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14. ABSTRACT

So far this project has published the original finding that the anti-progesterone and glucocorticoid receptor antagonist mifepristone, when administered systemically, reduces reconsolidation of a cue-conditioned fear response in rats, and that the beta-adrenergic blocker propranolol blocks this mifepristone effect. We have produced the original discovery that alpha-2-adrenergic agonist clonidine also reduces reconsolidation of a cue-conditioned fear response in rats in a dose-dependent manner. We have produced the original discovery that the mammalian target of rapamycin (mTOR) kinase-dependent signaling mediates stabilization of fear conditioning-produced synaptic strengthening in the conditioned stimulus pathways following memory recall in rats, thus providing a postretrieval memory update mechanism. We have achieved steady progress in the implementation of two randomized, double-blind, placebo-controlled studies in humans: one of post-reactivation mifepristone's ability to reduce psychophysiologic responding during traumatic imagery in trauma-exposed subjects, and the other of six sessions of post-reactivation propranolol for the treatment of PTSD. The blinds for these human studies have not yet been broken.

15. SUBJECT TERMS

Stress disorders, post-traumatic; reconsolidation; imagery, psychotherapy; pharmacology; psychophysiology (all MeSH terms)

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1. INTRODUCTION

The aim of this project is to develop post-reactivation (PR) pharmacologic interventions that may serve as novel treatments for posttraumatic stress disorder (PTSD). The underlying theory is that candidate drugs, when given following the reactivation of a conditioned fear response in animals, or a traumatic memory in humans, will reduce the strength of the conditioned response or traumatic memory. We plan to test such drugs, either alone or in combination, for their possible reconsolidation-blocking properties in a hierarchy of experiments. Drugs that show promise at a given stage of investigation will be advanced to the next stage. In Stage I, we will evaluate the ability of candidate drugs to reduce freezing in a Pavlovian cue-conditioned fear task in rats. In Stage II, we will evaluate the ability of candidate drugs to reverse fear conditioning-induced synaptic enhancement in rat amygdala slices using whole-cell electrophysiologic recording. In Stage III, we will test the ability of a single session of PR candidate drug to reduce subsequent psychophysiologic responding during script-driven imagery of the traumatic event in trauma-exposed human subjects. In Stage IV, we will test the ability of a series of PR candidate drug therapy sessions to reduce symptoms in PTSD patients.

The animal reconsolidation experiments will entail three phases: 1.) single-trial fear conditioning; 2.) presenting the conditioned stimulus (reactivation), followed by PR drug; and 3.) measuring the conditioned response in a test trial, followed in certain cases by sacrificing the animal for electrophysiologic measurements. If the drug is an amnestic (i.e., reconsolidation-blocking) agent, the test conditioned response should be reduced in animals that previously received the drug. Because the (past) traumatic event itself represents the (phase 1) conditioning event, the human experiments will only have the last two stages: 2.) single or multiple sessions of traumatic memory reactivation followed by candidate drug; and 3.) measuring a.) psychophysiologic responses during script-driven imagery of the traumatic event, and/or b.) PTSD symptoms.

In order to rule out the possibility that nonspecific drug effects account for any findings, the experiments will incorporate non-reactivation (NR) drug control groups, as well as PR vehicle/placebo control groups

2. BODY

2.1. Animal work

2.1.1. Massachusetts General Hospital (MGH)

2.1.1.1. Published study. This project period saw the publication of our positive results with blocking the reconsolidation of cue-conditioned fear (Pitman et al , 2011a below-reprint attached) that were a direct result of this grant. An abstract of this publication follows:

2.1.1.1.2 Abstract. Reducing reconsolidation of reactivated traumatic memories may offer a novel pharmacological treatment for posttraumatic stress disorder (PTSD). Preclinical research is needed to identify candidate drugs. We evaluated the ability of postreactivation mifepristone (RU38486, a glucocorticoid antagonist), alone and in combination with propranolol (a beta-adrenergic blocker), both given systemically, to reduce cue-conditioned fear in rats. On Day 1, a 30-s tone conditioned stimulus (CS) was paired with an electric shock unconditioned stimulus (US). On Day 2, the CS was presented without the US (reactivation), and the freezing conditioned response (CR) was measured. This was immediately followed by subcutaneous injection of vehicle, mifepristone 30 mg/kg, propranolol 10 mg/kg, or both. On Day 3, the CR was again measured as a test of postreactivation long-term memory (PR-LTM). On Day 10, the CR was again measured to evaluate spontaneous recovery. On Day 11, the US was presented alone (reinstatement). On Day 12, the CR was again measured. A fifth group received

mifepristone without the CS presentation (nonreactivation) on Day 2. A sixth group was tested four hours after the Day 2 mifepristone injection to measure postreactivation short-term memory. Postreactivation, but not nonreactivation, mifepristone produced a decrement in the CR that did not undergo spontaneous recovery and underwent only modest reinstatement. Mifepristone did not exert its effect when administered concurrently with propranolol. Postreactivation mifepristone did not impair short-term memory. Systemic mifepristone blocks the reconsolidation of cue-conditioned fear in rats. Concurrent administration of propranolol prevents this effect. Postreactivation mifepristone may be a promising treatment for PTSD, but not necessarily in combination with propranolol

2.1.1.2. Published commentary. This project period also saw the publication of a commentary that was inspired in part by results of the above study (Pitman et al , 2011b-reprint attached).

2.1.1.3. Unpublished work

2.1.1.2.1. Midazolam, morphine, nabilone. We have been exploring the potential of several other drugs and combinations to be similarly implemented for reconsolidation blockade. As noted in the initial application, we are only interested in drugs that have been approved for human use and can be administered systemically. Therefore, as far as possible we select the dosage we use in rodents to reflect an appropriate human dosage. We previously obtained negative results with midazolam and morphine. We previously reported promising results with nabilone and oxytocin. Unfortunately, we have been unable to replicate these latter results. We have also been unable to obtain positive results with ondansetron, scopolamine, losartan, busprione, spironolactone, and sulfasalazine.

2.1.2. McGill University. As part of the animal behaviour research of this project, our goal is to assess the ability of different candidate drugs (or combinations thereof) to block reconsolidation of auditory fear memories. A typical reconsolidation experiment entails conditioning rats on day 1 to fear a tone paired with a footshock. The next day rats receive a presentation of the tone in order to reactivate the memory, immediately followed by an injection of the drug or its vehicle. Then on day 3 and day 10, the rats receive another presentation of the tone and the freezing behaviour (the conditioned response (CR) we are measuring) is quantified. If the drug is an amnestic (i.e., reconsolidation-blocking) agent, the CR should be reduced in animals that received the candidate drug.

During the 03 project year that just ended, we focused our efforts on investigating the reconsolidation blockade of auditory fear memories using the α-2-adrenergic agonist, clonidine, injected immediately after a reactivation session in male and female rats. Our results showed a significant impairment of the CR in the clonidine-treated animals compared to the controls at various doses (50, 100, 200 µg/kg), hence, confirming clonidine's effectiveness as a reconsolidation blocker (figure 1). For all tested doses, results showed no main effect of sex or interactions, a significant main effect of treatment ($p<0.05$) and days ($p<0.05$) and a significant treatment x day interaction for the 100 µg/kg dose ($p<0.05$). Clonidine disrupted the fear-related memory in a dose-dependent manner, reaching its maximum potential at 100µg/kg; increasing the dose further did not lead to a greater impairment of the CR.

Furthermore, we evaluated clonidine's selectivity by testing its effect on short-term memory. If the observed CR impairment is due to reconsolidation blockade, then animals should not show a decreased CR 4 hours after reactivation but should be impaired 24 hours later. Consistent with our prediction, the results revealed that post-reactivation administration of clonidine did not impair short-term fear memories (figure 2a). Clonidine-treated rats showed

similar CR to the vehicle group when tested 4 hours after reactivation, but showed impaired behaviour a day after injection ($p< 0.001$). We have also investigated clonidine's effects on non-reactivated memories. If clonidine's ability to impair fear-related CR is selective to reactivated memories, then animals that did not receive a reactivation session should show an intact fear response. Again, our results were consistent with this prediction, confirming that clonidine selectively impairs reactivated fear memories (figure 2b). No main effect of sex was observed.

In order to rule out the possibility that clonidine could induce a permanent learning impairment, we re-conditioned animals to fear a new tone using a different auditory fear protocol. After receiving a post-reactivation injection of clonidine (200µg/kg) or vehicle, and a test of memory retention one day and 7 days later, rats were trained again and tested for memory to the new tone. We hypothesized that if the clonidine-related memory impairment is selective to reconsolidation blockade, then the fear response of the previously treated animals should be similar to the controls. Our data demonstrated that post-reactivation administration of clonidine did not impair the ability to learn new fear memories as both groups exhibited similar CR on test day (figure 3).

More recently, we explored the effect of clonidine (100µg/kg) in combination with a pre-reactivation injection of D-cycloserine (15mg/kg), a partial NMDA agonist. This choice was motivated by a recent publication from Bustos et al. (2010), in which they show that D-cycloserine can enhance memory lability and make a resistant memory more susceptible to disruption by amnestic agents. We injected D-cycloserine 30 minutes prior and clonidine immediately after reactivation, and then tested for memory retention the next day and a week later. We hypothesized that this drug combination would induce a more pronounced decrease of the fear-related memory response than clonidine alone. Our results again showed reconsolidation blockade by clonidine, whereas administration of D-cycloserine alone did not have any effect on the memory at the same time points compared to the control group. Interestingly, the combination of D-cycloserine and clonidine did not provide a greater impairment than clonidine alone; rather DCS appeared to reverse the effect of clonidine (figure 4). Animals treated with the drug combination showed a similar CR to controls both one day and one week after receiving the treatment. Overall, the results show a significant main effect of day ($p<0.0001$) and a treatment x day interaction ($p<0.05$), but not a significant main effect of treatment ($p=0.055$). We concluded that this drug combination is not effective at blocking memory reconsolidation in the specific task and parameters we used in this study. Consequently, we decided not to pursue the use of this combination any longer. We have a manuscript in preparation that reports all of the above results.

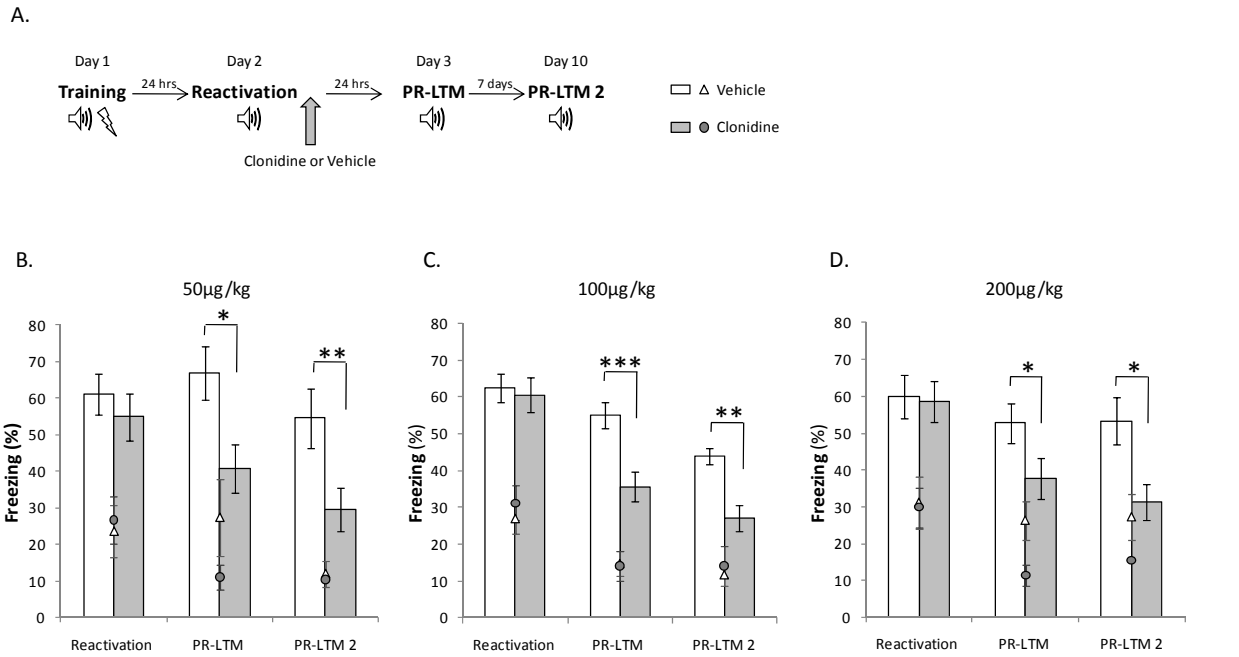

Figure 1. Post-reactivation administration of clonidine impairs reconsolidation of auditory fear memories. (A) Schematic of the experimental design. Rats received a single systemic injection of clonidine or its vehicle immediately after a reactivation session and were tested for post-reactivation long-term memory one day (PR-LTM) and 1 week later (PR-LTM 2). A dose of 50µg/kg (B; n=12), 100µg/kg (C; n=25) and 200µg/kg (D; n=14) was effective at impairing memory reconsolidation compared to the vehicle group (respectively n=10, n=25, n=14) as shown by an impaired conditioned response (freezing) at both time points. Bars represent mean ± s.e.m of freezing to the tone. Markers represent the mean ± s.e.m of freezing prior to the onset of the tone. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

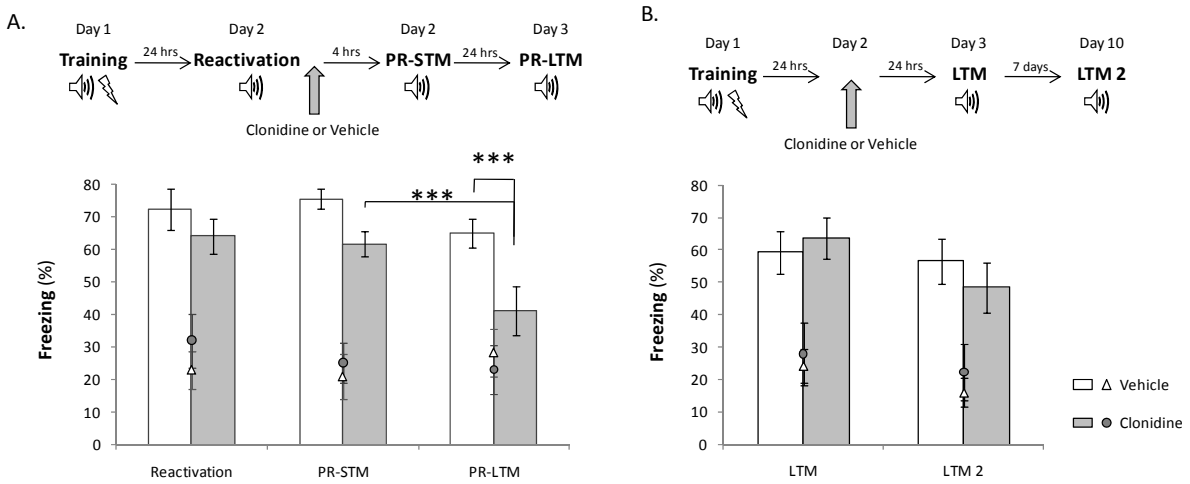

Figure 2. Reconsolidation blockade by clonidine does not impair short-term memory and is selective to reactivated fear memories. (A) Rats received a single systemic injection of clonidine (100µg/kg) or its vehicle immediately after a reactivation session and were tested for post-reactivation short-term memory (PR-STM) 4 hours later and for post-reactivation long-term memory (PR-LTM) 1 day later. Clonidine-treated rats (n=12) showed similar conditioned

response (freezing) than the vehicle group (n=12) when tested 4 hours after reactivation, but showed impaired behaviour a day and a week after injection. (B) Rats received a single systemic injection of clonidine (100µg/kg) or its vehicle without a reactivation session and were tested for long-term memory (LTM) retention 1 day and 1 week later. Clonidine-treated rats (n=12) showed similar conditioned response (freezing) than the vehicle group (n=12) when tested 24 hours or a week after injection. Bars represent mean ± s.e.m of freezing to the tone. Markers represent the mean ± s.e.m of freezing prior to the onset of the tone. Statistical significance: ***p < 0.001.

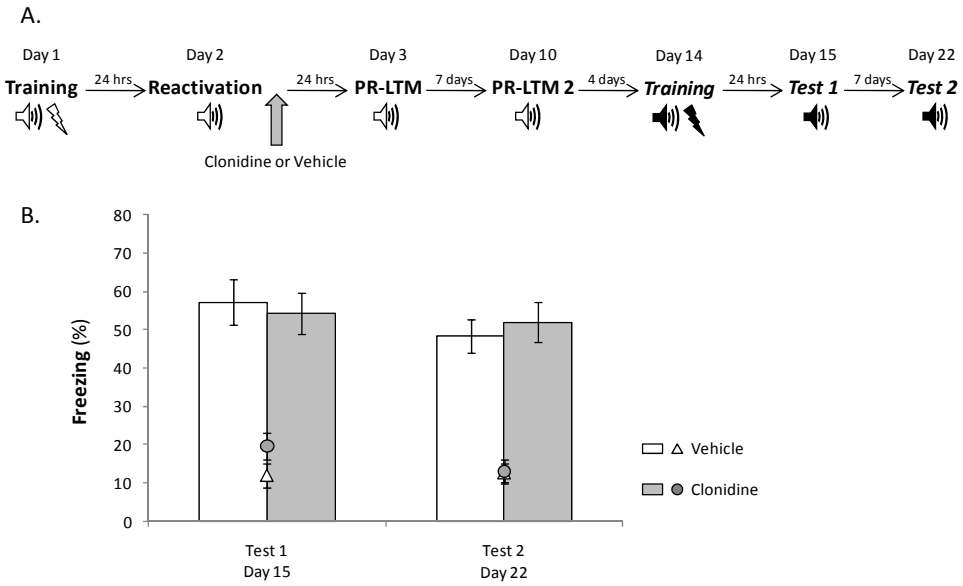

Figure 3. Post-reactivation administration of clonidine does not impair the ability to learn new fear memories. (A) Schematic of the experimental design. After receiving a post-reactivation injection of clonidine (200µg/kg) or vehicle, and being tested for memory retention 1 day and a week later, rats were re-conditioned to fear a new tone using a different auditory fear protocol. (B) Rats that previously received clonidine (n=12) showed intact fear behaviour (freezing) compared to the vehicle-treated animals (n=12). Bars represent mean± s.e.m of freezing to the tone. Markers represent the mean ± s.e.m of freezing prior to the onset of the tone.

