sections. Rats with electrodes in structures other than the IL were excluded.

Fear Conditioning

Rats were fear conditioned and extinguished in a standard 25- × 29- × 28-cm operant chamber (Coulbourn Instruments, Allentown, PA). The conditioned stimulus (CS) was a 30-s tone at 4 kHz, with an intensity of 80 dB. The interval between successive tone presentations averaged 4 min (range: 2–6 min). The unconditioned stimulus was a 0.5-mA scrambled footshock, 0.5 s in duration, that co-terminated with the tone. The experiment took place over 3 days. On Day 1, rats received five trials of tone alone (habituation phase) immediately followed by five trials of tone paired with footshock (conditioning phase). After conditioning, experimental groups were matched for acquired freezing levels. On Day 2, rats were returned to the same operant chamber and were given eight trials of tone alone (extinction phase) either without IL stimulation (unstimulated group) or paired with electrical stimulation of IL at one of three latencies (as described subsequently). A total of eight IL stimuli were given (one per tone). On Day 3, all rats were given two tones to test for extinction recall in the absence of IL stimulation.

Brain Stimulation

A pulse generator with constant current output (Grass Instruments, Quincy, MA) delivered a 300-ms train of square pulses (0.2-ms pulse width, 100 μA, 100 Hz). Three experimental groups differed in the latency of IL stimulation with respect to tone onset. The first group received IL trains 1.0 s before tone onset (−1.0-s group; $n = 12$). The second group received IL trains 0.1 s after tone onset (+0.1-s group; $n = 11$). The third group received IL trains 1.0 s after tone onset (+1.0-s group; $n = 8$). A fourth group was implanted with stimulating electrodes but never stimulated ($n = 11$). An additional group received IL trains 0.1 s after tone onset at a lower frequency (20 Hz; $n = 10$).

Data Analysis

Freezing was used as the measure of conditioned fear. Freezing is the cessation of all movements other than respiration (Blanchard & Blanchard, 1972). The total time spent freezing during the 30-s tone was scored from videotape with a digital stopwatch. Freezing values were examined in repeated measures analyses of variance (ANOVAs), and the Tukey honestly significant difference method was used in making post hoc comparisons (Statistica; StatSoft, Tulsa, OK).

Results

Immediate Effects of IL Stimulation on Tone-Induced Freezing

At the end of conditioning, all groups were freezing approximately 80% to the tone CS (see Figure 1A). The following day,

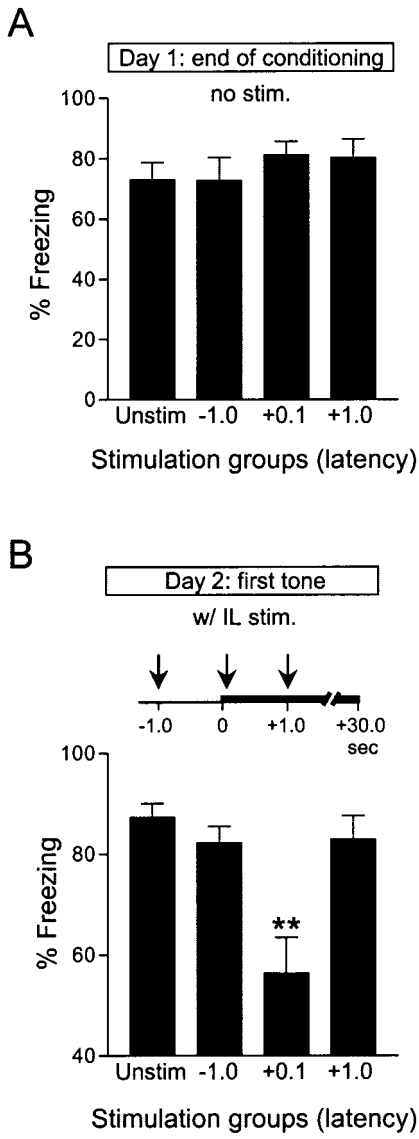

Figure 1. Results showing that infralimbic (IL) stimulation (stim.) at tone onset reduced conditioned freezing to the tone. A: Mean (± *SEM*) acquired freezing levels for all groups during the last conditioning trial on Day 1. B: Schema of stimulation onset with respect to the tone (top) and mean (± *SEM*) percentage of freezing during the first extinction trial on Day 2 (bottom). Stimulation groups were as follows: 1 s before tone onset (−1.0), 0.1 s after tone onset (+0.1), 1 s after tone onset (+1.0), and unstimulated (Unstim). Note that freezing to the tone was significantly reduced only in the +0.1-s group (**$p < .01$).

