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13. SUPPLEMENTARY NOTES Note: The previous report was deemed unacceptable for the following reason. "There is no data in the body of this report and data in the appended manuscript are not cited in the body of the report to support key research accomplishments. In a revised report, please summarize the data from the previous year. The appended publication can be used as supporting material as long as figures are referenced in the body of the progress report." The following revised report now contains data in the body of the report, which are indicated by yellow highlighting.					
14. ABSTRACT- 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.					
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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 amnesic (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). Since the publication of our positive results with blocking the reconsolidation of cue-conditioned fear with mifepristone, described in the last report, we have been unable to demonstrate successful reconsolidation with any further agents approved for human use. With the conclusion of the 04 year, we have terminated this work. The reason we are not continuing this into the no-cost extension period is that we believe the best use of our remaining funds will be continuing the work with human PTSD subjects.

2.1.2. McGill University. We successfully completed our reconsolidation study with clonidine. The following is a summary of the findings. Exposure to traumatic events can lead to posttraumatic stress disorder (PTSD). Current PTSD treatments typically only produce partial improvement. Hence, there is a need for preclinical research to identify new candidate drugs and to develop novel therapeutic approaches. Animal studies have indicated that fear memories can be weakened by blocking restabilization after retrieval, a process known as reconsolidation. Furthermore, evidence suggests that there are important alterations of the noradrenergic system in PTSD, and hence it may be of interest to study drugs that target this pathway. Here, we investigated the efficacy of clonidine, an α_2 -adrenoreceptor agonist, to block reconsolidation in an animal model of persistent traumatic memories. Using an auditory fear conditioning

paradigm in rats, we tested the efficacy of clonidine to weaken fear memory retention when administered systemically after retrieval. We evaluated dosage, number of treatments, and specificity in reconsolidation blockade. We found that postretrieval administration of clonidine disrupts fear-related memories in a dose-dependent manner and that two treatments are sufficient for maximal memory impairment. Furthermore, we determined that this effect is long lasting and specific to reconsolidation processes as shown by the selectivity to affect reactivated memories and the absence of spontaneous recovery and of postreactivation short-term memory impairment. Our results demonstrate the efficacy of systemic administration of clonidine following retrieval to persistently disrupt fear memory retention through reconsolidation blockade. This study provides important preclinical parameters for future therapeutic strategies involving clonidine to block reconsolidation as a novel treatment for PTSD symptoms.

The data from this study appear in the following five figures, with explanatory footnotes.

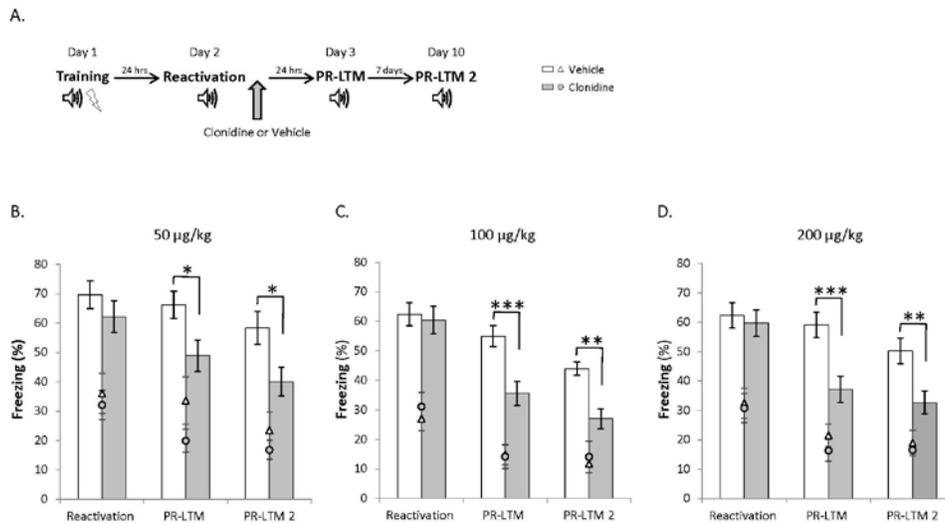


Figure 1. Post-reactivation administration of clonidine impairs reconsolidation of auditory fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine or its vehicle immediately after a reactivation session, and were tested for post-reactivation long-term memory 1 day (PR-LTM) and 1 week later (PR-LTM 2). A dose of 50 µg/kg (b; n=20), 100 µg/kg (c; n=25) and 200 µg/kg (d; n=20) was effective at impairing memory reconsolidation compared to the vehicle group (respectively n=16, n=25, n=20) as shown by an impaired conditioned response (freezing) at both time points. Bars represent mean ± s.e.m freezing to the tone. Markers represent the mean ± s.e.m freezing prior to the onset of the tone. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