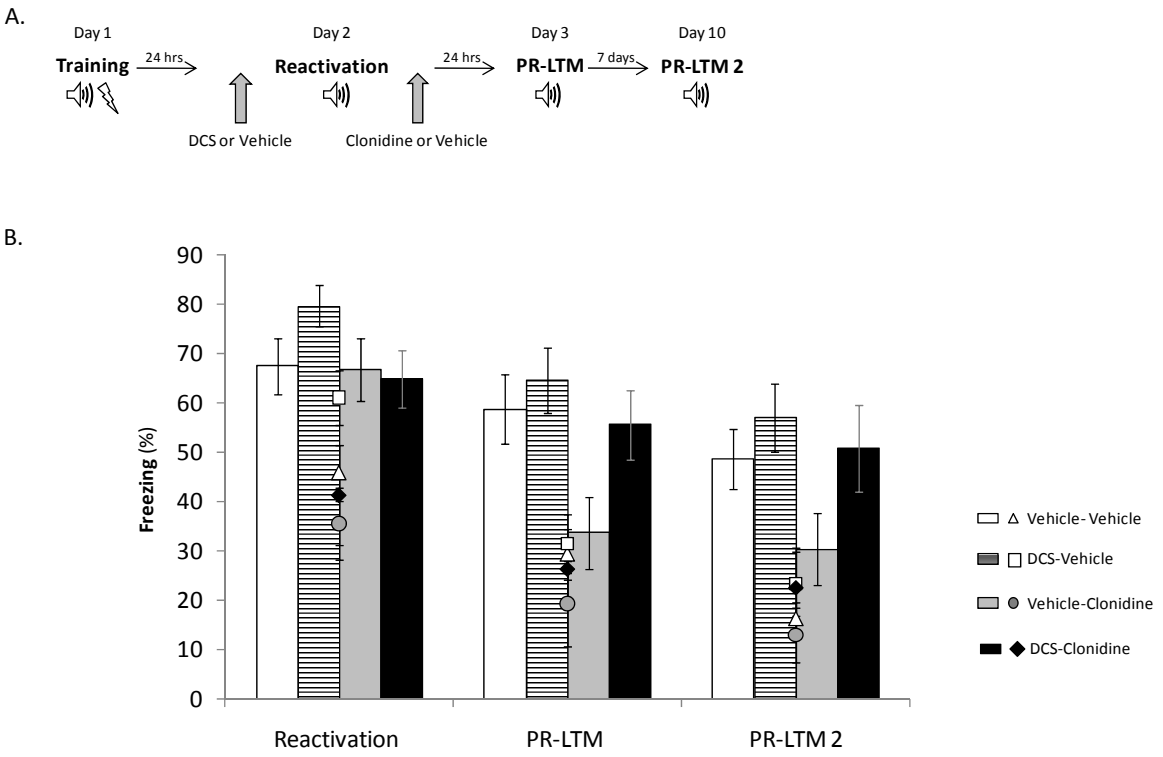

Figure 4. Pre-reactivation administration of D-cycloserine (DCS) combined with post-reactivation administration of clonidine does not impair reconsolidation of fear memories. (A) Schematic of the experimental design. Rats received a pre-reactivation injection of DCS (15mg/kg) or its vehicle and a post-reactivation injection of clonidine (200µg/kg) or vehicle, and were tested for post-reactivation long-term memory retention 1 day (PR-LTM) and 1 week later (PR-LTM 2). Vehicle-clonidine-treated rats (n=11) showed an impaired conditioned response (freezing) compared to the vehicle-vehicle group (n=12) when tested 24 hours or a week after injection. The DCS-vehicle (n=11) and DCS-clonidine (n=11) groups exhibited an intact conditioned response when compared to the control group. Bars represent mean ± s.e.m of freezing to the tone. Markers represent the mean ± s.e.m of freezing prior to the onset of the tone.

2.1.3. <u>McLean Hospital.</u> In our experiments, we are training rats in the auditory fear-conditioning paradigm and then relating changes in synaptic transmission in afferent inputs to the amygdala to fear memory following fear conditioning and fear memory reconsolidation. We are testing the ability of different compounds, blocking fear memory reconsolidation, to prevent changes in synaptic transmission in inputs to the amygdala associated with fear memory recall. Spague-Dawley rats (250-300 g) were trained in a single-trial fear-conditioning paradigm. The rats were conditioned on the training day and tested at 24 h post-training in the second context. One hour later, the rats were used for electrophysiological recordings. In these experiments, we confirmed that synaptic strength in thalamic input to the LA, as assessed by input-output curves for AMPA receptor-mediated EPSCs, is significantly increased in slices from fear-conditioned rats compared to control animals. The fear learning-associated increases in synaptic function at thalamo-LA synapses were not accompanied by changes in membrane excitability of neurons in the LA. These findings are consistent with the notion that the acquisition of fear memory to auditory conditioned stimuli (CS) is associated with synaptic strengthening in the CS pathways.

2.1.3.1. Published manuscript. This project period saw the publication of basic electrophysiological findings relevant to two different forms of long-term potentiation in the amygdala (Shin et al, 2010-reprint attached), which was partially supported by this grant. An abstract of this publication follows:

2.1.3.1.1. Abstract. Synaptic rules that may determine the interaction between coexisting forms of long-term potentiation (LTP) at glutamatergic central synapses remain unknown. Here, we show that two mechanistically distinct forms of LTP could be induced in thalamic input to the lateral nucleus of the amygdala (LA) with an identical presynaptic stimulation protocol, depending on the level of postsynaptic membrane polarization. One form of LTP, resulting from pairing of postsynaptic depolarization and low-frequency presynaptic stimulation, was both induced and expressed postsynaptically ("post-LTP"). The same stimulation in the absence of postsynaptic depolarization led to LTP, which was induced and expressed presynaptically ("pre-LTP"). The inducibility of coexisting pre- and postsynaptic forms of LTP at synapses in thalamic input followed a well-defined hierarchical order, such that pre-LTP was suppressed when post-LTP was induced. This interaction was mediated by activation of cannabinoid type 1 receptors by endogenous cannabinoids released in the lateral nucleus of the amygdala in response to activation of the type 1 metabotropic glutamate receptor. These results suggest a previously unknown mechanism by which the hierarchy of coexisting forms of long-term synaptic plasticity in the neural circuits of learned fear could be established, possibly reflecting the hierarchy of memories for the previously experienced fearful events according to their aversiveness level.

2.1.3.2. Manuscript under review. This project period also saw the submission of a manuscript directly related to the objectives of this project which was fully funded by this grant. The manuscript has been accepted for a full review in *Science*. An abstract of this manuscript follows:

2.1.3.2.1. Abstract. Retrieval of stored memories renders them labile, activating the protein synthesis-dependent processes of memory reconsolidation. The underlying cellular mechanisms of postretrieval memory reconsolidation are not completely understood. Here, we show that the mammalian target of rapamycin (mTOR) kinase-dependent signaling mediates stabilization of fear conditioning-produced synaptic strengthening in the conditioned stimulus pathways following memory recall, thus providing a postretrieval memory update mechanism.

2.1.3.2.2. Figures. The two figures below present the substance of the findings.

FIGURE LEGENDS

Fig. 1. mTOR activity is necessary for reconsolidation of conditioned auditory freezing and maintaining stability of fear conditioning-induced synaptic enhancements. (**A**) A schematic representation of the experimental design. Rats were trained in a single-trial fear conditioning paradigm (*8*) and tested at 24 h (PR-LTM 1) or 48 h (PR-LTM 2) after reactivation trials. (**B**) Percent freezing observed in fear-conditioned rats (CS-US, Paired) and in rats that received CS only (CS-US, n = 23 rats; CS-only, n = 17 rats. 7 rats from the Paired group and 5 rats from the CS-only group received a second re-test (PR-LTM2). (**C**) Left, averaged EPSCs evoked in thalamic input to the LA by presynaptic stimuli of increasing intensity in slices from Naïve (10 rats), CS-only, and Paired groups of rats. Traces are averages of 10 EPSCs. Right, synaptic input-output curves obtained in thalamic input to the LA (Naïve, n = 26 neurons; CS-only, n = 16 neurons; Paired, n = 14 neurons). Peak amplitudes of the EPSCs were significantly different between Naïve, CS-only, and Paired groups (two-way ANOVA, $F_{2,313} = 11.35$, $P < 0.001$). *Post hoc* Bonferroni's simultaneous multiple comparisons revealed significant differences in the EPSC amplitudes between Naïve and Paired groups ($P < 0.001$), and between CS-only and Paired groups ($P = 0.003$). Thus, synaptic strength in thalamic input was enhanced in fear conditioned rats (Paired group). (**D**) In cortical input, peak amplitudes of the EPSCs also differed significantly between Naïve (n = 16), CS-only (n = 8), and Paired (n = 13) groups (two-way ANOVA, $F_{2,213} = 14.48$, $P < 0.001$). EPSC amplitudes were larger in the Paired group compared with either Naïve ($P < 0.001$) or CS-only group ($P < 0.001$; Bonferroni's simultaneous multiple

comparisons). (**E**) A schematic representation of the experiments where fear-conditioned rats received a postretrieval injection of rapamycin (RAP; 20 mg/kg, i.p.) or vehicle (VEH). (**F**) Rapamycin impairs reconsolidation of auditory fear conditioning. There was no significant difference in percent freezing between VEH-treated (n = 29) and RAP-treated (n = 29) rats during memory reactivation (*t*-test, $P = 0.74$). A significant impairment was observed during the PR-LTM test (see text for details). (**G**) Left, averaged EPSCs evoked in thalamic input to the LA by stimuli of increasing intensity in slices from fear-conditioned VEH and RAP rats. Right, synaptic input-output curves obtained in thalamic input in slices from both groups of rats (VEH, n = 17 neurons; RAP, n = 16 neurons (two-way ANOVA, $F_{1,138} = 101.4$, $P < 0.001$ for VEH group versus RAP group of conditioned rats). (**H**) Experiments were analogous to G, but the EPSCs were recorded in cortical input to the LA (VEH, n = 13 neurons; RAP, n = 12 neurons; two-way ANOVA, $F_{1,104} = 27.58$, $P < 0.001$). Results are shown as means ± SEM.

Fig. 2. Mechanisms of fear conditioning-induced synaptic strengthening in inputs to the LA. (**A**) Left, examples of EPSCs evoked in thalamic input to the LA with paired presynaptic stimuli in slices from behaviorally naïve, CS-only and fear-conditioned (CS-US) rats. The interstimulus interval was 50 ms. Traces are averages of 10 paired EPSCs. Right, summary plot of the paired-pulse stimulation experiments. Paired pulse ratio (PPR) was calculated as the ratio of the second EPSC amplitude to the first EPSC amplitude. Naïve group of rats, n = 23 neurons; CS-only group, n = 21 neurons; Paired group, n = 14 neurons. The magnitude of PPR in the Paired group of rats was significantly decreased compared to Naïve (*t*-test, $P = 0.03$) and CS-only (*t*-test, $P = 0.02$) control rats. There was no difference in PPR values between Naïve and CS-only groups (*t*-test, $P = 0.45$). (**B**) Experiments were analogous to A, but the EPSCs were recorded in cortical input to the LA. Naïve group, n = 20 neurons; CS-only group, n = 11 neurons; Paired group, n = 13 neurons (*t*-test, $*P = 0.039$ and $**P = 0.009$ versus Naïve and CS-only rats, respectively); no difference between Naïve and CS-only rats (*t*-test, $P = 0.1$). (**C**) Traces of the asynchronous quantal EPSCs evoked by stimulation of thalamic input (V_H= –70 mV) in slices from the CS-only and Paired rats. In these experiments, Sr^{2+} was substituted for extracellular Ca^{2+}. (**D**) Top, cumulative amplitude histograms of asynchronous quantal events recorded in thalamic input to the LA in slices from the CS-only and Paired groups. Bottom, summary plot of asynchronous EPSCs data (mean amplitude; CS-only, n = 10 neurons; Paired, n = 9 neurons; *t*-test, $P = 0.34$). (**E** and **F**) Experiments were analogous to C and D, but the asynchronous EPSCs were recorded in cortical input to the LA (CS-only, n = 5 neurons; Paired, n = 7 neurons; *t*-test, $P = 0.73$). Error bars indicate SEM.

Fig. 3. Synaptic mechanisms of postretrieval stabilization of conditioning-induced potentiation in inputs to the LA. (**A**) Left, examples of EPSCs evoked in thalamic

input to the LA with paired stimuli in slices from fear-conditioned rats that received one injection of either rapamycin (RAP; 20 mg/kg, i.p.) or vehicle (VEH) immediately after the fear memory test (memory was retrieved at 24 h post-conditioning). Recordings were performed shortly after the re-activation (24 h after the memory test; see Fig. 1E). Right, summary plot of PPR data (vehicle, n = 21 neurons; rapamycin, n = 19 neurons; *t*-test, *P* = 0.79). (**B**) Experiments were analogous to A, but the EPSCs were recorded in cortical input to the LA (VEH, n = 13 neurons; RAP, n = 11 neurons; *t*-test, *P* = 0.31). (**C**) Traces of the asynchronous quantal EPSCs evoked by stimulation of thalamic input in slices from VEH or RAP groups. (**D**) Top, cumulative amplitude histograms of asynchronous quantal events recorded in thalamic input to the LA in slices from VEH or RAP rats. Bottom, summary plot of asynchronous EPSCs data (mean amplitude; VEH, n = 5 neurons; RAP, n = 7 neurons; *t*-test, **P* = 0.024). (**E** and **F**) The experiments were analogous to C and D, but the asynchronous EPSCs were recorded in cortical input to the LA (VEH, n = 5 neurons; RAP, n = 6 neurons; *t*-test, **P* = 0.013). Error bars indicate SEM.

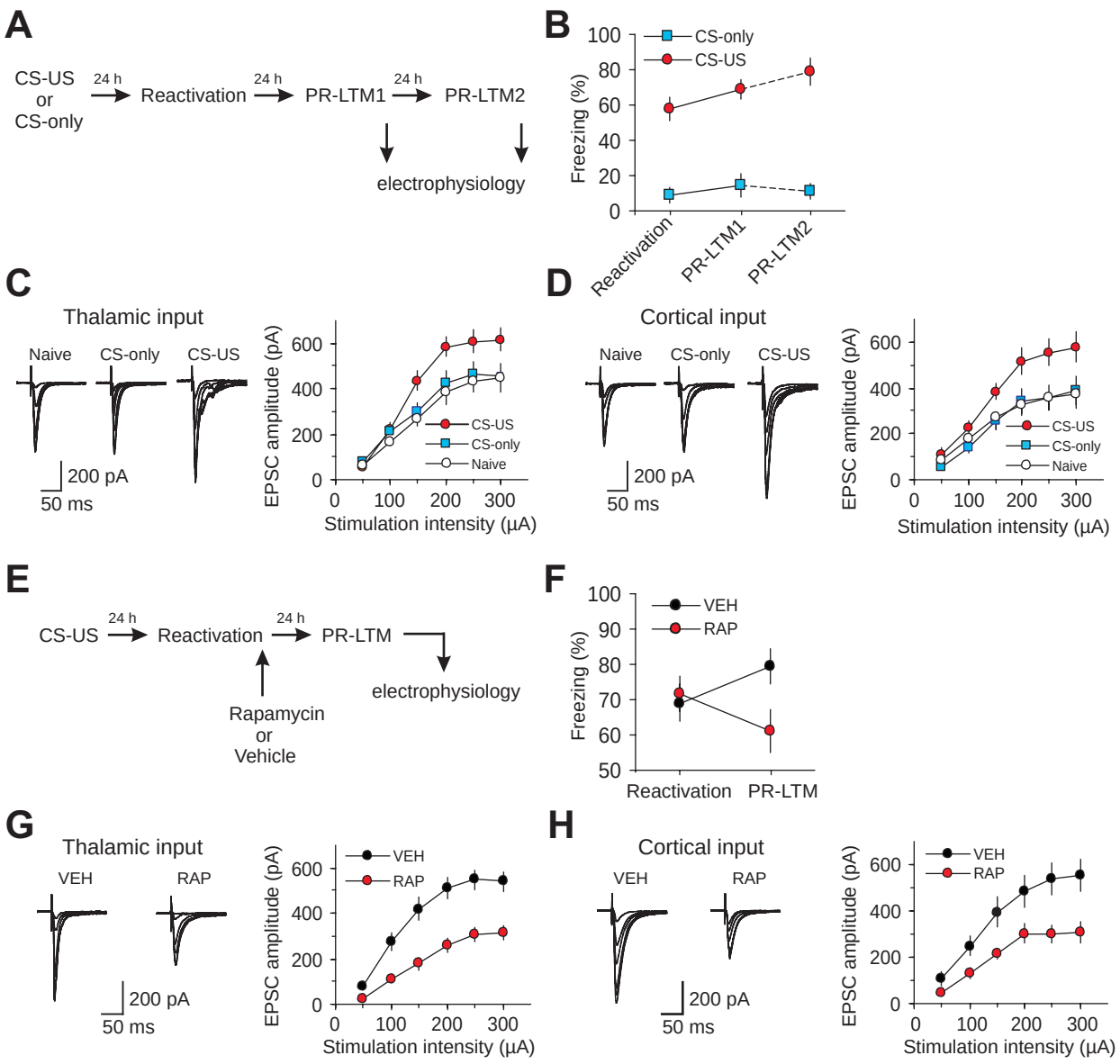
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Fig. 1