extinction tones were paired with IL stimulation. Stimulation of IL 0.1 s after tone onset significantly reduced freezing in the very first trial, replicating our previous observation (Milad & Quirk, 2002). Rats stimulated at 0.1 s showed 56.3% freezing in Trial 1, as compared with 87.3% in the unstimulated control group (see Figure 1B). In contrast, stimulation given either 1.0 s before tone onset or 1.0 s after tone onset did not reduce freezing on Trial 1 (82.2% and 82.9% for the –1.0- and +1.0-s groups, respectively). A one-way ANOVA revealed a significant effect of stimulus timing, $F(3, 38) = 9.06$, $p < .001$, and post hoc analysis showed that the +0.1-s group showed significantly less freezing than all other groups (all ps $< .01$). Thus, the modulation of freezing on Trial 1 by IL stimulation showed a surprisingly narrow window of effectiveness, starting at tone onset and extending less than 1 s into the tone.

As extinction progressed, rats in the +0.1-s group ended the session with only 17.4% freezing, as compared with 48.0% for the unstimulated controls (Figure 2A). In contrast, the +1.0-s group never differed from unstimulated controls in any trial block. An ANOVA of freezing values on Day 2 revealed significant main effects of group, $F(3, 38) = 7.09$, $p < .001$, and trial block, $F(3, 114) = 59.81$, $p < .001$, as well as a significant interaction, $F(9, 114) = 2.62$, $p < .01$. Post hoc analyses showed that the +0.1-s group had significantly lower values than both the unstimulated ($p < .001$) and +1.0-s ($p < .001$) groups in all trial blocks. Surprisingly, stimulation at −1.0 s, which had no effect on Trials 1 and 2, accelerated the rate of extinction on subsequent trials. Post hoc analysis showed that the −1.0-s group dropped significantly faster than the unstimulated group in Blocks 2–4 ($p < .01$; see Figure 2A).