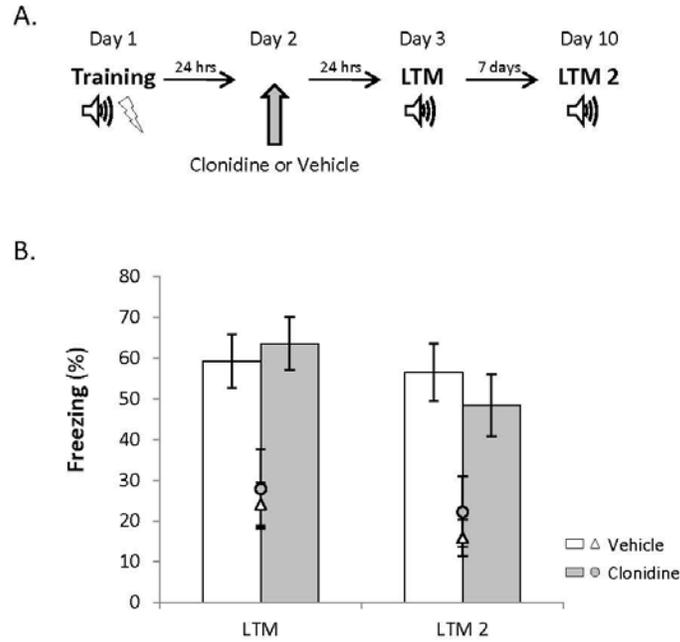


Figure 2. Clonidine does not impair retention of non-reactivated fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine (100 $\mu\text{g}/\text{kg}$) or its vehicle without a memory reactivation session and were tested for long-term memory retention 1 day (LTM) and 1 week later (LTM 2). (b) Clonidine-treated rats ($n=12$) showed a similar conditioned response (freezing) to the vehicle group ($n=12$) when tested 24 hours or 1 week after injection. Bars represent mean \pm s.e.m freezing to the tone. Markers represent the mean \pm s.e.m freezing prior to the onset of the tone.

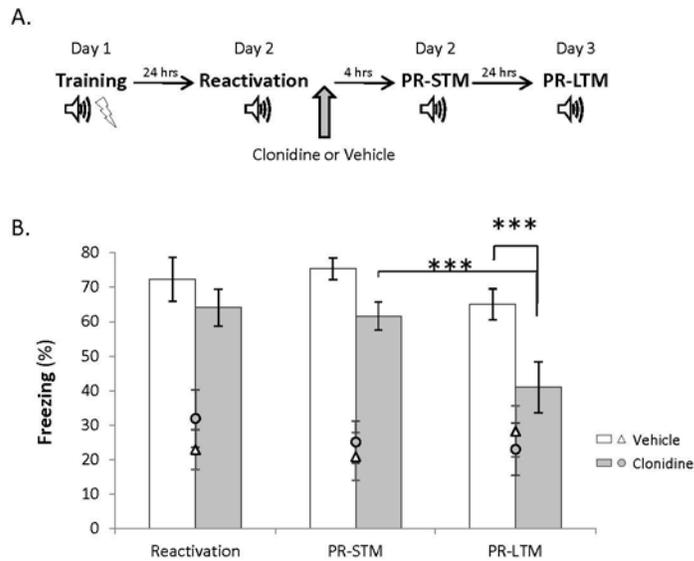


Figure 3. Post-reactivation administration of clonidine does not impair short-term fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine (100 $\mu\text{g}/\text{kg}$) or its vehicle immediately after a reactivation session and were tested 4 hours later for post-reactivation short-term memory (PR-STM) and 1 day later for post-reactivation long-term memory (PR-LTM). (b) Clonidine-treated rats ($n=12$) showed a similar conditioned response (freezing) to the vehicle group ($n=9$) when tested 4 hours after reactivation, but reduced freezing behavior 1 day after injection. Bars represent mean \pm s.e.m freezing to the tone. Markers represent the mean \pm s.e.m freezing prior to the onset of the tone. Statistical significance: *** $p < 0.001$.