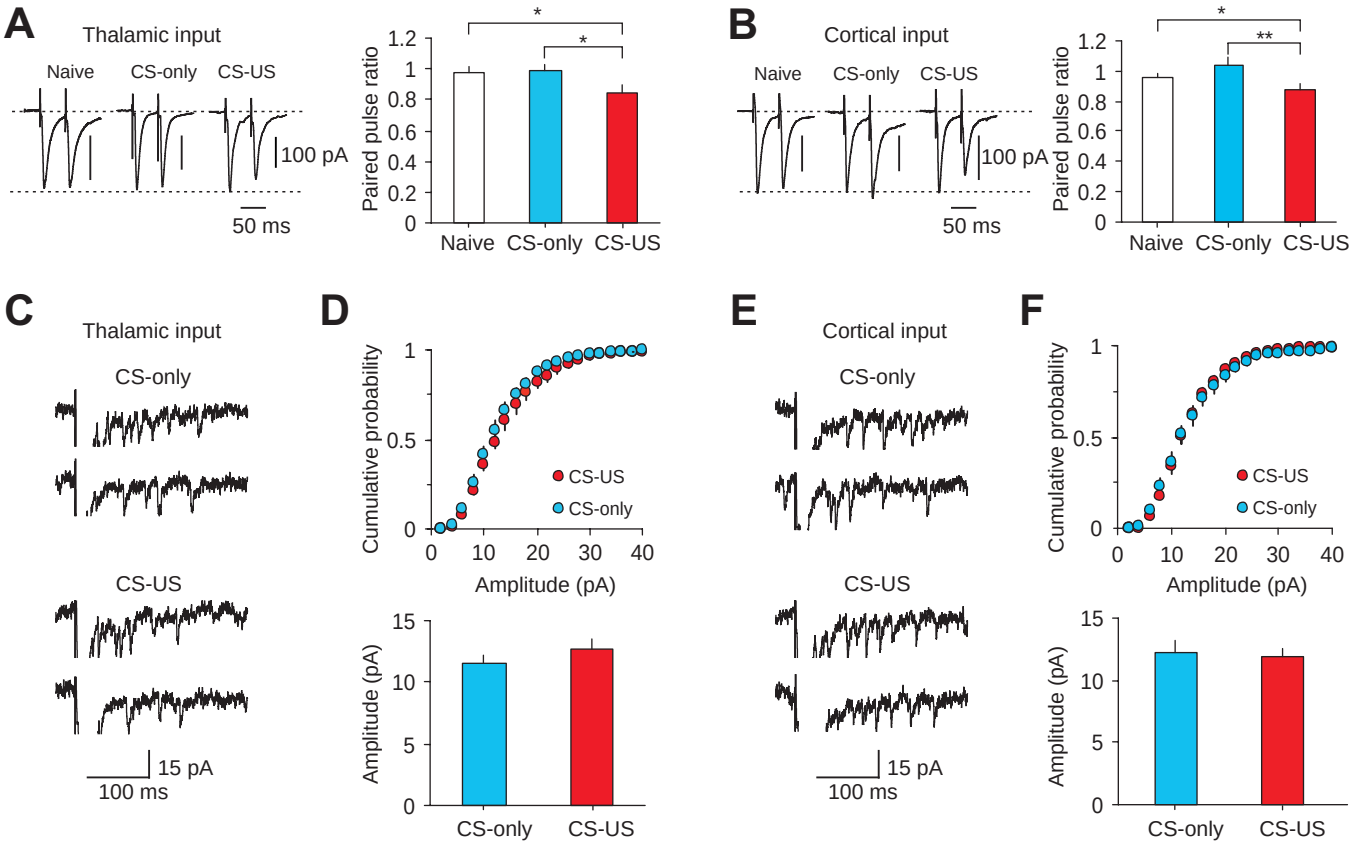
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Fig. 2

2.4. Human work

2.4.1 MGH. On the basis of the animal results reported in §2.1.1.1 and §2.1.2.1 above, we decided to perform a double-blind, placebo-controlled study of post-reactivation mifepristone's ability to reduce psychophysiologic responding during traumatic imagery in trauma-exposed human subjects. At the time of the last annual report, we had succeeded in obtaining an investigational new drug (IND) approval from the FDA for this novel post-marketing application of mifepristone, and we had obtained all necessary IRB approvals for this study. We had also completed negotiated a contract between Danco Laboratories and MGH to provide the drug at cost. Finally, we had completed running 3 subjects. During the 03 year, we completed running 21 additional subjects, for a total number of recruited subjects to date of 24. Two more subjects are in the midst of participation. Three more subjects are scheduled to be studied in the near future. We have not yet broken the blind.

2.4.2. McGill University/Douglas Mental Health University Institute

2.4.2.1 Background for current study. We decided to undertake with a double-blind, randomized, placebo-controlled trial of a series of six sessions of post-reactivation propranolol for the treatment of PTSD. Several considerations motivated this decision. First, an influential article published in early 2009 succeeded in demonstrating that propranolol blocked the reconsolidation of a conditioned fear memory in normal humans (Kindt al, 2009), in a sense bypassing the need for further confirmatory rat studies. Second, in previously published work, we succeeded in demonstrating that a single session of propranolol following reactivation of the traumatic memory in PTSD patients significantly reduced a biological PTSD marker, viz., physiologic responding during subsequent script-driven imagery of the event (Brunet et al, 2008). Third, an analysis of a previously collected data set from an open label, six session, post-reactivation propranolol case series in 32 PTSD patients yielded promising results. Results from that work serve as the basis for the double-blind, randomized, placebo-controlled trial that is now underway. The study is looking at the therapeutic effects of six weekly treatment sessions consisting of reactivating the trauma memory while under the influence of either propranolol or placebo. The therapeutic effects are measured in two ways: (1) PTSD symptoms before, during and up to four months after the treatment, and psychophysiologic responding to script-driven imagery depicting the person's traumatic event (post-treatment and at follow-up).

2.4.2.1.1. Progress to date. At the time of the last annual report, 9 patients had completed the treatment protocol. During the 03 year, and additional 16 patients completed the protocol. Overall, a total of 60 participants have been screened since study startup, 36 of whom received some treatment, and 25 of whom have completed the protocol. An additional 4 subjects are in the midst of study participation. We have not yet broken the blind.

2.4.2.2. Published open label study. During the 03 year, we also published the results of 3-open-label propranolol reconsolidation studies (Brunet et al, 2011-reprint attached). A summary of the results follows:

2.4.2.2.1. Results. In three independent studies that took place in three different countries with men and women, six brief trauma reactivation sessions under the influence of propranolol brought about large PTSD symptom improvements. Such results extend our previous placebo-controlled psychophysiological results (Brunet et al, 2008) in two important ways. First, recalling one's traumatic experience under the influence of propranolol received on six occasions, rather than just once, produced a much larger symptom reduction, thereby

demonstrating more clearly the clinical potential of this novel approach. The effect sizes reported compare favorably to those produced by exposure-based psychotherapies, yet they were obtained using a different approach that involves fewer and shorter sessions and virtually no side effects. Second, the treatment effects were shown to persist over time. One explanation for our results is that propranolol blocked the reconsolidation of the traumatic memory, which in turn led to symptom reduction. Another potential explanation for the present findings is that the intervention induced extinction. Although we cannot rule out such an explanation, extinction-based treatment sessions are typically prolonged and involve a greater number of sessions. In fact, brief exposures may exacerbate symptoms.The conclusion that propranolol was necessary for symptom improvement must await results of a double-blind, randomized, placebo-controlled trial (which is currently underway as part of the present project as described in §2.4.2.1)

3. KEY RESEARCH ACCOMPLISHMENTS

3.1. Published original discovery that the anti-progesterone and glucocorticoid receptor antagonist mifepristone, when administered systemically, reduce reconsolidation of a cue-conditioned fear response in rats. Further original discovery that the beta-adrenergic blocker propranolol blocks this mifepristone effect. (Status: published)

3.2. Original discovery that the post-reactivation administration of clonidine impairs reconsolidation of auditory fear memories in rats. (Status: in preparation for publication).

3.3. Original discovery that the mammalian target of rapamycin (mTOR) kinase-dependent signaling mediates stabilization of fear conditioning-produced synaptic strengthening in the conditioned stimulus pathways following memory recall, thus providing a postretrieval memory update mechanism. (Status: under full review in *Science)*.

3.4. Progress in studying human subjects in a double-blind controlled study of post-reactivation mifepristone's ability to reduce psychophysiologic responding during traumatic imagery in trauma-exposed human subjects. (Status: study underway, blind not yet broken).

3.5. Progress in studying human subjects in a randomized, double-blind controlled study of six sessions of post-reactivation propranolol for the treatment of PTSD. (Status: study underway, blind not yet broken).

4. REPORTABLE OUTCOMES

Brunet A, Poundja1 J, Tremblay J, Bui E, Thomas E, Orr SP, Azzoug A, Birmes P, Pitman RK. Trauma reactivation under the influence of propranolol decreases PTSD symptoms: 3 open-label trials. *Journal of Clinical Psychopharmacology* 2011;31:547-550.

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5. CONCLUSION

Animal and human studies offer promise for the development of a novel treatment for PTSD based upon pharmacological blockade of memory reconsolidation. We have identified three promising candidate drugs that are approved for human use, viz., propranolol, mifepristone, and clonidine. Randomized, placebo-controlled, double-blind clinical trials are underway to test two of these drugs (propranolol and mifepristone).

6. REFERENCES (in addition to those already presented in §4 above)

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7. APPENDICES/SUPPORTING DATA

Supporting data are presented within the body of §2 above.

48 and 56. Retrospective chart review revealed that this participant has a history of weight instability and also experienced an alteration in her psychotropic medication regimen that corresponded with the period of weight gain. It was noted that the other participant who gained weight (4 kg) had been participating in community reintegration activities outside the hospital during the period of weight gain, where the participant's diet was not controlled.

DISCUSSION

The present findings offer encouragement that a modest contribution to weight management for patients on psychotropic polypharmacy regimens may be provided by metformin, irrespective of minimal participation in exercise programming. Furthermore, the data show that weight loss continued over a 40-week period. However, metformin's effect on weight is modest and perhaps not sufficient to stave off the high risk of early mortality in this population. Indeed, it remains to be seen whether metformin's effect can be sustained in the long term for SMI patients, especially in regard to delaying the onset of T2D and metabolic syndrome as has been demonstrated with healthy adults in the ADA's Prevention of Diabetes Program.[10]

Given our duty as health care providers to reduce the risk of our treatments (ie, psychiatric medications) to our patients, we are obligated to take an aggressive approach to weight management. Metformin therapy seems to be a good starting point. However, although group data from this performance improvement project demonstrate a positive effect of metformin on weight for almost 1 year, at the individual level, not all participants benefited from the intervention. Consequently, we would suggest that combined trials of metformin and other weight-modulating drugs be initiated (see Maayan et al[11] for a review of the efficacy of various weight-reducing drugs), with the goal of increasing the range of medications available for those patients who do not respond to metformin therapy. Future work on the genetics of responsiveness to metformin may eventually allow for its more efficient use. Indeed, Zhang et al[12] has provided evidence for the involvement of the leptin promotor in clozapine-associated weight gain, and Fernandez et al[6] has provided evidence for the association of the leptin promoter in metformin response. Thus, future research on the pharmacogenomics of metformin may prove especially helpful for guiding metformin therapy.

AUTHOR DISCLOSURE INFORMATION

The authors declare no conflicts of interest.

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Trauma Reactivation Under the Influence of Propranolol Decreases Posttraumatic Stress Symptoms and Disorder
3 Open-Label Trials

To the Editors:

The β-adrenergic receptor blocker propranolol, when administered shortly after the reactivation of conditioned fear and other memories, can reduce the strength of those memories through blockade of reconsolidation in animals.[1] Propranolol also can reduce the strength of newly acquired emotional memories in healthy participants[2] and in some[3,4]—but not all[5]—trauma-exposed clinical samples. Propranolol also may attenuate the emotional strength of long-standing traumatic memories in posttraumatic stress disorder (PTSD) and, therefore, represent a novel treatment approach. In a small randomized controlled trial,[6] postreactivation propranolol produced a decrease 1 week later in physiological responding during traumatic script-driven imagery. Propranolol also reduced PTSD symptoms, but the advantage over placebo, 19% versus 11%, was not significant. However, only 1 dose of treatment was provided in this proof-of-concept study. We wondered whether a greater number of treatment sessions would lead to a clinically meaningful improvement, and whether this improvement would be long lasting. We report on the results of 3 independent open-label trials designed to examine these questions.

METHOD

Participants

Participants were aged 18 to 65 years and met *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text*

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Systemic Mifepristone Blocks Reconsolidation of Cue-Conditioned Fear; Propranolol Prevents This Effect

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Reducing reconsolidation of reactivated traumatic memories may offer a novel pharmacological treatment for posttraumatic stress disorder (PTSD). Preclinical research is needed to identify candidate drugs. We evaluated the ability of postreactivation mifepristone (RU38486, a glucocorticoid antagonist), alone and in combination with propranolol (a beta-adrenergic blocker), both given systemically, to reduce cue-conditioned fear in rats. On Day 1, a 30-s tone conditioned stimulus (CS) was paired with an electric shock unconditioned stimulus (US). On Day 2, the CS was presented without the US (reactivation), and the freezing conditioned response (CR) was measured. This was immediately followed by subcutaneous injection of vehicle, mifepristone 30 mg/kg, propranolol 10 mg/kg, or both. On Day 3, the CR was again measured as a test of postreactivation long-term memory (PR-LTM). On Day 10, the CR was again measured to evaluate spontaneous recovery. On Day 11, the US was presented alone (reinstatement). On Day 12, the CR was again measured. A fifth group received mifepristone without the CS presentation (nonreactivation) on Day 2. A sixth group was tested four hours after the Day 2 mifepristone injection to measure postreactivation short-term memory. Postreactivation, but not nonreactivation, mifepristone produced a decrement in the CR that did not undergo spontaneous recovery and underwent only modest reinstatement. Mifepristone did not exert its effect when administered concurrently with propranolol. Postreactivation mifepristone did not impair short-term memory. Systemic mifepristone blocks the reconsolidation of cue-conditioned fear in rats. Concurrent administration of propranolol prevents this effect. Postreactivation mifepristone may be a promising treatment for PTSD, but not necessarily in combination with propranolol.

Keywords: memory, conditioning, classical, fear, mifepristone, propranolol (all MeSH terms)

Reconsolidation is a memory process that has been studied largely during the last decade. It has long been recognized that when something is first learned, for example a conditioned fear response, its trace exists in an unstable state in the brain. In order for its memory to be retained, it must be converted to a stable state through a process known as consolidation. Reconsolidation theory holds that when the stabilized memory is reactivated (retrieved) under certain circumstances, it returns to an unstable state, from which it must be reconsolidated if it is to endure (Nader & Hardt, 2009). The reconsolidation process has mainly been revealed through its blockade. When certain drugs are administered shortly after reactivation, subsequent testing finds the memory to be diminished (Abrari, Rashidy-Pour, Semnanian, & Fathollahi, 2008; Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Muravieva & Alberini, 2010; Nader, Schafe, & Le Doux, 2000; Przybyslawski, Roullet, & Sara, 1999; Taubenfeld, Riceberg, New, & Alberini, 2009).

In contrast to reconsolidation, extinction is a process whereby new learning inhibits the expression of old learning, for example, learning to no longer fear a previously feared object or situation (Milad, Rauch, Pitman, & Quirk, 2006; Quirk & Mueller, 2008). Although the original learning is no longer behaviorally evident, its continuing presence is revealed under certain circumstances. An

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extinguished behavior may return with the passage of time (spontaneous recovery; Quirk, 2002). It may also become evident when testing takes place in a context other than that in which it was extinguished (renewal), and it may be made to return by readministration of the unconditioned stimulus (US) alone (reinstatement; Bouton, 2002). Because memories that have undergone reconsolidation blockade putatively do not undergo spontaneous recovery (Bustos, Maldonado, & Molina, 2006; Duvarci & Nader, 2004; Lin, Mao, & Gean, 2006; Jin et al., 2007), renewal (Duvarci & Nader, 2004), or reinstatement (Bustos et al., 2006; Duvarci & Nader, 2004; Lin et al., 2006), it is inferred that they have been erased, although this is not universally accepted (McGaugh, 2004).