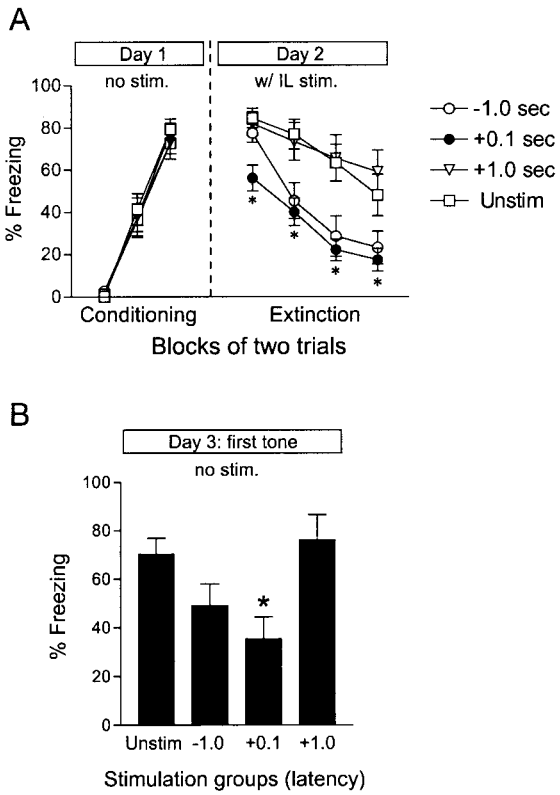

Figure 2. Results showing that infralimbic (IL) stimulation (stim.) at tone onset reduced freezing throughout extinction training as well as during extinction recall. A: Mean (± *SEM*) percentage of freezing for all groups across extinction training, shown in blocks of two trials. Stimulation at +0.1 s significantly reduced freezing in all trial blocks (*$p < .01$). Note that the −1.0-s group, which did not show reduction of freezing during the first extinction block, extinguished significantly faster than controls on Day 2. Unstim = unstimulated. B: Mean (± *SEM*) percentage of freezing during the first extinction trial on Day 3. Only rats that received stimulation at +0.1 s had significantly lower values than unstimulated controls (*$p < .05$).

Recall of Extinction on Day 3 in the Absence of IL Stimulation

On Day 3, the +0.1-s group continued to have significantly lower values than controls, showing 30% freezing as compared with 62.1% for the control group (see Figure 2B), replicating our previous observation that IL stimulation strengthens extinction memory (Milad & Quirk, 2002). A one-way ANOVA on Day 3 revealed a significant main effect of group, $F(3, 38) = 4.46$, $p < .01$, with post hoc tests confirming that the +0.1-s group had significantly lower values than the unstimulated group ($p < .05$) as well as the +1.0-s group ($p < .01$). Despite its rapid drop on Day 2, the −1.0-s group did not differ significantly from unstimulated controls on Day 3 ($p = .29$), suggesting that the reduction in freezing on Day 2 in this group was a temporary effect of stimulation.

Stimulation of IL at a Lower Frequency

In the experiment described here, as in our previous study, IL was stimulated at a frequency of 100 Hz. This rate is higher than the firing rate of typical IL neurons, which fired at a maximal rate of 20 Hz in the first 400 ms after tone onset (Milad & Quirk, 2002). We therefore repeated the experiment in a separate group of rats using 20 Hz instead of 100 Hz stimulation (+0.1 latency). As shown in Figure 3, the lower rate of stimulation was equally effective in reducing freezing at all time points. A two-way ANOVA with repeated measures revealed significant main effects of treatment, $F(2, 29) = 12.36$, $p < .01$, and trial block, $F(4, 116) = 44.69$, $p < .01$. Post hoc analysis showed that the 20 Hz group had significantly less freezing than unstimulated controls in all trial blocks on Day 2 (all ps $< .01$), as well as Day 3 ($p < .01$), and never differed from the 100 Hz group. These findings indicate that very little IL stimulation is needed to reduce freezing, in that 20 Hz produces only six pulses within 300 ms. Stimulation sites for all groups are illustrated in Figure 4.

Discussion

We replicated our earlier findings (Milad & Quirk, 2002) that pairing conditioned tones with brief IL stimulation reduces conditioned freezing and strengthens extinction memory. We extended these findings by demonstrating a high degree of temporal specificity of these effects. Whereas stimulation 0.1 s after tone onset reduced freezing to the tone, stimulation either 1.0 s before or 1.0 s after tone onset did not. Thus, there is a brief window after tone onset during which IL activity can modulate freezing to a tone CS.

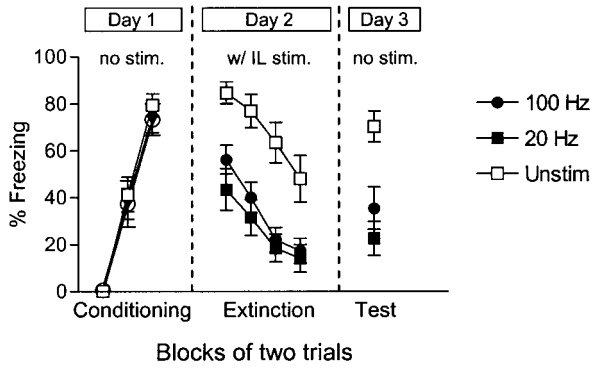

Figure 3. Infralimbic (IL) stimulation (stim.) at 20 Hz versus 100 Hz. Stimulation at 20 Hz (six pulses) was as effective as 100 Hz in reducing conditioned freezing and strengthening extinction memory. All stimulation was delivered at 0.1 s after tone onset. Data are means (± *SEM*). Unstim = unstimulated.