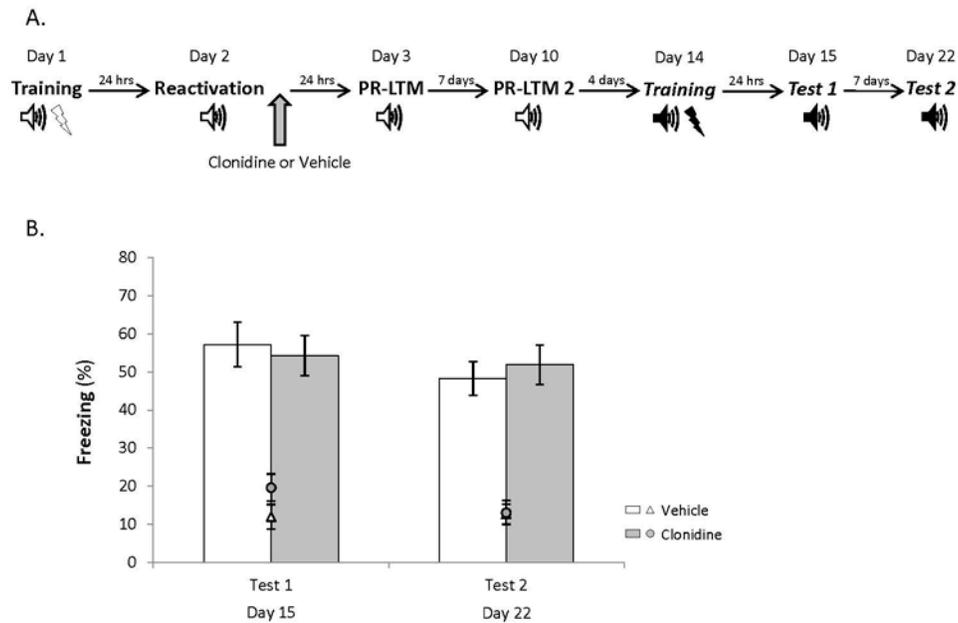


Figure 4. Post-reactivation administration of clonidine does not impair the ability to learn new fear memories. (a) Schematic of the experimental design. After receiving a post-reactivation injection of clonidine (200 $\mu\text{g}/\text{kg}$) or vehicle, and being tested for memory retention 1 day (PR-LTM) and 1 week later (PR-LTM 2), rats were conditioned to fear a different tone using a different auditory fear protocol. (b) Rats that previously received clonidine ($n=12$) showed intact fear behavior (freezing) compared to the vehicle-treated animals ($n=12$) when tested 1 day (Test 1) or 1 week later (Test 2). Bars represent mean \pm s.e.m freezing to the tone. Markers represent the mean \pm s.e.m freezing prior to the onset of the tone.