Reports of studies of reconsolidation blockade in animals not infrequently conclude with the suggestion that this mechanism could lead to a novel translational treatment for posttraumatic stress disorder (PTSD). A central feature of PTSD is an overly strong, distressing memory of the causal traumatic event. If these memories could be weakened, substantial suffering might be alleviated. Given that declarative memory and conditioning are mediated by different brain systems and hence are at least partly dissociable, the ideal outcome would be for the patient to retain the declarative ("factual") memory of the traumatic event but lose the associated intense emotion, which has been conceptualized as a conditioned response (CR). Although such a scenario may appear far-fetched, two of the few preclinical human reconsolidation blockade studies, which employed fear conditioning, showed that following the administration of systemic propranolol (a beta-adrenergic blocker that has been reported to block reconsolidation in some animal studies) at the time of memory reactivation, subjects no longer showed the conditioned fear response, but they retained declarative knowledge of the learned contingency (Kindt, Soeter, & Vervliet, 2009; Soeter & Kindt, 2010).

In the only study to date that has attempted to apply reconsolidation blockade to traumatic memories, chronic PTSD patients described their traumatic events, thereby reactivating the memory (Brunet et al., 2008). Shortly afterward, they were given propranolol or placebo. A week later, they engaged in script-driven mental imagery of the event while physiological responses were recorded. Patients who had received postreactivation propranolol showed significantly smaller responses than those who had received placebo, consistent with weakening of the traumatic memory's emotional component. Although this study did not employ sufficient controls to conclude that reconsolidation blockade was the underlying mechanism, it is a viable explanation. One question that emerges from this line of translational research is whether other drugs could possess even stronger reconsolidation-blocking effects and, therefore, be candidates for trials in PTSD either alone or in combination with propranolol. Unfortunately most rat reconsolidation studies employ drugs that either are administered intracerebrally or are too toxic for humans, usually both. Candidate drugs for human use must be capable of safe, systemic administration. It is also desirable that the drug, or drug combination, has been shown to block reconsolidation in animals.

One such candidate drug is the glucocorticoid receptor blocker mifepristone, or RU38486 (most familiar for its use as an abortifacent). Both intraamygdala (Jin et al., 2007) and systemic (Taubenfeld et al., 2009) mifepristone have been shown to block reconsolidation of fear learning in an inhibitory avoidance paradigm in rats. Although inhibitory avoidance may be relevant to PTSD, cue conditioning may be of greater relevance. Psychological distress and physiological reactivity to trauma-related cues have been encoded as *DSM–IV* PTSD criteria B.4 and B.5, respectively.

The present study attempted further to explore in rats the potential of postreactivation mifepristone as a novel treatment for PTSD by testing whether this drug can block reconsolidation of cue-conditioned fear. Additionally, mifepristone was tried with and without concurrently administered propranolol, in order to explore whether the combination of these two drugs would have stronger reconsolidation-blocking effects than either alone. In PTSD, cue and context are usually not so easily separated as they can be in animal research. For example, a Vietnam veteran may be more likely to become distressed at the sight of an Asian male (cue) at night (context). PTSD veterans' fear responses have been found to be excessively augmented by dangerous contexts (Grillon, Morgan, Davis, & Southwick, 1998). For this reason, unlike in many animal studies, the rats underwent conditioning, reactivation, and testing in the same experimental chamber.

Method

Rats

The procedures were approved by the Subcommittee on Research Animal Care (SRAC) of the Massachusetts General Hospital in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Equal numbers of male and female Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing ~250g were cohoused (two of the same gender per cage) at the Massachusetts General Hospital Center for Comparative Medicine in transparent polyethylene cages and maintained on a 12-hr light (day)/dark (night) schedule with free access to food and water. They were transported to our laboratory for the study's procedures, which were performed in the early afternoon, and returned to the housing facility at the end of each day. On each of the two days prior to the experiment, rats were handled for five minutes and then placed in the conditioning chamber for five minutes of habituation. Each experimental Plexiglas chamber (Coulburn Instruments, Whitehall, PA) measured $25 \times 29 \times 29$ cm and was situated inside a sound-attenuated box (Med Associates, Burlington, VT).

Drugs

Mifepristone (Sigma, St Louis, MO) in a dose of 7.5 mg (approximately 30 mg/kg) was dissolved in 0.5 ml propylene glycol vehicle. Racemic propranolol (Sigma) in a dose of 2.5 mg (approximately 10 mg/kg) was dissolved in 0.1 ml saline vehicle. Drugs were administered subcutaneously.

Experimental Procedures

On each experimental day, rats were placed in the chamber for 2 min. Then a 4-kHz, 80 dB SPL tone (conditioned stimulus, CS) was presented for 30 sec. Duration of freezing served as the CR and was measured via motion-sensing computer software (FreezeScan, Clever Systems, Reston, VA). Scores are presented as percentage of the total duration of the CS. On Day 1,

rats were trained with a single 1-s 0.75mA shock (US) that was delivered via the grid floor and coterminated with the tone. The rats then remained in the chamber for 1 min and then returned to their home cages. On Day 2 the CS was presented without the US (reactivation). Immediately thereafter the rats were removed from the testing chamber and injected with postreactivation (PR) drug. Drugs were not administered on any other day. However, some rats on Day 2 received nonreactivation (NR) mifepristone without being placed in the chamber. On Days 3 and 10 (one and eight days after reactivation respectively) the CS was again presented without the shock, and the CR was calculated as a measure of PR long-term memory (PR-LTM). Here "long-term" means at least one day following memory reactivation. On Day 11 the US was presented in the absence of the CS (reinstatement). On Day 12, the CS again was presented without the shock, and the CR was calculated as a measure of postreinstatement PR-LTM. There were four PR drug groups: Vehicles alone (VEH), mifepristone (MIF), propranolol (PROP), and both mifepristone and propranolol (MIF + PROP). A fifth group received NR mifepristone (NR_MIF) but did not undergo the reinstatement procedure. A sixth group was tested 4 (instead of 24) hrs after the mifepristone injection in order to measure PR short-term memory (PR-STM). Each of the foregoing groups consisted of 12 male and 12 female rats, with the exception that the reinstatement MIF group comprised only half the original number (i.e., six males and six females).

Data Analysis

The raw dependent measure consisted of percent freezing during each CS presentation, that is, the CR. For testing LTM, percent freezing scores were analyzed by means of a repeated-measures, four-factor analysis of variance (ANOVA) with Gender, MIF (present or absent), and PROP (present or absent) as between-rats effects, and DAY as a repeated measure. LTM after nonreactivated mifepristone, and STM after mifepristone, were analyzed by parallel, three-factor ANOVAs. The experiment-wise alpha of 0.05 (two-tailed) was partitioned in the following manner. There were two major, independent, a priori hypotheses: first that mifepristone would block reconsolidation, and second that mifepristone would interact with propranolol in blocking reconsolidation. For tests subsumed under each of these hypotheses, the threshold for statistical significance was $p < .02$. Given that no study drug was administered on Day 2, interactions with Day were expectable under the a priori hypotheses. For analyses not involving the a priori hypotheses, including all gender main effects and interactions, we divided the remaining alpha of 0.01 by the number of results generated by the four-factor ANOVA unrelated to the two major hypotheses, which was 10, yielding a significance threshold of $p < .001$. For the additional ANOVAs described below, a parallel approach was taken.

Results

Postreactivation Long-Term Memory

Figure 1 displays percent freezing for each group on each test day collapsed across Gender. The four-factor ANOVA on percent freezing scores yielded a significant main effect of gender: $F(1, 112) = 10.7$, $p = .001$; least square means with standard errors in parentheses were: male: 59.6 (2.8), female 46.7 (2.8). However, gender did not significantly interact with any other factor The four-factor ANOVA also yielded a significant DAY x MIF x PROP interaction: $F(3, 112) = 11.5$, $p < .0001$. Stratified by DAY, there was a significant MIF x PROP interaction on Day 3: $F(1, 88) = 5.7$, $p < .02$, and on Day 10: $F(1, 88) = 7.4$, $p = .01$. The MIF x PROP interaction was not significant on Day 2 (when testing was conducted prior the study medication) nor on Day 12 (postreinstatement). Inspection of the Figure 1 Day 3 data indicates the only group that showed attenuated freezing was the MIF group. Stratified by PROP, the mifepristone effect was significant in the absence: $F(1, 44) = 13.2$, $p = .001$, but not in the presence: $F(1, 44) = 0.2$, $p = .64$, of propranolol. The Day 10 data show a similar pattern. Stratified by PROP, the mifepristone effect was again significant in the absence: $F(1, 44) = 13.8$, $p = .001$, but not in the presence: $F(1, 44) = 0.1$, $p = .74$, of propranolol.

Comparison of least square means indicated that freezing in the MIF group decreased from Day 2 to Day 3: $t(88) = 10.9$, $p < .0001$, consistent with blockade of memory reconsolidation. The further (nonsignificant) decrease from Day 3 to Day 10 indicates no spontaneous recovery of the CR. To evaluate whether freezing in the MIF group underwent reinstatement, we tested the difference in least square means between Day 10 (prereinstatement) and Day 12 (postreinstatement), which was significant $t(88) = -2.6$, $p = .01$. However, percent freezing in the MIF group on Day 12 was still significantly lower than it had been on Day 2: $t(88) = 5.5$, $p < .0001$. These results indicate only partial reinstatement of the CR in the MIF group.

Nonreactivation Long-Term Memory

Figure 2 displays mean percent freezing on each test day collapsed across gender in rats that were (PR-MIF) versus were not (NR-MIF) presented with the CS prior to mifepristone. A three-factor ANOVA with GENDER and REACTIVATION as between-rat effects and DAY (Days 3 and 10–Day 2 data are unavailable in nonreactivated rats, and Day 12 reinstatement was not studied) as a repeated measure yielded a main effect of REACTIVATION: $F(1, 44) = 26.2$, $p < .0001$. Inspection of Figure 2 indicates that only when mifepristone was preceded by memory reactivation was there a substantial subsequent decrement in conditioned freezing.

Postreactivation Short-Term Memory

Figure 3 displays percent freezing collapsed across gender following the CS presentation during Day 2 reactivation and again either 4 hrs (PR-STM) or 24 hrs (PR-LTM) later in the MIF group. A three-factor analysis of variance with GENDER and memory TERM (PR-STM or PR-LTM) as between-rat effects and DAY (Days 2 and either Day 2 + 4 hr or Day 3–Days 10 and 12 were not studied) as a repeated measure yielded a main effect of memory TERM: $F(1, 44) = 27.4$, $p < .0001$. Rats in the PR-STM group showed virtually no decrease in freezing.

Discussion

The results of the present study replicate and extend those of an earlier inhibitory avoidance study (Taubenfeld et al., 2009)

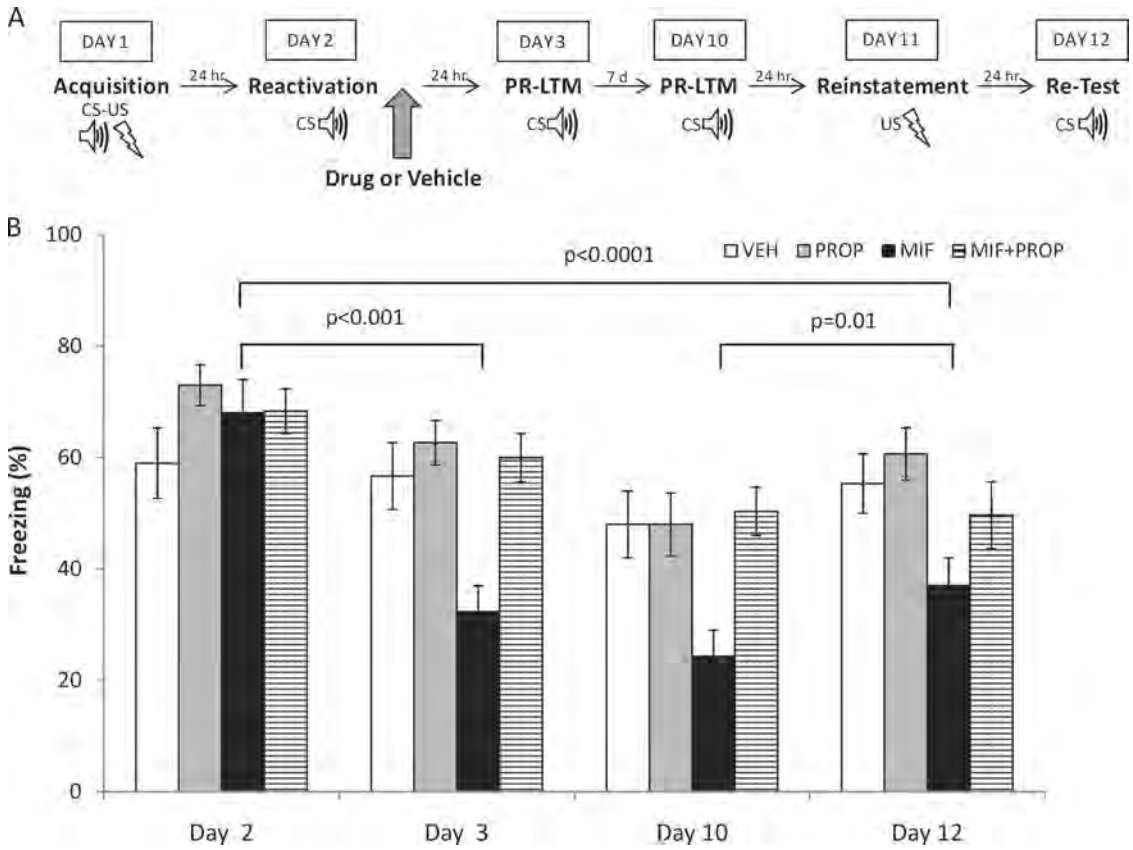

Figure 1. Postreactivation long-term memory (PR-LTM) in the four drug groups. Group mean percentage of freezing to the tone (i.e., conditioned fear response) on Day 2 (reactivation followed by drug), Days 3 and 10 (test days), and Day 12 (test day following reinstatement). See text for details. VEH = vehicle; PROP = propranolol 10 mg/kg; MIF = mifepristone 30 mg/kg; MIF + PROP = both mifepristone and propranolol; Bars = standard error.

by showing that mifepristone administered systemically to rats following the presentation of a previously conditioned fear cue significantly reduced subsequent cue-induced conditioned responding, as manifest in a shorter duration of freezing. The present design incorporated controls necessary to infer that reconsolidation blockade was the mechanism behind this effect. First, the (partial) amnesia for the CS-US association induced by postreactivation mifepristone was relatively long-lasting (for rats), namely, 10 days, that is, there was no evidence of spontaneous recovery. Second, there was only modest reinstatement of the CR in rats that had received mifepristone. Third, nonreactivation mifepristone, that is, drug in the absence of memory reactivation, produced no amnesia. Fourth, when measured four hours following postreactivation mifepristone, the CR was still fully present, whereas it was reduced the next day. Like consolidation, reconsolidation is a time-dependent process that affects long- but not short-term memory.

The present results further suggest that mifepristone is worth exploring in human reconsolidation blockade studies, including as a potential novel treatment for PTSD. A paradoxical result, however, was that concurrent postreactivation propranolol prevented the memory reconsolidation-blocking effect of mifepristone. Propranolol is known to antagonize the memory consolidation-*enhancing* effect of corticosterone by blocking a final common pathway of hormonal modulation of memory, namely, noradrenergic innervation of the basolateral amygdala (Roozendaal et al., 2006). It has been found that basolateral amygdala lesions block not only the memory consolidation-enhancing effect of the glucocorticoid agonist RU28362 (administered intrahippocampally) on inhibitory avoidance, but also the memory consolidation-reducing effect of mifepristone (Roozendaal & McGaugh, 1997). Similar results have been obtained with intraamygdala beta-blockade (Roozendaal B, personal communication of unpublished data). The present results extend these findings to reconsolidation, in that we found that systemic propranolol blocked the reconsolidation-*reducing* effect of mifepristone. This finding suggests that a permissive level of (nor)adrenergic activity is required not only for the memory-enhancing effects of glucocorticoids but also for the memory-reducing effects of their antagonists. The mechanism of this permission remains to be elucidated. From a translational standpoint, the finding that propranolol prevents rather than enhances the reconsolidation-blocking effect of mifepristone, at least in the doses used here, militates against attempting to combine these two drugs in a reconsolidation-blockade treatment approach to PTSD.