The ineffectiveness of pretone stimulation rules out a prolonged anxiolytic effect of the brief IL stimulation we used. Pairing IL stimulation with the tone appears to be essential. This suggests that IL acts by inhibiting downstream structures involved in processing conditioned tones. A likely downstream structure is the amygdala. IL projects to the basolateral amygdala (BLA) and to the capsular division of the amygdala central nucleus (Hurley, Herbert, Moga, & Saper, 1991; McDonald, Mascagni, & Guo, 1996; Sesack, Deutch, Roth, & Bunney, 1989), which contains gamma-aminobutyric acid (GABA)-ergic intercalated (ITC) cells. The medial division of central nucleus (CeM) receives excitatory input from BLA (Paré, Smith, & Paré, 1995; Pitkänen, Savander, & LeDoux, 1997) and projects to the midbrain and hypothalamic sites that mediate fear responses (Bellgowan & Helmstetter, 1996; Davis & Whalen, 2001; De Oca, DeCola, Maren, & Fanselow, 1998; LeDoux, 2000). Therefore, IL could suppress tone-evoked responses of BLA neurons through local inhibitory interneurons within the BLA (Rosenkranz, Moore, & Grace, 2003). Alternatively, IL could suppress CeM output by activating ITC cells, which act as an inhibitory interface between BLA and CeM (Royer, Martina, & Paré, 1999; Royer & Paré, 2002). Although either route is possible, projections from IL to ITC cells are particularly robust in the rat (McDonald et al., 1996; Sesack et al., 1989) and monkey (Freedman, Insel, & Smith, 2000).

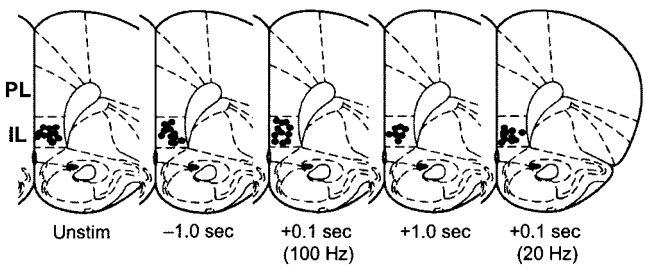

Figure 4. Placement of stimulating electrodes in infralimbic (IL) cortex for all experimental groups. PL = prelimbic; Unstim = unstimulated. Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Figure 8, Copyright 1998, with permission from Elsevier.

Figure 5 diagrams how IL could influence the expression of conditioned freezing during recall of extinction. Before extinction, when rats are recalling conditioning, tone stimuli strongly activate BLA neurons (Collins & Paré, 2000; Maren, 2000; Quirk, Repa, & LeDoux, 1995), which leads to increased CeM activation (Toyomitsu, Nishijo, Uwano, Kuratsu, & Ono, 2002) and expression of fear. During recall of extinction, tone stimuli also activate IL, which inhibits CeM output through activation of ITC cells (Royer et al., 1999). This model agrees with several findings. First, IL neurons signal tones only after extinction training, when rats are recalling extinction (Milad & Quirk, 2002). Second, cells in IL and BLA respond to tones at similar latencies (Jodo, Suzuki, & Kayama, 2000; Milad & Quirk, 2002; Repa et al., 2001; Toyomitsu et al., 2002), suggesting that IL activity would arrive in time to cancel BLA activation of CeM. Third, IL stimulation inhibits the responsiveness of CeM neurons to BLA stimulation (Quirk, Likhtik, Pelletier, & Paré, 2003). Finally, tone-induced bursts in BLA neurons are brief, typically ending within 200 ms after tone onset (Quirk et al., 1995; Repa et al., 2001; Toyomitsu et al., 2002). Thus, stimulation of IL 1.0 s after tone onset would arrive too late to prevent BLA bursts from activating CeM and would therefore fail to reduce freezing (as we observed). It is important to note, however, that IL also projects directly to the hypothalamic and midbrain sites that generate freezing (Floyd, Price, Ferry,