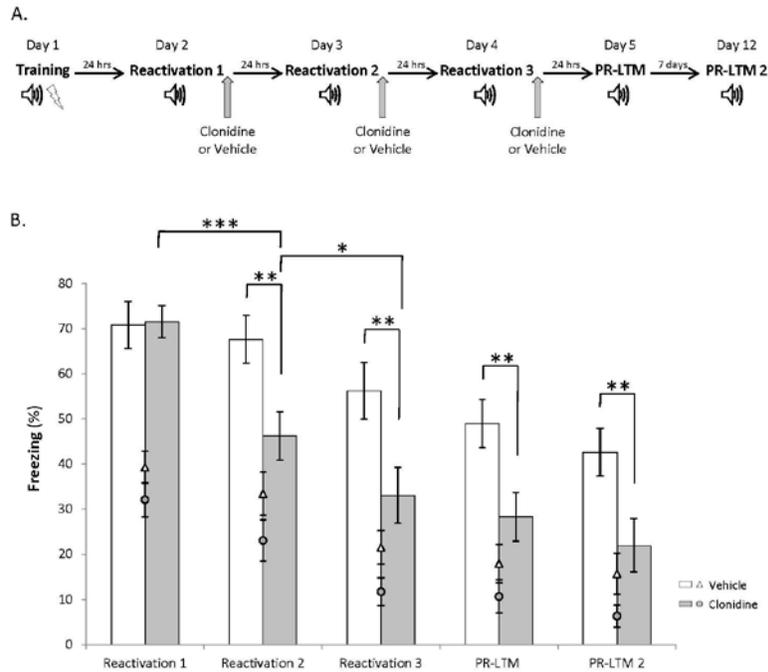


Figure 5. Two post-reactivation clonidine treatments are sufficient to maximally impair fear memory retention. (a) Schematic of the experimental design. Rats received a systemic injection of clonidine (100 $\mu\text{g}/\text{kg}$) or its vehicle immediately after a reactivation session for 3 consecutive days and were tested for post-reactivation long-term memory 1 day (PR-LTM) and 1 week later (PR-LTM 2). (b) Clonidine-treated rats ($n=16$) showed an impaired conditioned response (freezing) as compared to the vehicle group ($n=15$) at each test session. Memory disruption was observed after the first clonidine treatment and reached its maximum after 2 treatments at day 3. Bars represent mean \pm s.e.m freezing to the tone. Markers represent the mean \pm s.e.m freezing prior to the onset of the tone. Statistical significance: $p < 0.05$, $**p < 0.01$, $***p < 0.001$

This article has been published (Gamache et al, 2012) and is listed under Reportable Outcomes below.

2.1.3. McLean Hospital. The manuscript described in the previous report was not accepted by *Science* or *Nature*. We have made improvements and submitted it to *Proceedings of the National Academy of Sciences*. Reviews were favorable, and we were invited to resubmit a revised manuscript. A summary of the findings follows. Synaptic mechanisms underlying memory reconsolidation after retrieval are largely unknown. Here we report that synapses in projections to the lateral nucleus of the amygdala (LA) implicated in auditory fear conditioning, which are potentiated by learning, enter a labile state after memory reactivation and must be re-stabilized through a postsynaptic mechanism implicating the mammalian target of rapamycin (mTOR) kinase-dependent signaling. Fear conditioning-induced synaptic enhancements were primarily presynaptic in origin. Reconsolidation blockade with rapamycin, inhibiting mTOR kinase activity, suppressed synaptic potentiation in slices from fear-conditioned rats. Surprisingly, this reduction of synaptic efficacy was mediated by post- but not presynaptic mechanisms. These findings suggest that different plasticity rules may apply to the processes underlying the acquisition of original fear memory and post-reactivational stabilization of fear conditioning-induced synaptic enhancements mediating fear memory reconsolidation.

The data from this study appear in the following four figures, with explanatory footnotes.