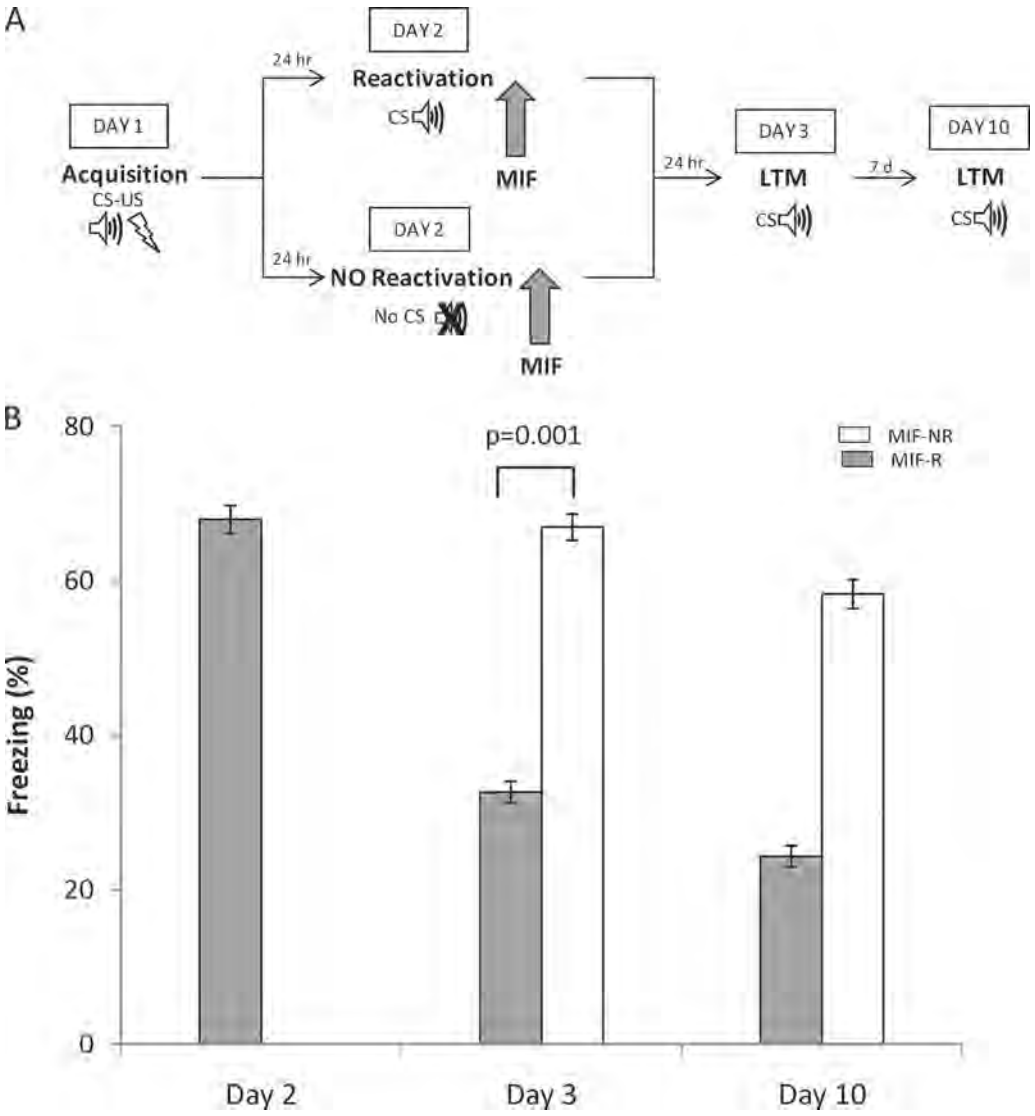

Figure 2. Postreactivation long-term memory (PR-LTM) in the nonreactivated versus reactivated mifepristone groups. Group mean percentage of freezing (i.e., conditioned fear response) on Day 2 (mifepristone preceded or not preceded by reactivation) and Days 3 and 10 (test days). MIF = mifepristone 30 mg/kg; NR = nonreactivation; R = reactivation. No Day 2 data are shown for the NR group because the conditioned stimulus was not presented to this group on that day. Bars = standard error.

In the present study, systemic postreactivation propranolol alone did not block reconsolidation of conditioned fear. This negative result is partially at odds with results of some previously published studies that used the same 10 mg/kg dose as in the present study (Debiec & Ledoux, 2004; Muravieva & Alberini, 2010; Przybyslawski et al., 1999) or nearly the same dose (5 mg/kg; Abrari et al., 2008). The discrepancy might be explained by design and methodological differences. The present study used a cue-conditioning procedure whereas one of these previous positive studies employed inhibitory avoidance (Przybyslawski et al., 1999) and one employed context conditioning (Abrari et al., 2008). Of the two studies reporting that propranolol blocked reconsolidation of cue conditioning, one (Muravieva & Alberini, 2010) used Long Evans, rather than Sprague–Dawley rats as herein. In both cue-conditioning studies (Debiec & Ledoux, 2004; Muravieva & Alberini, 2010), the conditioned responses were acquired in one experimental chamber (context), but reactivated and then tested in another chamber. For reasons of clinical applicability described above, in the present study all procedures were performed in the same chamber.

Interestingly, in the last of the two above studies (Muravieva & Alberini, 2010), propranolol failed to block the reconsolidation of inhibitory avoidance, whereas systemic mifepristone had previously succeeded in doing so in a study in the same laboratory (Taubenfeld et al., 2009). In addition to the present results, this suggests that, compared to propranolol, mifepristone may be a superior reconsolidation blocker of conditioned fear across various designs and may ultimately turn out to be a more useful treatment

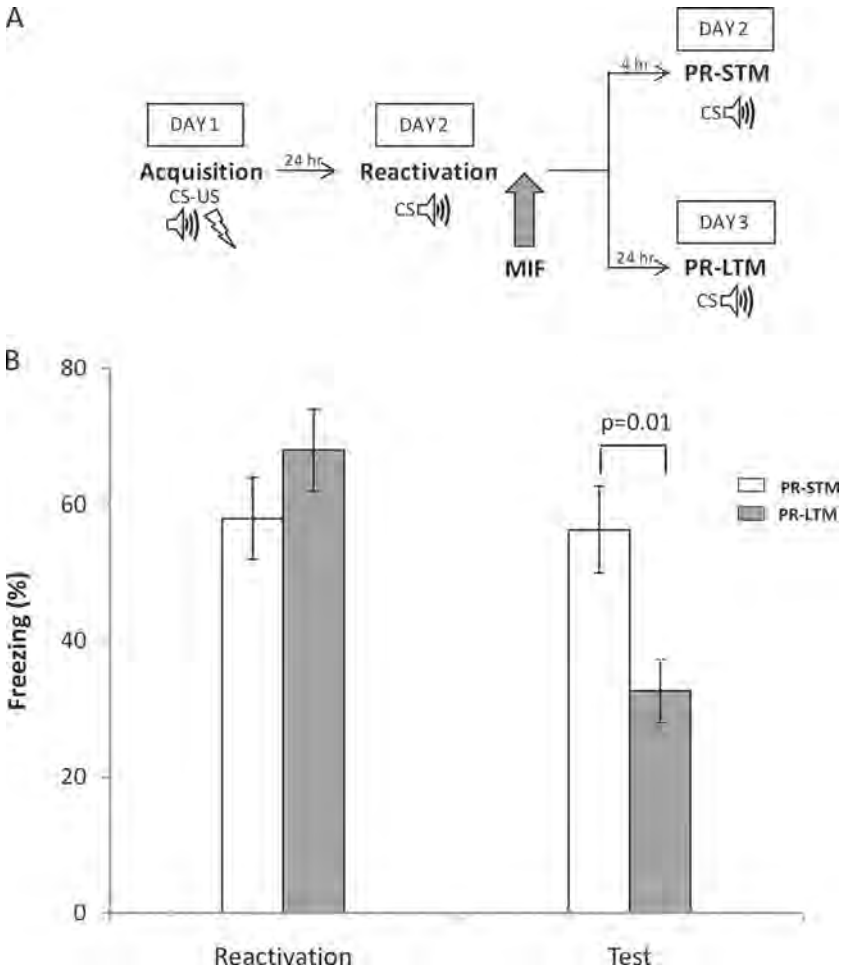

Figure 3. Postreactivation short-term memory (PR-STM) versus postreactivation long-term memory (PR-LTM) in mifepristone groups. Group mean percentage of freezing (i.e., conditioned fear response) on Day 2 (reactivation followed by mifepristone 30 mg/kg) and again either 4 (MIF_PR-STM) or 24 (MIF_PR-LTM) hrs later. Bars = standard error.

for PTSD. At any rate, results of translational studies in animals can only identify effects that deserve further investigation in humans; one-to-one correspondence is not assured.

This study has several limitations. For reasons discussed in the introduction, CRs were only tested in a single context (chamber). Consequently, renewal could not be assessed. Due to the lack of a quantification of freezing to the context prior to the CS presentation, the possibility that context conditioning played some role in the observed results cannot be ruled out. The present design employed only single doses of mifepristone (30 mg/kg) and propranolol (10 mg/kg). These doses were chosen on the basis of their having most often been used in relevant published rat studies, and the consideration that higher doses on a translational mg/kg basis could be prohibitive in humans. The possibilities that different doses of each drug might produce greater reconsolidation blockade, and that different doses of the two drugs in combination might allow mifepristone to block reconsolidation cannot be ruled out.

It could be that the mifepristone-propranolol interaction observed in the present study was pharmacokinetic rather than pharmacodynamic in nature. In other words, one of the drugs may have increased or decreased metabolism of the other, thereby affecting blood levels. However, this explanation is unlikely given that such a pharmacokinetic interaction has not been previously reported and that the metabolism of mifepristone and propranolol rely upon different cytochrome P450 enzymes (Jang, Wrighton, & Benet, 1996; Yoshimoto, Echizen, Chiba, Tani, & Ishizaki, 1995). The relatively high dose of propranolol used here could have caused effects other beta-adrenergic (e.g., serotonergic). Although the mifepristone results have been interpreted within the framework of glucocorticoid receptor blockade, this drug has other, especially antiprogesterone, properties which could partially underlie its observed effect. Because mifepristone is currently the only suitable glucocorticoid receptor blocker approved for human use, this limitation was unavoidable. Although the underlying mechanism of action is of scientific interest, the nature of this action may not be of great concern from a clinical standpoint. The primary objective of the present study was to test

reconsolidation-blockers as potential candidates for treating PTSD, regardless of their mechanisms of action.

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Revision, criteria for chronic PTSD. Exclusion criteria included a history of traumatic brain injury; a current or past psychotic, bipolar, or substance dependence disorder; a previous adverse reaction to a β-blocker; current use of a medication that could involve dangerous interactions with propranolol, including antidepressants that are cytochrome P450 2D6 inhibitors (antidepressants with no such interactions were not an exclusion criteria); a medical condition that contraindicated the administration of propranolol (eg, asthma, heart problems, and diabetes); pregnancy or breast-feeding; or participation in any form of psychotherapy other than supportive. Participants gave written informed consent. All procedures were approved by the local ethics committee.

Study Design and Procedures

Study 1 involved 28 participants recruited via the newspaper in Montreal (Qc), Canada. These participants reported the following index traumatic events: motor vehicle accident (3), participation in a military UN peacekeeping mission (3), physical assault (5), assault with a weapon (2), sexual abuse (3), incest (5), severe physical abuse during childhood (3) or other (4). The study comprised a pretreatment assessment, 6 treatment sessions, a posttreatment assessment, and a 6-month follow-up. Participants were 96% whites and 68% women, with a mean age of 37.9 years (SD, 9.5), and a mean time elapsed since the traumatic event of 201.4 months (SD, 178.3; range, 3–540; median, 180). Comorbidity determined from a structured interview[7] included major depressive disorder (8), social phobia (8) obsessive-compulsive disorder (6), generalized anxiety disorder (5), panic disorder with (2) and without (5) agoraphobia, agoraphobia without panic (2) bulimia (3) and anorexia nervosa (1).

Study 2 involved 7 participants recruited by word of mouth in Boston, Mass. These participants reported the following index traumatic events: motor vehicle accident (2), physical assault (1), assault with a weapon (1), rape (1), witness to family member's fatal illness (1), and military combat (1). The study comprised a pretreatment assessment, 6 treatment sessions, a posttreatment assessment, and a 6-month follow-up. Participants were 100% white and 71% women, with a mean age of 40.1 years (SD, 11.8) and a mean time elapsed since the traumatic event of 120.0 months (SD, 118.0; range, 36–312; median, 132). Comorbidity[7] included major depressive disorder (3) and panic disorder without agoraphobia (2).

Study 3 involved 32 participants taking part in an ongoing longitudinal study examining the long-term outcome of an industrial disaster that had occurred in Toulouse, France.[8] Seven participants were treated with propranolol, and 25 refused treatment but agreed to serve as controls by taking part in 6-month postdisaster (performed previously), pretreatment, posttreatment, and 6-month follow-up assessments. The treated participants completed the assessments and 6 treatment sessions (as in studies 1 and 2). Treated participants were 71% women and 100% whites, with a mean age of 46.7 (SD, 18.3) years. The controls were 52% men and 100% whites, with a mean age of 47.9 (SD, 15.7) years. The 2 groups did not significantly differ on sex or age. The time elapsed since the traumatic event for all participants was 78 months. Comorbidity[7] in the treated participants included MDD (3), social phobia (1), obsessive-compulsive disorder (1), generalized anxiety disorder (1), and agoraphobia without panic (2). Comorbidity data for the untreated controls are unavailable because of their more limited involvement.

PTSD Measures

The PTSD symptom score and diagnosis was determined by the Clinician-Administered PTSD Scale (CAPS)[9] before and after treatment and at follow-up in studies 1 and 2. Intersession improvement was measured weekly in study 1 with the PTSD Checklist (PCL)[10] before each treatment. In study 3, PTSD severity was measured by the PCL administered 6-months after disaster, before and after treatment, and at follow-up. In the treated group, PTSD was diagnosed with the Structured Clinical Interview for *DSM-IV.*[11]

Study Medication

Propranolol hydrochloride is a nonselective synthetic β_1- and β_2-adrenoreceptor antagonist that crosses the blood brain barrier. Study 1 used a dose of 0.67 mg/kg short-acting (SA) oral propranolol in the first session. Ninety minutes later, an additional 1 mg/kg of long-acting (LA) oral propranolol was administered, provided that systolic blood pressure had not fallen by 10 mm Hg or more to lower than 100 mm Hg and that the SA dose was well tolerated, all of which were the case for every participant. In the subsequent sessions, the SA and LA doses were given simultaneously. The modal dose used was 40 mg SA and 60 mg LA, with means of 47.8 and 71.1 mg, respectively. Study 2 used the same protocol as study 1 but with fixed doses of 40 mg SA and 80 mg LA. Study 3 used 40 mg SA in the first session, followed 90 minutes later by 80 mg LA. In the subsequent sessions, only the 80 mg LA was administered. There were no serious adverse events and very few side effects, essentially limited to mild sedation.

Treatment Protocol

Ninety minutes after their first dose of propranolol, participants provided a written (studies 1 and 3) or oral (study 2) account of the index event that led to their current PTSD. During subsequent treatment sessions, 90 minutes after ingesting propranolol, they read aloud (or renarrated in study 2) their trauma account to the interviewer "as if they were back in the experience again." Treatment sessions were purposefully kept short (<15–20 minutes) to minimize extinction.

Data Analysis

Study 1 data were analyzed using repeated-measures analysis of variance (ANOVA) with 9 measurement times (pretreatment, treatment sessions 1–6, posttreatment, and follow-up) and PCL score as the outcome measure. The data also were analyzed using a repeated-measures ANOVA, with 3 measurement times (pretreatment, posttreatment, and follow-up) and the CAPS score as the outcome measure. Study 2 data were analyzed using repeated-measures ANOVA with 3 measurement times (pretreatment, posttreatment, and follow-up), with the CAPS score as the outcome measure. Study 3 data were analyzed using a group (treated vs untreated) × time (6-month postdisaster, pretreatment, posttreatment, and follow-up) repeated-measures ANOVA and PCL score as the outcome measure. Degrees of freedom were Greenhouse-Geisser corrected. A 2-sided P value of less than 0.05 conferred statistical significance. Effect sizes were calculated as if the measures were independent, not paired, providing a more conservative estimate of treatment effect.

RESULTS

Study 1

As shown in Figure 1, mean (SD) PCL total scores at pretreatment, treatment sessions 1–6, posttreatment, and follow-up were as follows: 60.4 (11.4), 53.3 (11.6), 49.8 (13.5), 46.8 (13.2), 45.7 (14.0), 44.0 (13.1), 40.5 (15.8), 37.9 (14.9), and 36.0 (15.1), respectively. The decrease in PTSD scores across time was highly significant ($F_{4.05,\ 109.40} = 22.31$, $P < 0.001$). The pretreatment versus posttreatment contrast

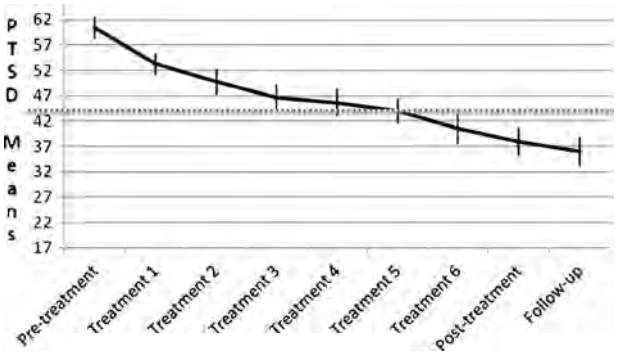

FIGURE 1. Mean symptomatic improvement as a function of treatment and at follow-up among 28 individuals with long-standing PTSD (study 1). The vertical axis indexes the PCL score for the last 7 days, which ranges from 17 (no symptoms at all) to 85. The dashed line score represent the clinical cutoff of 44, above which a diagnosis of PTSD is likely.[9] The bars represent standard error. Note: PCL score was assessed before each session. Sessions from pretreatment to posttreatment were weekly, and the follow-up was 6-months after pretreatment.

was significant ($t_{27} = 8.81$, $P < 0.001$), as was the pretreatment versus follow-up contrast ($t_{27} = 8.22$, $P < 0.001$). These contrasts translated into very large effect sizes of Cohen $d = 1.70$ and $d = 1.82$. This corresponded to symptomatic improvements of 52% and 56%, respectively. Mean (SD) CAPS scores at pretreatment, posttreatment, and follow-up assessments were 71.8 (18.6), 45.8 (21.9), and 42.7 (24.6), respectively. The decrease in PTSD scores across time was highly significant ($F_{2, 54} = 38.05$, $P < 0.001$). On the CAPS, 20 (71%) of 28 participants no longer met the full criteria for PTSD at follow-up.