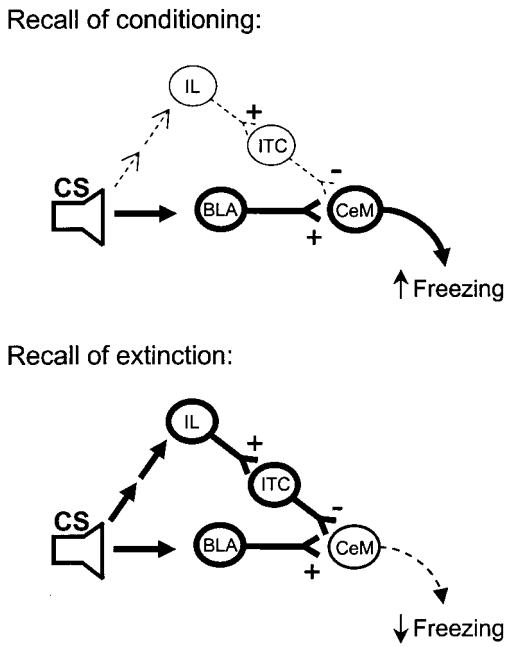

Figure 5. Model of infralimbic–amygdalar interactions. During recall of conditioning, increased output of the basolateral amygdala (BLA) activates the medial division of the central nucleus (CeM) to produce conditioned fear responses. During recall of extinction, parallel activation of infralimbic cortex (IL) excites (plus sign) amygdala intercalated cells (ITC), which dampen (minus sign) the output of CeM and reduce fear. Boldface lines indicate increased activity; dashed lines indicate decreased activity. Upward arrow indicates increased freezing; downward arrow indicates decreased freezing. CS = conditioned stimulus.

Keay, & Bandler, 2000; Freedman et al., 2000; Hurley et al., 1991) and could modulate freezing independently of the amygdala.

Stimulation 1 s before tone onset did not reduce freezing to the initial conditioned tones but did accelerate the rate of within-session extinction. One possible explanation for this unexpected finding is that pretone stimulation of IL may have triggered prolonged firing in a small subset of ITC cells (Royer, Martina, & Paré, 2000), resulting in some degree of inhibition in CeM. Early in extinction, robust BLA tone responses would override this minor inhibition. However, as extinction progresses and amygdala responses decline (Repa et al., 2001; Quirk et al., 1995), the inhibitory effect would become more apparent. The next day (in the absence of stimulation) freezing would rebound, as we observed. In contrast, rats receiving IL stimulation at 0.1 s latency showed improved recall of extinction on Day 3, suggesting strengthening of extinction memory. The frequency of stimulation we used could potentiate synapses within either IL (Herry & Garcia, 2002) or IL's targets in the amygdala. This is consistent with previous studies suggesting that *N*-methyl-D-aspartate (NMDA) receptors in BLA are necessary for long-term retention of extinction (Walker & Davis, 2002; Walker, Ressler, Lu, & Davis, 2002), and ITC cells exhibit NMDA-dependent synaptic plasticity when significantly depolarized (Royer & Paré, 2002).

In conclusion, we have demonstrated that IL stimulation can modulate the expression of conditioned fear to a tone CS, but only within a brief temporal window around tone onset. These findings add to a growing literature on the role of mPFC in fear extinction by suggesting that IL may gate the output of downstream structures such as the amygdala. Understanding prefrontal-amygdala interactions in fear conditioning has potential clinical implications. For example, PTSD is associated with reduced vmPFC activity together with exaggerated amygdala responses (Bremner et al., 1999; Pitman, Shin, & Rauch, 2001; Rauch et al., 2000; Shin et al., 2001). Perhaps pairing traumatic reminders with activation of mPFC (Eschweiler et al., 2000) could strengthen extinction memory and improve clinical outcomes of exposure therapy for PTSD (Foa, 2000; Rothbaum, Kozak, Foa, & Whitaker, 2001).

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