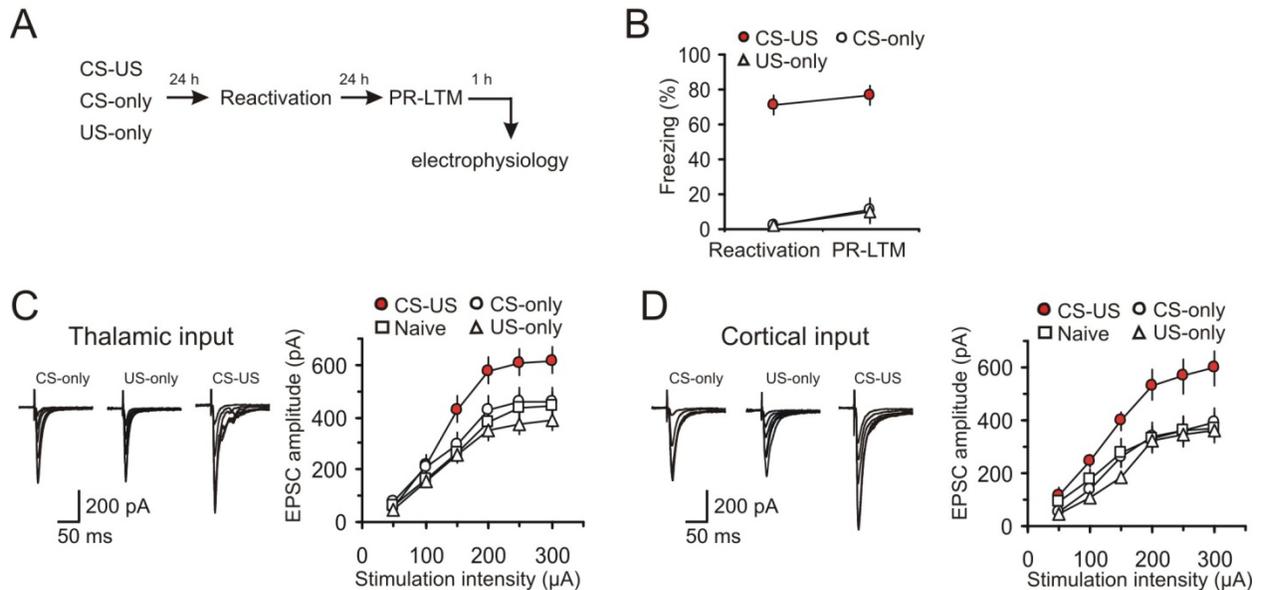


Figure 1. Fear conditioning leads to synaptic enhancements in cortical and thalamic inputs to the LA. (A) A schematic representation of the experimental design. Rats were trained in a single-trial fear conditioning paradigm and tested at 24 h (PR-LTM) after reactivation trials. (B) Percent freezing observed in fear-conditioned rats (CS-US, Paired) and in rats that received CS or US only (CS-US, $n = 22$ rats; CS-only, $n = 20$ rats; US-only, $n = 6$ rats). (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, US-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; US-only = 12 neurons; Paired, $n = 14$ neurons). Peak amplitudes of the EPSCs were significantly different between Naïve, CS-only, US-only and Paired groups (two-way ANOVA, $F_{3,379} = 11.57$, $P < 0.001$). Post hoc Bonferroni's simultaneous multiple comparisons revealed significant differences in the EPSC amplitudes between Naïve and Paired groups ($P < 0.001$), between CS-only and Paired groups ($P < 0.01$), and between US-only and Paired groups ($P < 0.001$). 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 ($n = 8$), US-only ($n = 12$) and Paired ($n = 12$) groups (two-way ANOVA, $F_{3,279} = 17.66$, $P < 0.001$). EPSC amplitudes were larger in the Paired group compared with either Naïve ($P < 0.001$), CS-only ($P < 0.001$), or US-only group ($P < 0.001$; Bonferroni's simultaneous multiple comparisons). Results are shown as means \pm SEM.