Study 2

Mean (SD) CAPS total scores at pretreatment, posttreatment, and follow-up were 68.4 (15.8), 35.6 (31.2), and 34.1 (33.2), respectively. The repeated-measures ANOVA revealed a significant time effect ($F_{2, 12} = 14.03$, $P < 0.01$). Pretreatment CAPS scores were significantly higher than posttreatment ($t_6 = 3.96$, $P < 0.01$) and follow-up scores ($t_6 = 3.79$, $P < 0.01$). These contrasts translated into large effect sizes of $d = 1.33$ and $d = 1.32$, respectively, and improvements of 48% and 50%, respectively. Furthermore, 5 (71%) of the 7 participants no longer met full criteria for PTSD at follow-up.

Study 3

Mean (SD) PCL scores at 6 months postdisaster, pretreatment, posttreatment, and follow-up for the treatment group were as follows: 60.9 (5.3), 60.7 (4.1), 41.0 (4.3), and 38.4 (3.6), respectively, and those for the control group were as follows: 59.7 (2.5), 61.7 (2.3), 58.7 (2.7), and 58.7 (2.8), respectively. There was a significant group × time interaction ($F_{1.83, 54.99} = 11.61$, $P < 0.001$). As expected, the groups did not differ significantly from each other at the 6 months postdisaster ($t_{30} = 0.21$, $P = 0.83$) and pretreatment ($t_{30} = 0.20$, $P = 0.84$) assessments but did differ at the posttreatment ($t_{30} = 3.16$, $P < 0.01$) and follow-up ($t_{30} = 3.57$, $P < 0.01$) assessments. The treatment effect sizes were $d = 1.77$ in the propranolol versus $d = 0.24$ in the control group at posttreatment, and Cohen d at follow-up were as follows: $d = 2.19$ versus $d = 0.23$. Pretreatment to posttreatment PTSD symptom improvement in the propranolol group was 45% versus 7% in the controls. At follow-up, these improvements were 51% versus 7%. Six (86%) of the 7 treated participants no longer met the criteria for PTSD at follow-up, compared with 2 (8%) of the 25 untreated participants ($P < 0.001$; Fisher exact test).

DISCUSSION

In 3 independent studies, 6 brief trauma reactivation sessions under the influence of propranolol brought about large PTSD symptom improvements. Such results extend our previous placebo-controlled psychophysiological results[6] in 2 important ways. First, recalling one's traumatic experience under the influence of propranolol received on 6 occasions, rather than just once, produced a much larger symptom reduction, thereby demonstrating more clearly the clinical potential of this novel approach. The effect sizes reported compare favorably to those produced by exposure-based psychotherapies,[12] yet they were obtained using a different approach that involves fewer and shorter sessions and virtually no side effects. Second, the treatment effects were shown to persist over time.

The studies took place in 3 different countries with men and women, but a lack of participants of minority ethnicity limits the generalizability of the findings. In study 3, the control group improved minimally over the course of the 6 months. However, conclusions based on this comparison group are limited by factors such as their self-selection against treatment and unmeasured comorbidity.

One explanation for our results is that propranolol blocked the reconsolidation of the traumatic memory, which in turn led to symptom reduction. Although our study lacked the necessary controls to show that reconsolidation blockade was the active therapeutic mechanism, a recent experimental study supported reconsolidation blockade by propranolol as the mechanism underlying the observed reduction in conditioned fear.[13] Another potential explanation for the present findings is that the intervention induced extinction. Although we cannot rule out such an explanation, extinction-based treatment sessions are typically prolonged and involve a greater number of sessions. In fact, brief exposures may exacerbate symptoms.[14] Still, this possibility could be examined in future studies by using a placebo reactivation condition. Until then, the conclusion that propranolol was necessary for symptom improvement must await results of a double-blind, randomized, placebo-controlled trial. The current data make a compelling case for launching a rigorous randomized clinical trial. Positive results would add to the growing literature targeting neuroplasticity as a novel treatment approach for mental disorders.[15]

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The authors declare no conflicts of interest.

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Sertindole-Associated Deep Venous Thrombosis

To the Editors:

It has been documented that venous thromboembolism risk has a 7-fold increase among users of conventional antipsychotic agents who were younger than 60 years and free of major risk factors.[1] In clinical practice, conventional antipsychotics are sometimes changed to atypical antipsychotics because of their favorable side effects and efficiency on negative symptoms. Although clear evidence is lacking, possible thromboembolic effects of atypical antipsychotics are observed in case reports and clinical investigations.[2–5] Sertindole, a newly marketed atypical antipsychotic, after phase 4 investigation in our country, is a nonsedating atypical antipsychotic agent with a high selectivity for dopaminergic neurons in the mesolimbic system and also with affinity for serotonin 5-HT_{2A} and 5-HT_{2C}, and α_1-adrenoreceptors.[6]

We describe a case of venous thromboembolism during sertindole treatment in a woman diagnosed with schizophrenia.

CASE REPORT

Ms M. is a 37-year-old single woman, elementary school graduate, and unemployed. She has a 5-year history of schizophrenia and presented to the Firat University School of Medicine, Department of Psychiatry, with complaints of reference delusions, avulsion, alogia, voices commenting on behaviors, suicidal ideation, and affective flattening. She was admitted to the inpatient clinic without any resistance to admission. She had been followed by our clinic for nearly 2 years and had been admitted 2 times. She had no history of substance abuse and other Axis I disorders. There was also no history of family psychiatric disorder. She was otherwise in good general physical health and had no personal or familial history of venous thromboembolism or oral contraceptives, acetylsalicylic acid, or any anticoagulant use such as heparin or rivaroxaban. Although the patient had a body mass index of 25 kg/m^2, neither her weight nor her level of physical activity had significantly changed with antipsychotic medication. She had no identified cardiovascular risk factors including smoking. She had taken antipsychotics including typical and atypical antipsychotics during her illness period including haloperidol, olanzapine, and amisulpride. She was off medication for nearly 4 months. She was hospitalized in our inpatient clinic with the complaints mentioned above. The last treatment was discontinued, and sertindole 4 mg/d was started and titrated to 16 mg/d within 9 days. Her level of motion continued in a normal pattern, and she was not bedridden. On day 11 of the hospitalization, she complained of right leg edema. Two days later, redness and pain appeared in her right leg when she was walking. We immediately consulted with the department of cardiovascular surgery. Disabled coagulation profile, with abnormal concentrations of C-reactive protein and fibrinogen,

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Will reconsolidation blockade offer a novel treatment for posttraumatic stress disorder?

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Many articles about memory reconsolidation conclude with its therapeutic implications for posttraumatic stress disorder (PTSD). A core feature of PTSD is the memory of a traumatic event that is characterized by excessive strength, immalleability, and persistence. We found that Korean and World War II veterans with PTSD showed elevated physiological responses during mental imagery of their personal combat events as long as 40 years later (Orr et al., 1993). We have hypothesized that traumatic memories in PTSD become "overconsolidated" under the influence of stress hormones stimulated by the traumatic event (Pitman, 1989). Traditional theory holds that once a memory has been consolidated, i.e., placed into long-term storage, it exists as a permanent trace. According to this view, the most one can hope for therapeutically would be to inhibit the memory's expression through a mechanism such as extinction, but this inhibition is fragile, and the associated distress and arousal may return. Years ago we consulted on the case of a veteran who was admitted to the hospital for low back pain. Following World War II, he had experienced a year of nightmares and flashbacks of his combat experiences. With time these symptoms remitted, and he had been symptom free for 30 years. The medical work-up for his back pain revealed carcinoma of the prostate metastatic to the vertebrae, a fatal condition. The night after the patient was presented with this diagnosis, he experienced nightmares, not of his cancer or its future consequences, but of combat. This reinstatement of his combat memories by the stress of his cancer diagnosis indicated that they had not been erased but only had become latent.

Recent animal research has challenged the permanence of consolidated memory traces by suggesting that reactivation (retrieval) of a memory can return it to an unstable state from which it must be "reconsolidated" if it is to persist. Blocking reconsolidation offers the therapeutic possibility of weakening traumatic memories in PTSD. A recent Pavlovian differential conditioning study in normal humans employed memory reactivation accompanied by the beta-adrenergic blocker propranolol (Kindt et al., 2009). After that intervention, the previously acquired conditioned stimulus (CS) could no longer be made to elicit a skin conductance response. In contrast, the declarative memory of the contingency survived, suggesting that only the memory's fear component had been erased – an ideal scenario from the clinical standpoint. Another recent normal human conditioning study substituted a behavioral intervention (Schiller et al., 2010) and used potentiated startle as the measure of fear. A single CS trial was followed by a 10-min delay, and then by further extinction trials. Following this intervention, the conditioned fear response was not merely inhibited but permanently eliminated. It was argued that the delay provided sufficient time for the reactivated fear memory trace to return to an unstable state, so that the remaining CS presentations occurred during a "reconsolidation window." This allowed the original fear memory to be modified or "updated" to incorporate the new information that the CS was no longer dangerous. The investigators suggested that such a delay tactic could be incorporated into cognitive-behavioral therapy (CBT) to increase its efficacy. However, such a delay may already be a component of CBT, given that sessions typically go on for an hour or longer following the initial exposure (i.e., memory reactivation). Foa and Kozak (1986) have characterized the mechanism behind exposure therapy as the incorporation of "corrective information."

Although the preclinical animal and normal human studies are encouraging, the translational gap to clinical application is huge. Critical differences between PTSD and laboratory experiments include (in the former) the stronger unconditioned stimulus (US, e.g., a gunshot wound vs. a mild electric shock), greater and more sustained arousal at the time of the traumatic event (i.e., a stronger unconditioned emotional response), the more complex nature of the CS (e.g., a firefight vs. a colored shape), the possible presence of multiple conditioning events, and the longer duration between the memory's formation and the intervention (e.g., years vs. days). Erasing or updating the memory of a conditioned response acquired 1 day earlier under the influence of a mild US might be likened to the effect of a firecracker, whereas achieving the same for a deeply engraved traumatic memory of a life-threatening event in PTSD might be likened to the effect of an atomic bomb. It remains to be seen whether such a device can be constructed.

Lang (1985) proposed that emotion is defined by a specific information structure in memory, whose content consists of three primary categories: (1) information about prompting external stimuli and the context in which they occur (stimulus propositions); (2) information about responding in this context, including expressive verbal behavior, overt acts, and the visceral and somatic events that mediate arousal and action (response propositions); and (3) information that defines the meaning of the stimulus and response data (meaning propositions). These propositions are organized into an associative network which, when a critical number of propositions are accessed, is processed as a unit. We suggested that PTSD consists of one or more traumatic emotion networks that, when activated, produce its characteristic symptomatology (Pitman, 1988; Pitman and Orr, 1990). If a PTSD associative network could be reactivated in its entirety, and then have its reconsolidation blocked in entirety, this could simplify clinical application. Unfortunately research with second-order conditioning suggests that this may not be

so simple. Dębiec et al. (2006) conditioned rats to a tone pattern (CS1) by pairing it with a shock US (first-order conditioning). Then they conditioned rats to different tone pattern (CS2) by pairing it with the CS1 (second-order conditioning). They found that blocking reconsolidation of the first-order association with the protein-synthesis inhibitor anisomycin reduced the freezing (fear) response to both the CS1 and the CS2. In contrast, blocking reconsolidation of the second-order association reduced the freezing response only to the CS2; the freezing response to the CS1 remained intact. These findings suggest that successful reconsolidation blockade or memory updating in PTSD will require accessing the original, core traumatic associations; merely addressing secondary, peripheral associations will not suffice. Moreover, under certain circumstances (Eisenberg et al., 2003), pharmacological intervention could succeed not in blocking reconsolidation of the fear association but rather in blocking consolidation of extinction learning, possible resulting in an antitherapeutic effect.

Unfortunately from the therapeutic application standpoint, animal evidence indicates memories that have been formed under stressful conditions (Bustos et al., 2010), as well as memories that have aged for long periods (Suzuki et al., 2004), are more resistant to being made to undergo reconsolidation. For reconsolidation blockade, or updating, to be successful, two steps are required. First, the problematic memory must be destabilized. Second, its restabilization (reconsolidation) must then be prevented or modified (updated). Resistance may be encountered during the first of these stages. Specifically, activation of NR2B NMDA-receptor subunits appears to be required for reactivation-induced memory destabilization, and their downregulation may prevent this. Recent animal research suggests that administration of the NMDA agonist D-cycloserine (DCS) may prepare a memory for destabilization and facilitate pharmacological reconsolidation blockade that would otherwise not take place (Bustos et al., 2010). Given that both the formation of memories under stressful conditions, and the age of such memories, characterize PTSD, for a reconsolidation-based treatment to work, pharmacological or other assistance with memory destabilization may be required, in addition to subsequent pharmacological blockade of reconsolidation, or behavioral updating, once destabilization has occurred. This suggests a second possible, and different, application of DCS to PTSD therapy, in addition to its possible role in the strengthening of extinction retention (Cukor et al., 2009).

The only published reconsolidation blockade-like study in PTSD to date did succeed in producing evidence that propranolol administered at the time of traumatic memory reactivation diminished the memory's emotional component, as manifest in smaller psychophysiological responses during subsequent script-driven traumatic imagery (Brunet et al., 2008). However, this study lacked sufficient controls to permit the inference that reconsolidation blockade was the underlying mechanism. Moreover, these results are preliminary, and many additional studies will be required to determine whether the therapeutic promise of reconsolidation blockade or modification will be fulfilled.

Finally, there is no reason to assume that if the therapeutic PTSD bomb can eventually be constructed, its ultimate ingredient will be propranolol. In unpublished research with rats, we have found that the glucocorticoid receptor antagonist mifepristone, in addition to blocking reconsolidation of inhibitory avoidance learning (Taubenfeld et al., 2009), has substantially stronger cue-induced-fear reconsolidation-blocking properties than propranolol. This drug has yet to be tested in human reconsolidation experiments. Other drugs may exist that are stronger still. However, for any drug to be clinically useful, it must be approved for human use and capable of systemic administration. Moreover, the efficacy of any drug has yet to be compared with the efficacy of behavioral memory updating techniques. All these questions and more will need to be addressed within a large research and development Manhattan project for PTSD. The translation from preclinical work to clinical application may prove long and difficult, and even unsuccessful. However, given the importance of PTSD as a public mental health problem, it is worth pursuing.

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Hierarchical order of coexisting pre- and postsynaptic forms of long-term potentiation at synapses in amygdala

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Synaptic rules that may determine the interaction between coexisting forms of long-term potentiation (LTP) at glutamatergic central synapses remain unknown. Here, we show that two mechanistically distinct forms of LTP could be induced in thalamic input to the lateral nucleus of the amygdala (LA) with an identical presynaptic stimulation protocol, depending on the level of postsynaptic membrane polarization. One form of LTP, resulting from pairing of postsynaptic depolarization and low-frequency presynaptic stimulation, was both induced and expressed postsynaptically ("post-LTP"). The same stimulation in the absence of postsynaptic depolarization led to LTP, which was induced and expressed presynaptically ("pre-LTP"). The inducibility of coexisting pre- and postsynaptic forms of LTP at synapses in thalamic input followed a well-defined hierarchical order, such that pre-LTP was suppressed when post-LTP was induced. This interaction was mediated by activation of cannabinoid type 1 receptors by endogenous cannabinoids released in the lateral nucleus of the amygdala in response to activation of the type 1 metabotropic glutamate receptor. These results suggest a previously unknown mechanism by which the hierarchy of coexisting forms of long-term synaptic plasticity in the neural circuits of learned fear could be established, possibly reflecting the hierarchy of memories for the previously experienced fearful events according to their aversiveness level.

synaptic transmission | glutamate | plasticity | endocannabinoids

Fear conditioning is one of the best experimental models of associative learning, which results from memorizing the temporal association between biologically neutral conditioned stimuli (CS) and aversive unconditioned stimuli (US) during behavioral training (1, 2). In the course of auditory fear conditioning, signals produced by the acoustic conditioned stimulus enter the lateral nucleus of the amygdala (LA) through projections originating in the auditory thalamus (thalamic input) and indirect projections from the auditory cortex (cortical input) (3). The acquisition of fear memory to auditory stimulation is mediated by long-term potentiation (LTP)-like synaptic enhancements in the CS pathways, including both cortical and thalamic inputs to the LA (4–9). Different forms of LTP could be observed, however, at synapses in the amygdala (7, 8, 10–12) as well as in other regions of the brain (13, 14), depending on the presynaptic activity levels and degree of postsynaptic depolarization. Thus, conventional pairing-induced LTP and spike timing-dependent LTP in thalamic projections to the LA are expressed postsynaptically and may implicate trafficking of AMPA receptors at stimulated synapses ("post-LTP") (8, 15), whereas LTP in cortical input to the LA is expressed presynaptically, resulting from an increase in the probability of neurotransmitter release ("pre-LTP") (7). Little is known, however, about whether the coexisting forms of LTP at glutamatergic synapses interact with each other during the induction process, and if they do, how such interactions could be mediated. It prompted us to ask which synaptic mechanisms determine the order in which the coexisting forms of LTP in the CS projections to the LA are induced.

Here, we report that the induction of LTP in thalamic input to the LA, which is both induced and expressed postsynaptically, suppresses the mechanisms of pre-LTP coexisting at the same synapses, thus potentially preventing situations where different forms of synaptic plasticity are simultaneously expressed.