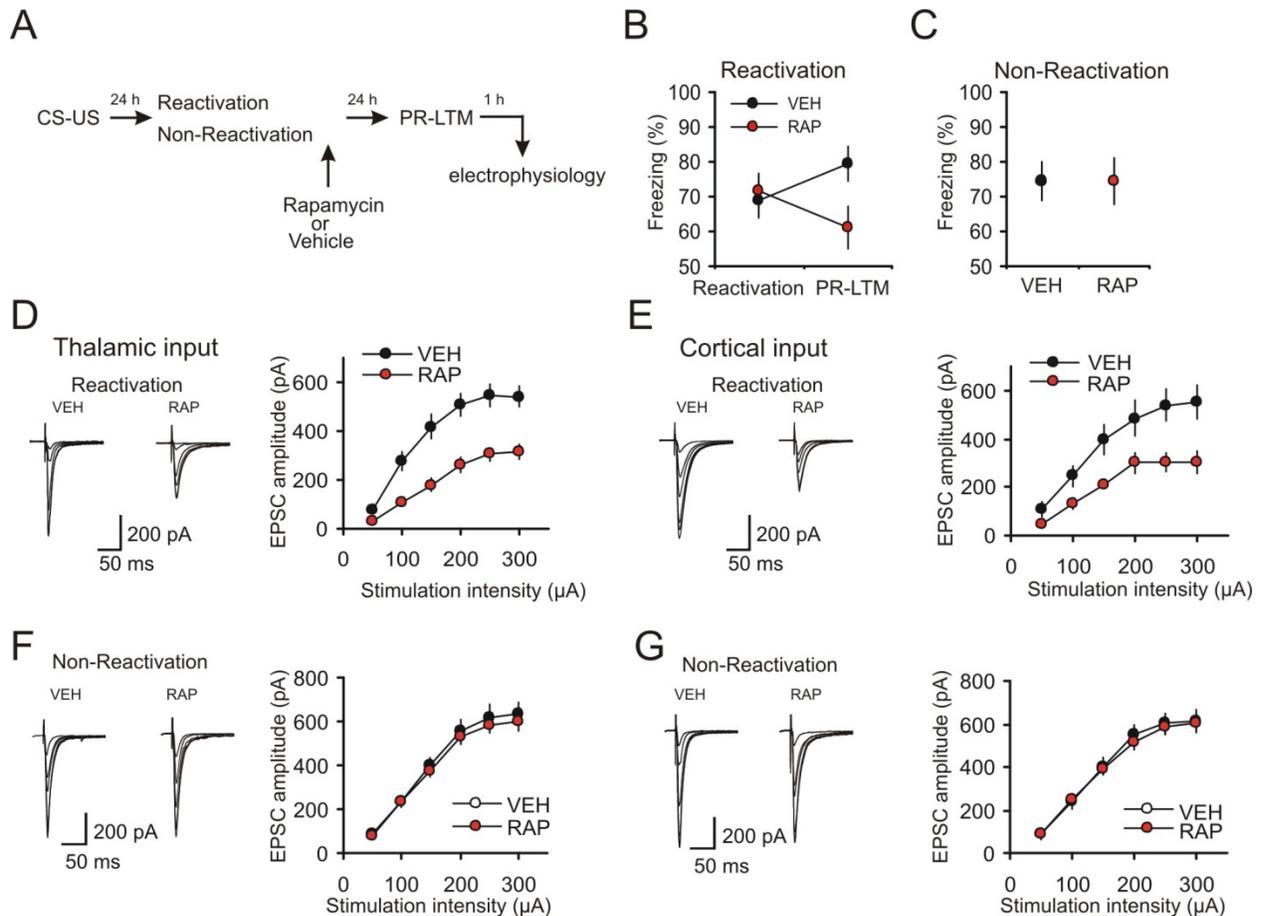


Figure 2. Post-retrieval rapamycin impairs reconsolidation of fear memory and suppresses conditioning-induced synaptic enhancements. (A) A schematic representation of the experiments where fear-conditioned rats received a postretrieval injection of rapamycin (RAP; 20 mg/kg, i.p.) or vehicle (VEH). (B) 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 in RAP rats during the PR-LTM test (see text for details). (C) Rapamycin had no effect on conditioned freezing in “non-reactivated” control rats. Rats in Non-reactivation group received rapamycin or vehicle injections at 24 h post-conditioning without memory reactivation and PR-LTM was tested 24 h after the injections (RAP, $n = 16$ rats; VEH, $n = 8$ rats; t test, $P = 0.9$ for VEH group vs. RAP group). (D) Left, averaged EPSCs evoked in thalamic input to the LA by stimuli of increasing intensity in slices from fear-conditioned rats which received post-reactivation injections of VEH or RAP. Right, synaptic input-output curves obtained in thalamic input in slices from both groups of rats (VEH, $n = 12$ neurons; RAP, $n = 13$ neurons (two-way ANOVA, $F_{1,138} = 101.4$, $P < 0.001$ for VEH group versus RAP group of conditioned rats). (E) Experiments were analogous to D, but the EPSCs were recorded in cortical input to the LA (VEH, $n = 12$ neurons; RAP, $n = 8$ neurons; two-way ANOVA, $F_{1,104} = 27.58$, $P < 0.001$). (F) Rapamycin or vehicle were injected at 24 h post-conditioning without memory reactivation and synaptic input-output curves were obtained in thalamic input 24 h after the injections (VEH, $n = 14$ neurons; RAP, $n = 23$ neurons; two-way ANOVA, $F_{1,120} = 1.20$, $P = 0.275$). (G) Experiments were analogous to F but the EPSCs were recorded in cortical input (VEH, $n = 9$ neurons; RAP, $n = 19$ neurons; two-way ANOVA, $F_{1,168} = 0.43$, $P = 0.515$). Results are shown as means \pm SEM.

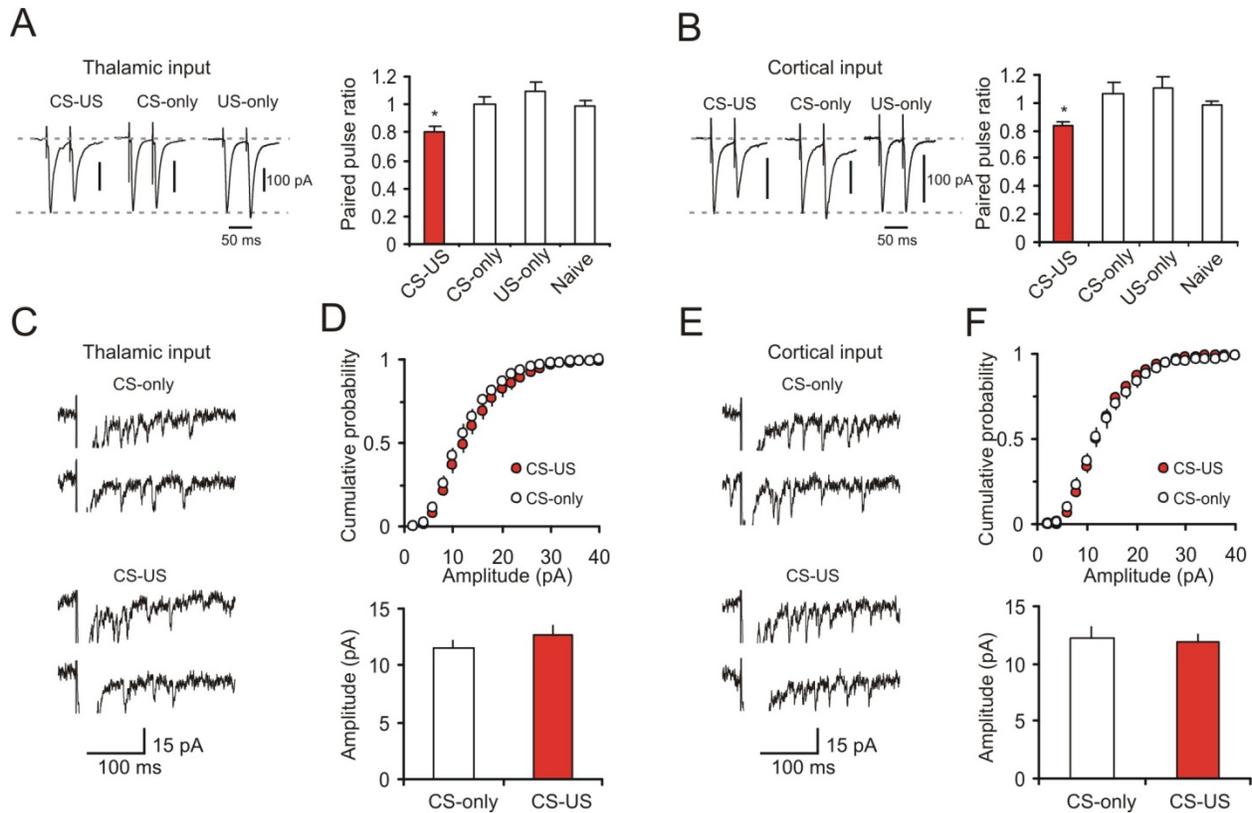


Figure 3. Fear conditioning-induced synaptic strengthening in inputs to the LA is primarily presynaptically-mediated. (A) Left, examples of EPSCs evoked in thalamic input to the LA with paired presynaptic stimuli in slices from CS-only, US-only and fear-conditioned (CS-US) rats. The interstimulus interval 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. CS-only group of rats, $n = 10$ neurons; US-only group, $n = 12$ neurons; Naïve group, $n = 17$ neurons; CS-US group, $n = 9$ neurons. The magnitude of PPR in the Paired group of rats (CS-US) was significantly decreased compared to Naïve, CS-only or US-only rats (one-way ANOVA, $F_{3,44} = 4.02$, $P = 0.013$). There was no difference in PPR values between Naïve and CS-only ($P = 0.45$) or US-only groups ($P = 0.203$). (B) Experiments were analogous to A, but the EPSCs were recorded in cortical input to the LA. CS-only group, $n = 8$ neurons; US-only group, $n = 9$ neurons; Naïve group, $n = 18$ neurons; Paired group, $n = 7$ neurons. The magnitude of PPR in the Paired group was significantly decreased compared to Naïve, CS-only or US-only rats (one-way ANOVA, $F_{3,38} = 3.37$, $P = 0.028$). There was no difference between Naïve and CS-only rats ($P = 0.1$) or US-only rats ($P = 0.1$). (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, $n = 9$ neurons; Paired, $n = 10$ 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.