Results

GluR5 Kainate Receptor-Dependent Pre-LTP Is Readily Induced in Thalamic Input to the LA. To explore the interactions between different forms of LTP in the CS pathways, we recorded excitatory postsynaptic currents (EPSCs) in LA neurons evoked by stimulation of either cortical or thalamic inputs to the LA (1, 16). Stimulation of thalamic input for 2 min with paired pulses (50-ms interpulse interval) at 2 Hz frequency and a holding potential of −70 mV led to LTP of the thalamo-amygdala EPSC (Fig. 1 *A*, *B*, *D*, and *E* and Figs. S1*A* and S2 *A–E*), whereas the same induction protocol failed to induce LTP in the cortico-amygdala pathway (Fig. 1 *A*, *C*, and *D*), indicating that this form of LTP was pathway-specific. The inducibility of LTP under these conditions was insensitive to changes in GABA-mediated inhibition (Fig. S3). Unlike conventional pairing-induced and spike timing-dependent LTP (7, 17), this form of synaptic potentiation was not blocked by the Ca^{2+} chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA, 20 mM) in the recording pipette solution, and therefore, it did not require postsynaptic Ca^{2+} influx for its induction (Fig. 1 *F* and *H*). Pretreatment of slices for 30 min with the cell-permeable Ca^{2+} chelator 1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) blocked the induction of LTP (Fig. 1 *F* and *H* and Fig. S4), indicating that presynaptic Ca^{2+} influx might be implicated in the induction process. This form of LTP was insensitive to both the metabotropic glutamate receptors (mGluR) antagonist (RS)-α-Methyl-4-carboxyphenylglycine (MCPG, 500 μM) and the NMDA receptor antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV, 50 μM) (Fig. 1 *G* and *H*). LTP was completely blocked, however, by the selective antagonists of the GluR5 subunit-containing kainate receptors, (RS)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrimidine-2,4-dione (UBP296, 1 μM) (Fig. 1 *G* and *H*) or (S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-

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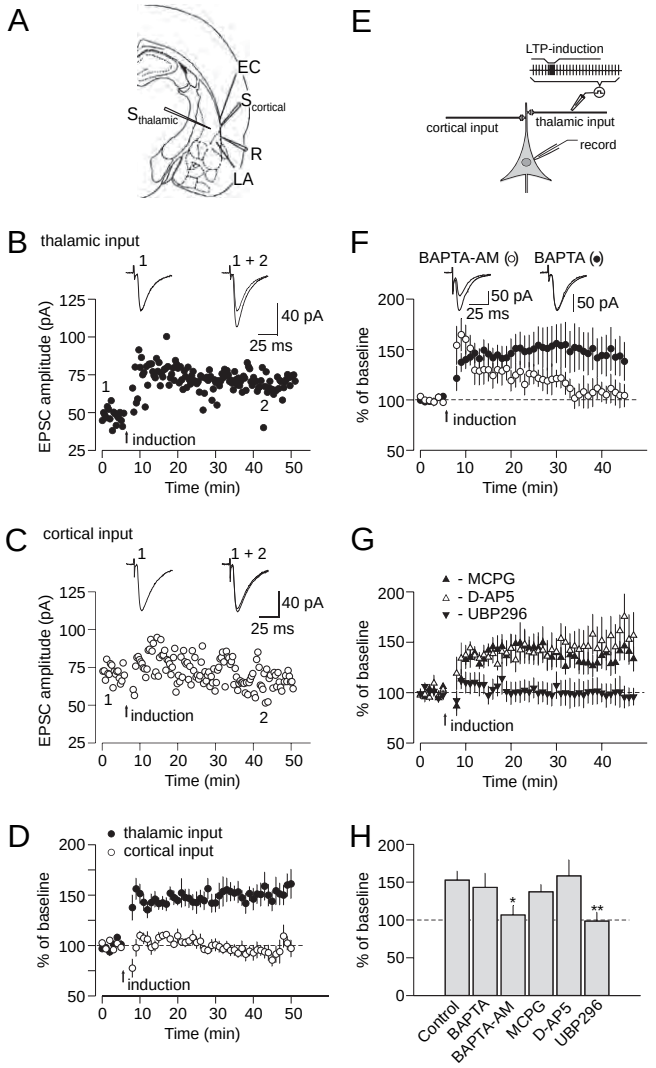

Fig. 1. Properties of pre-LTP in thalamic input to the LA. (*A*) Position of the stimulation ($S_{cortical}$ and $S_{thalamic}$) and recording (R) electrodes. EC, external capsule. (*B*) LTP of the EPSC in thalamic input to the LA was induced by the protocol consisting of a 2-min period of presynaptic stimulation with paired pulses (50-ms interpulse interval) delivered at the arrow (at −70 mV throughout the experiment). (*Insets*) Averaged EPSCs before (1) and after (2) the induction of LTP. (*C*) The same protocol did not induce LTP in cortical input. (*D*) LTP experiments as in *B* and *C* in cortical ($n = 6$) and thalamic ($n = 8$) inputs to the LA (*t* test, $P = 0.001$ between inputs). (*E*) The experimental design. (*F*) Normal LTP in thalamic input was observed when BAPTA (20 mM) was included in the pipette solution ($n = 10$), but LTP was blocked by membrane-permeable BAPTA-AM (50 μM; $n = 6$). Slices were incubated in BAPTA-AM–containing solution for >30 min before the recording. (*Insets*) Averaged EPSCs recorded before and 35 min after the delivery of LTP-inducing stimulation. (*G*) LTP in thalamic input in the presence of MCPG (500 μM; $n = 4$), D-AP5 (50 μM; $n = 6$), or UBP296 (1 μM; $n = 6$). (*H*) Summary of LTP experiments at thalamo-amygdala synapses (control, $n = 8$; BAPTA, $n = 10$, $P = 0.68$ vs. control LTP; BAPTA-AM, $n = 6$, $P = 0.019$ vs. control; MCPG, $n = 4$, $P = 0.41$ vs. control; D-AP5, $n = 6$, $P = 0.81$; UBP296, $n = 6$, $P = 0.007$). Error bars indicate SEM.

dione (ACET, 0.5 μM) (Fig. S1 *B* and *C*). GluR5 subunit-containing kainate receptors are highly expressed in the amygdala (18). Together, these findings indicate that this form of LTP in thalamic input to the LA required activation of GluR5-containing kainate receptors and presynaptic Ca^{2+} influx.

The observed kainate (KA) receptor-dependent form of LTP in the LA was associated with decreased paired-pulse facilitation (PPF), which is indicative of presynaptic enhancements (increased probability of neurotransmitter release) (Fig. S5 *A* and *B*) (19). Moreover, KA receptor-dependent LTP at the level of unitary EPSCs (7) resulted from the increased probability of successes (when a quantal synaptic event could be detected), without changes in the mean size of unitary EPSCs (potency) (7), also indicating a presynaptic site of expression (Fig. S5 *C–F*). Thus, similar to the mossy fiber LTP in the hippocampus, which also implicates activation of KA receptors in the induction process (20), the expression of the newly described form of LTP in thalamic input to the LA had a significant presynaptic component (pre-LTP).

Pre-LTP could be induced by activating GluR5 kainate receptors exogenously, because GluR5 subunit-specific agonist (RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid (ATPA, 1 μM) has produced potentiation of synaptic transmission in thalamic input when added to the external medium (Fig. 2*A*). ATPA-induced potentiation was expressed presynaptically, because it was associated with decreased paired-pulse facilitation (Fig. 2*B*). Moreover, ATPA-induced potentiation occluded LTP induced by electrical stimulation (Fig. 2*A*), indicating that these processes may share common mechanisms. Similar to electrically induced LTP, ATPA-induced potentiation also needed Ca^{2+} influx for its induction, because it could not be induced in a Ca^{2+}-free external solution; also, it was blocked by the GluR5 KA receptor antagonist UBP296 (Fig. 2 *C–E*). Thus, ATPA-induced potentiation and pre-LTP in thalamic input are mechanistically similar, and both require activation of presynaptic GluR5 subunit-containing KA receptors for their induction.

Pre-LTP in Thalamic Input to the LA Is Suppressed by the Induction of Post-LTP at the Same Synapses. In contrast, both conventional pairing-induced LTP and spike timing-dependent LTP in thalamic projections to the LA are expressed postsynaptically (post-LTP) (8, 15). We induced post-LTP in thalamic input to the LA by paring postsynaptic depolarization to +30 mV during the induction with the presynaptic stimulation, which was identical to the stimulation delivered for the induction of KA receptor-dependent pre-LTP (240 paired pulses at 2 Hz frequency) (Fig. 3*A*). Under these conditions, the magnitude of PPF was unaffected by the induction of LTP (Fig. S6 *A* and *B*). At the level of unitary synaptic responses, LTP was associated with a significant increase in potency, whereas the rate of failures was unchanged (Fig. S6 *C* and *D*). This confirms that the expression of the pairing-induced form of LTP in thalamic input is postsynaptic and not associated with increases in probability of release. The resulting pairing-induced LTP was blocked by BAPTA in the recording pipette and depended on postsynaptic Ca^{2+} influx through NMDA receptors and L-type Ca^{2+} channels (Fig. 3 *B–D*). Unlike LTP induced with the same stimulation protocol but without postsynaptic depolarization (Fig. 1), it was not blocked by the GluR5 antagonist UBP296 (Fig. 3 *C* and *D*). Therefore, two different forms of LTP coexist in thalamic input to the LA, and their inducibility may follow a certain order. Thus, the pre-LTP, which is induced in the absence of depolarization, was suppressed when post-LTP was induced. Consistent with this notion, we found that, when post-LTP is blocked by the Ca^{2+} chelator BAPTA in the recording pipette solution, pre-LTP (induced by the identical stimulation but without postsynaptic depolarization) could be observed at the same synapses (Fig. 4 *A* and *B*). These findings suggest that suppression of pre-LTP does not require a rise in postsynaptic Ca^{2+} concentration, unlike the induction of post-LTP.

Suppression of Pre-LTP Is Mediated by Activation of Cannabinoid Type 1 Receptors by Endogenous Cannabinoids. How could the induction of post-LTP suppress pre-LTP? The only difference between the two induction protocols, leading to mechanistically distinct forms of LTP at thalamo-amygdala synapses, is postsynaptic

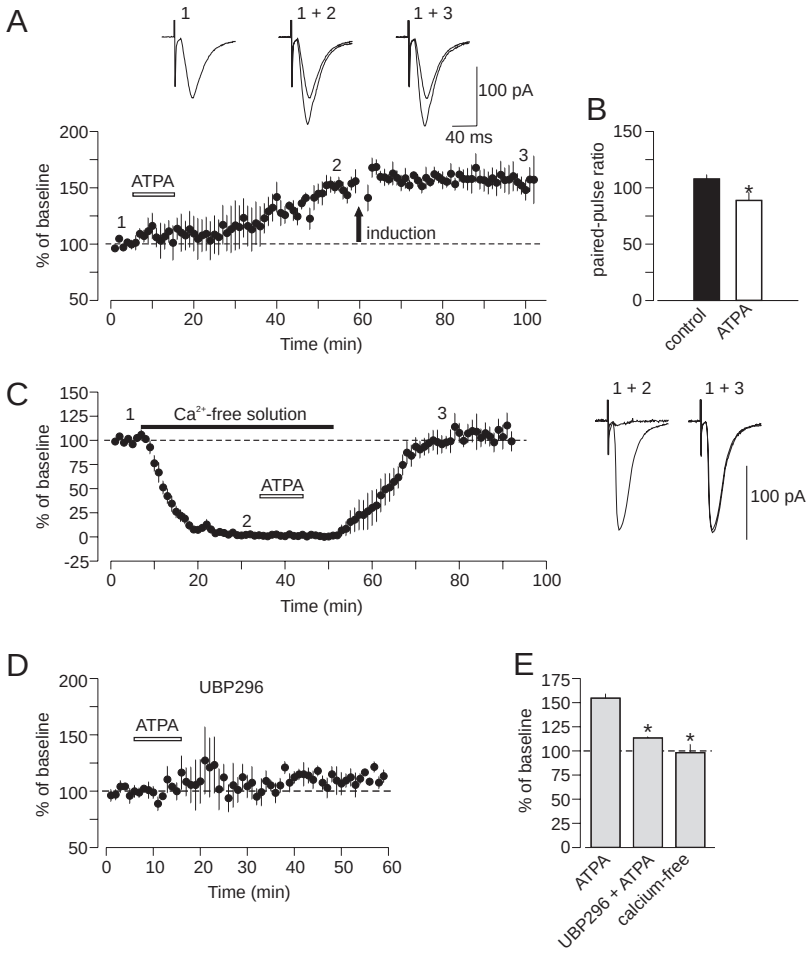

Fig. 2. ATPA-induced potentiation and pre-LTP in thalamic input are mechanistically similar. (*A*) The GluR5-specific agonist ATPA (1 μM) in the external solution potentiated the EPSC in thalamic input (n = 4; $P < 0.01$ vs. baseline). Potentiation induced by ATPA has occluded LTP in response to electrical stimulation (delivered at arrow). (*Insets*) Averaged thalamo-amygdala EPSCs recorded under control conditions (1), during ATPA-induced potentiation (2), and after the delivery of LTP protocol (3). (*B*) Paired-pulse facilitation (PPF; 50-ms interpulse interval) was decreased during ATPA-induced potentiation (n = 4, P = 0.017). (*C*) Potentiation was prevented when ATPA (1 μM) was applied in Ca^{2+}-free external solution (n = 6, P = 0.75 for post-ATPA vs. baseline). Traces (to the right) are averaged EPSCs recorded at different time points (shown as 1, 2, and 3) during the experiment. (*D*) UBP296 (1 μM) blocked potentiation of thalamo-amygdala EPSCs by ATPA (n = 3). (*E*) Summary of experiments with ATPA (ATPA, n = 4; UBP296 + ATPA, n = 3, P = 0.014 vs. ATPA alone; ATPA in Ca^{2+}-free solution, n = 6, P = 0.01 vs. ATPA alone). Error bars indicate SEM.

depolarization during the induction of post-LTP. Depolarization alone, however, preceding pre-LTP–inducing stimulation in the absence of BAPTA in the recording pipette solution had no effect on the magnitude of pre-LTP in thalamic input (Fig. S7). Depolarization in combination with activation of mGluRs could lead to the production of diffusible factors (e.g., endogenous cannabinoids) capable of affecting synaptic function (21). Importantly, there is evidence that endocannabinoids could be released in the amygdala through activation of mGluRs in a Ca^{2+}-independent manner (22). Therefore, we tested whether endocannabinoid signaling is implicated in the interaction between two forms of LTP in thalamic input to the LA. Consistent with the role of endogenous cannabinoids in such an interaction, we found that the post-LTP induction protocol led to LTP even with the intrapipette solution containing a high concentration of BAPTA (20 mM), which efficiently blocked LTP under control conditions (Fig. 3*B*), when delivered in the presence of the antagonist of cannabinoid type 1 (CB1) receptor, AM281 (0.5 μM) (Fig. 4 *C* and *E*). Similar to pre-LTP, LTP induced with the post-LTP induction protocol in the presence of AM281 in the external solution and 20 mM BAPTA in the pipette solution was expressed presynaptically (Fig. S8 *A* and *B*). The unmasked LTP was GluR5 kainate receptor-dependent, because it was suppressed by UBP296. Moreover, it occluded pre-LTP, providing evidence that two forms of plasticity might be mechanistically related (Fig. 5*A*). These findings indicate that blocking CB1 receptors, which are expressed in the amygdala (23), unmasked pre-LTP, despite postsynaptic depolarization, which would suppress it in the absence of AM281 in the external solution. It has been shown previously that the functional effects of mGluRs activation might be membrane potential-dependent (24, 25). This could explain the need for depolarization of a recorded neuron for the blocking effect on pre-LTP to occur.

If the production of endogenous cannabinoids, perhaps activating presynaptic CB1 receptors, during the induction of post-LTP leads to suppression of pre-LTP, then pre-LTP in thalamic input should be blocked after activation of CB1 receptors by specific agonists. Consistent with this prediction, we found that the pre-LTP–inducing stimulation (not involving postsynaptic depolarization) did not result in LTP of the thalamo-amygdala EPSCs when delivered in the presence of the endogenous agonist of CB1 receptors, anandamide (500 nM) (Fig. 4 *D* and *E*). Anandamide in this concentration had practically no effect on baseline synaptic transmission in thalamic input (Fig. S9). The endocannabinoid production during the induction of post-LTP,

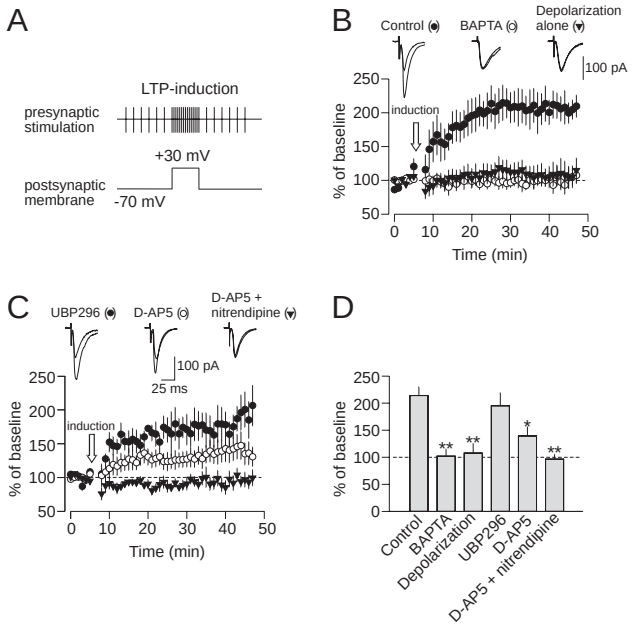

Fig. 3. Requirements for the induction of post-LTP at thalamo-amygdala synapses. (*A*) LTP in thalamic input was induced by presynaptic stimulation for 2 min with paired pulses (50-ms interpulse interval). Unlike pre-LTP, the recorded LA neuron here was voltage-clamped at +30 mV during the induction. (*B*) Post-LTP, observed under control conditions ($n = 6$), was blocked by 20 mM BAPTA in the recording pipette solution ($n = 10$). Postsynaptic depolarization to +30 mV without presynaptic stimulation did not result in LTP ($n = 5$). (*Insets*) Averaged EPSCs recorded before and 35 min after the time point when LTP-inducing stimulation was delivered. (*C*) Post-LTP in thalamic input in the presence of UBP296 (1 μM; $n = 4$), D-AP5 (50 μM; $n = 5$), or D-AP5 + nitrendipine (nitrendipine, 10 μM; $n = 5$). (*Insets*) Averaged EPSCs recorded before and 35 min after the delivery of LTP-inducing stimulation. (*D*) Summary of LTP experiments at thalamo-amygdala synapses (control, $n = 6$; BAPTA, $n = 10$, $P < 0.01$ vs. control LTP; depolarization alone, $n = 5$, $P < 0.01$ vs. control LTP; UBP296, $n = 4$, $P = 0.51$ vs. control; D-AP5, $n = 5$, $P = 0.01$ vs. control; D-AP5 + nitrendipine, $n = 5$, $P < 0.01$). Error bars indicate SEM.

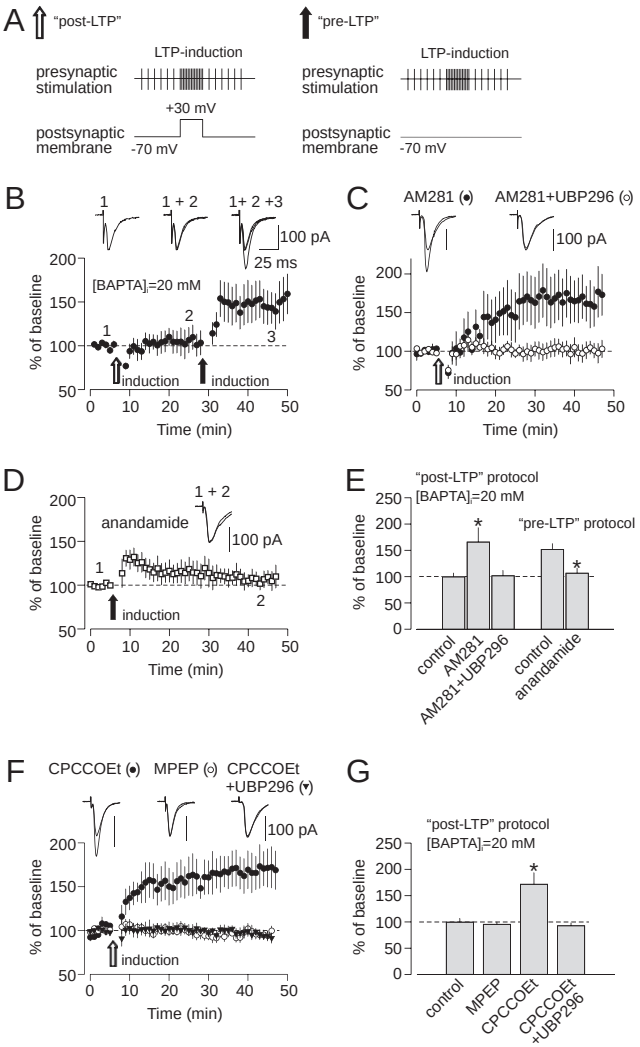

Fig. 4. Induction of post-LTP suppresses pre-LTP at thalamo-amygdala synapses through CB1 receptor activation. (*A*) Stimulation protocols for the induction of post-LTP (*Left*) and pre-LTP (*Right*). (*B*) When post-LTP in thalamic input was blocked by 20 mM BAPTA in pipette solution (empty arrow), pre-LTP could be induced in the same pathway (filled arrow; $n = 7$). (*Insets*) Averaged EPSCs recorded before (1), after the delivery of post-LTP protocol (2), and after the delivery of pre-LTP protocol (3). (*C*) Post-LTP protocol, delivered in the presence of 20 mM BAPTA in pipette solution, resulted in LTP when CB1 receptors were blocked by AM281 (0.5 μM; $n = 6$). The induction of this unmasked LTP was suppressed in the presence of GluR5 antagonist UBP296 (1 μM; $n = 7$). (*D*) In the presence of CB1 receptor agonist anandamide (0.5 μM), the induction of pre-LTP was blocked ($n = 4$). (*E*) Summary of LTP experiments with post- and pre-induction protocols. Post-LTP protocol in the presence of 20 mM BAPTA in the pipette solution (control, $n = 10$; AM281, $n = 6$, $P = 0.01$ vs. control; AM281 + UBP296, $n = 7$, $P = 0.038$ vs. control). Pre-LTP protocol (control, $n = 8$; anandamide, $n = 4$, $P = 0.025$ vs. control). (*F*) Post-LTP protocol, delivered in the presence of 20 mM BAPTA in the pipette solution, resulted in LTP when mGluR1 was blocked with CPCCOEt (50 μM; $n = 11$). LTP was not rescued in the presence of the mGluR5 antagonist MPEP (100 μM; $n = 7$). No LTP was observed when CPCCOEt was applied together with the GluR5 kainate receptor antagonist UBP296 (1 μM; $n = 6$). (*G*) Summary of LTP experiments with post-LTP induction protocol delivered in the presence of BAPTA in the pipette solution as in *F* (control, $n = 10$; MPEP, $n = 7$, $P = 0.57$ vs. control; CPCCOEt, $n = 11$, $P = 0.012$ vs. control; CPCCOEt + UBP296, $n = 6$, $P = 0.7$ vs. control). Error bars indicate SEM.

leading to suppression of the pre-LTP, was likely mediated by activation of the type 1 mGlu receptor (mGluR1). Thus, the KA receptor-dependent pre-LTP (as evidenced by its sensitivity to UBP296) could be induced with the post-LTP induction protocol when it was delivered in the presence of the mGluR1 antagonist CPCCOEt (50 μM), whereas the mGluR5 antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP, 100 μM) had no effect (Fig. 4 *F* and *G*). In these experiments, we prevented the induction of post-LTP, including a high concentration of BAPTA in pipette solution.

Therefore, CB1 receptor activation may mediate the interaction between pre- and post- forms of LTP in thalamic input to the LA, preventing situations when the different forms of LTP coexisting at the same synapses are simultaneously expressed (Fig. 6). Interestingly, pre-LTP and post-LTP could be induced simultaneously under certain conditions. Thus, the delivery of the post-LTP induction protocol in the presence of AM281 but without BAPTA in the pipette solution resulted in nearly doubled LTP (Fig. 5*B*), suggesting that pre-LTP and post-LTP might be additive if the endogenous cannabinoid cascade is inactivated.

Discussion

Our findings show that postsynaptically released cannabinoids may suppress a form of LTP in thalamic input to the LA, which is both induced and expressed presynaptically (pre-LTP). Moreover, this form of LTP required presynaptic Ca^{2+} influx for its induction. These results indicate that cannabinoid might be acting presynaptically to suppress pre-LTP. The mechanistic explanation of the observed interaction between different forms of LTP at the same synapses would require a detailed characterization of

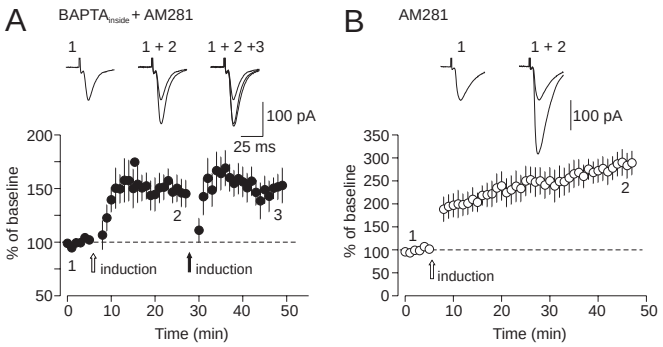

Fig. 5. Properties of LTP induced with post-LTP protocol in the presence of BAPTA in the pipette solution and AM281 in the external medium. (*A*) First, LTP was induced in thalamic input with the post-LTP protocol in the presence of AM281 (0.5 μM) in the bath and 20 mM BAPTA in the pipette solution. Subsequent delivery of the pre-LTP–inducing protocol (thalamic input was stimulated for 2 min with paired pulses at 2 Hz frequency at a holding potential of −70 mV) did not result in additional potentiation ($n = 6$, $P = 0.56$ vs. LTP with the post-LTP protocol). This was not caused by washout of pre-LTP, because it could be induced at ∼30 min after beginning the whole-cell recording (Fig. 4*B*). The observed occlusion of pre-LTP suggests that two forms of plasticity might be mechanistically similar. (*Insets*) Averaged EPSCs before (1) and after delivery of the first (2) and the second (3) LTP-inducing stimulation protocols. (*B*) Delivery of the post-LTP induction protocol in the presence of AM281 (0.5 μM) but without BAPTA in the pipette solution resulted in the very large LTP. The EPSC was potentiated to 289 ± 26% of its baseline amplitude ($n = 6$, $P = 0.0002$ vs. pre-LTP alone), suggesting that both pre-LTP and post-LTP might be induced simultaneously under conditions where the endogenous cannabionoids (eCB) cascade is blocked. (*Insets*) Averaged EPCS before (1) and after (2) the induction of LTP. Error bars indicate SEM.

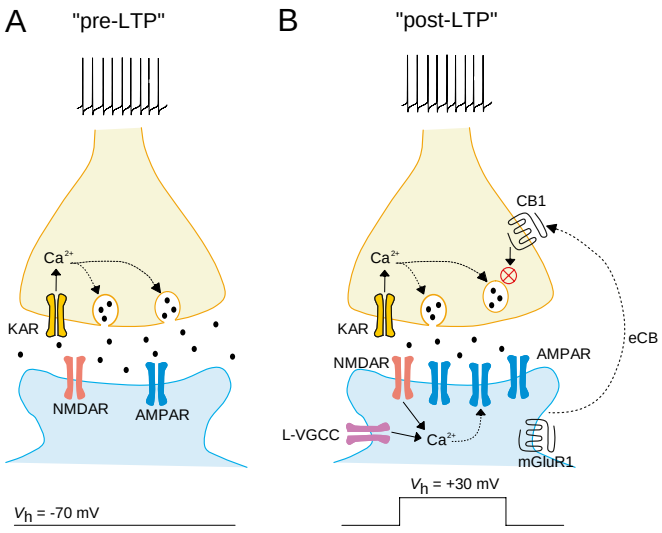

Fig. 6. Potential mechanisms of suppression of pre-LTP by the induction of post-LTP at thalamo-LA synapses. (*A*) Stimulation of thalamic input to the LA with paired pulses at 2 Hz frequency for 2 min without postsynaptic depolarization leads to the induction of LTP, which is both induced and expressed presynaptically (pre-LTP). This form of LTP depends on activation of presynaptic GluR5 subunit-containing kainate receptors and presynaptic Ca^{2+} influx. (*B*) Delivery of the identical presynaptic stimulation, but under conditions of postsynaptic depolarization to +30 mV, results in a form of LTP that is both induced and expressed postsynaptically (post-LTP). The induction of post-LTP depends on postsynaptic Ca^{2+} influx through NMDA receptors and L-type voltage-gated Ca^{2+} channels. Therefore, two mechanistically distinct forms of LTP coexist in thalamic input, but pre-LTP is suppressed by the induction of post-LTP at the same synapses. The observed interaction between coexisting forms of LTP in inputs to the LA may be mediated by activation of presynaptic CB1 receptors by eCB released in response to activation of the mGluR1, thus preventing the long-term enhancements of neurotransmitter release observed during pre-LTP.

the molecular events in nerve terminals associated with the induction and expression of pre-LTP in the thalamo-amygdala pathway. Although the processes underlying postsynaptically expressed forms of LTP at central synapses are reasonably well-characterized, relatively little is still known about the molecular mechanisms of presynaptic forms of LTP at glutamatergic synapses. Nevertheless, previous experiments suggested some interesting possibilities that might be tested in future studies. Thus, it has been proposed recently that persistent changes in the machinery of neurotransmitter release through cAMP/PKA signaling and the active zone protein RIM1α could represent a general mechanism underlying presynaptic forms of long-term plasticity at central synapses (26). For example, RIM1α was implicated in the induction of protein kinase A (PKA)-dependent forms of LTP at the mossy fiber synapses and CA3–CA1 synapses in the hippocampus (27, 28) and the parallel fiber to Purkinje cell synapses (27). Moreover, phosphorylation of RIM1α by PKA enhances neurotransmitter release (29). Because CB1 receptors are $G_{i/o}$-coupled and therefore, their activation reduces cAMP production, activation of CB1 receptors could affect the release machinery in an RIM1α-dependent manner, perhaps because of a decrease in RIM1α phosphorylation by PKA (26). Interestingly, it was shown that the expression of presynaptic LTP at cortico-LA synapses, induced by direct activation of cAMP/PKA-dependent pathways by the adenylyl cyclase activator forskolin, may implicate lasting increases in L-type Ca^{2+} channel-mediated glutamate release (30). Therefore, if activation of presynaptic CB1 receptors on thalamic terminals suppresses up-regulation of the cAMP/PKA pathway- and RIM1α-dependent modifications of the release machinery required for the induction of presynaptically expressed LTP, pre-LTP would be suppressed.

What might be the functional significance of mechanistically distinct forms of synaptic plasticity coexisting in thalamic input to the LA? Differences in the patterns of neuronal activity in behaving animals could produce different levels of postsynaptic depolarization, and thus, they recruit different forms of synaptic plasticity in inputs to the amygdala needed to encode conditioned fear memory and then, retrieve it. This situation is modeled in our slice experiments, where different levels of postsynaptic depolarization led to the induction of different forms of LTP. The form of LTP corresponding to the potentially more aversive US (associated with more depolarization; post-LTP) dominates. This might reflect the hierarchy of memories for the events having different biological significance, where more aversive events produce stronger memories. Our findings correspond well with the fact that the stronger US (foot shock) is often associated with the stronger freezing reactions in experimental animals at times when fear memory is tested. A role of cannabinoid release in fear-related behavior has been shown directly in a recent study describing the endocannabinoid CB1 receptor-dependent modulation of amygdala-dependent Pavlovian fear conditioning (31). The observed interaction between mechanistically distinct forms of LTP could potentially help in maintaining the link between specific forms of synaptic plasticity and the acquisition of memories for the specific types of behavioral experiences. It would be interesting to explore whether various forms of aversive learning are similar in their ability to implicate the described mechanism of interaction between coexisting forms of LTP at synapses in the neuronal circuits of learned behavioral responses.

Materials and Methods

Coronal brain slices containing the amygdala (250–300 μm) were prepared from 3- to 5-wk-old Sprague–Dawley rats with a vibratome. Slices were continuously superfused in solution containing (in mM) 119 NaCl, 2.5 KCl, 2.5

$CaCl_2$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$, 10 glucose, and 0.1 picrotoxin (unless noted otherwise) and equilibrated with 95% O_2 and 5% CO_2 (pH 7.3–7.4) at room temperature (22–24 °C). Whole-cell recordings of compound or unitary EPSCs were obtained from pyramidal neurons in the lateral amygdala under visual guidance (differential interference contrast/infrared optics) with an EPC-9 amplifier and Pulse v8.40 software (HEKA Elektronik). The cells were classified as principal neurons based on their appearance and their ability to show spike frequency adaptation to the prolonged depolarizing current injection. Synaptic responses were evoked by field stimulation of the fibers in either the external capsule (cortical input) or the internal capsule (thalamic input) at 0.05 Hz. The patch electrodes (3–5 MΩ resistance) contained (in mM) 120 K-methane-sulfonate, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 Hepes, 2 MgATP, and 0.1 NaGTP (adjusted to pH 7.2 with KOH). Currents were filtered at 1 kHz and digitized at 5 kHz. Unitary EPSCs were evoked by low-intensity current pulses (20–40 μA; 100 μs duration) applied through a fine-tipped (~2 μM), concentric stimulating electrode consisting of a patch pipette that was coated with silver paint. The two leads of the stimulus isolation unit (ISO-Flex, Master-8 stimulator; AMPI) were connected to the inside of the pipette and the external silver coat. The stimulating pipettes were positioned to activate either cortical or thalamic input to the LA (Fig. 1*A*). The recording was used if the mean EPSC amplitude showed a steep all-or-none threshold as a function of stimulating current intensity and if there was no change in potency (the mean size of responses, excluding failures of synaptic transmission) during double-pulse stimulation with a 50-ms interpulse interval, indicating stimulation of a single presynaptic input. The EPSC amplitude was measured as the difference between the mean current during a prestimulus baseline and the peak current over a 1- to 2-ms window. In all LTP experiments, the stimulus intensity was adjusted to produce synaptic responses with an amplitude that was ~20–25% of maximum amplitude EPSC. For induction of pre-LTP, 240 paired presynaptic stimuli (with 50-ms interpulse intervals) were delivered at 2 Hz to the presynaptic fibers at a holding potential of −70 mV. For induction of post-LTP, the same stimulation was delivered at a holding potential of +30 mV. Summary LTP graphs were constructed by normalizing data in 60s epochs to the mean value of the baseline EPSP.

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