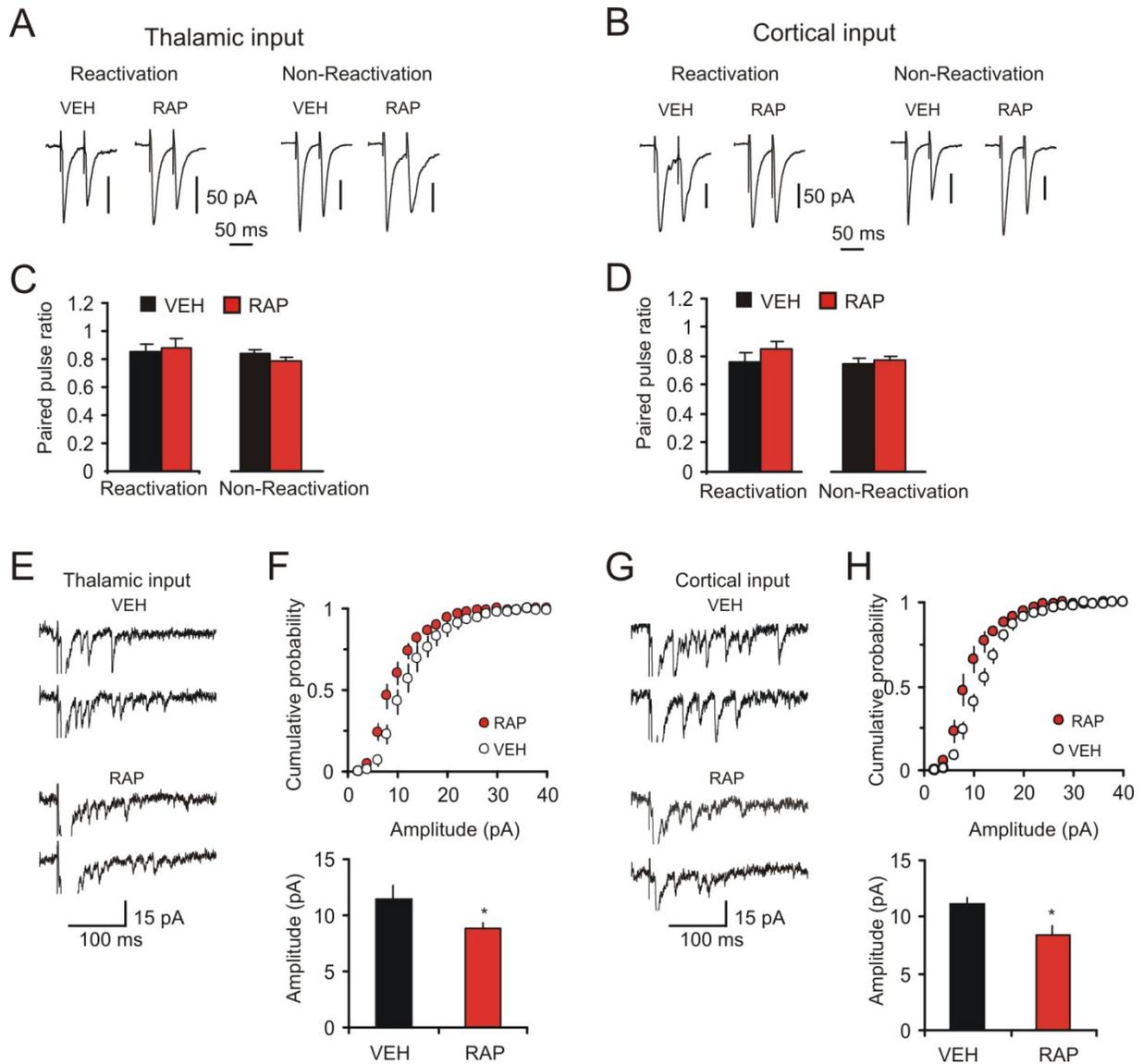


Figure 4. Postretrieval stabilization of conditioning-induced potentiation in inputs to the LA implicates postsynaptic mechanisms. (A) Reactivation (left), examples of EPSCs evoked in thalamic input to the LA with paired stimuli in slices from fear-conditioned rats that received one injection of either rapamycin (RAP; 20 mg/kg, i.p.) or vehicle (VEH) immediately after the fear memory reactivation (memory was retrieved at 24 h post-conditioning). Recordings were performed 24 h after the memory reactivation. Non-reactivation (right), examples of EPSCs recorded in slices from rats which received rapamycin or vehicle injections at 24 h post-conditioning without memory reactivation. Recordings were performed 24 h after the injections. (B) Analogous to A, but the EPSCs were recorded in cortical input. (C) Summary plot of PPR data in thalamic input (Reactivation: VEH, n = 19 neurons; RAP, n = 21 neurons; t test, P = 0.79; Non-Reactivation: VEH, n = 17 neurons; RAP, n = 24 neurons; t test, P = 0.19). (D) Summary plot of PPR data in cortical input (Reactivation: VEH, n = 11 neurons; RAP, n = 13 neurons; t test, P = 0.31; Non-reactivation: VEH, n = 10 neurons; RAP, n = 19 neurons; t test, P = 0.63). (E) Traces of the asynchronous quantal EPSCs evoked by stimulation of thalamic input in slices from VEH or RAP groups. (F) 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 = 0.048). (G and H) The experiments were analogous to E and F, but the asynchronous EPSCs were recorded in cortical input to the LA (VEH, n = 5 neurons; RAP, n = 6 neurons; t test, *P = 0.026). Error bars indicate SEM.

2.4. Human work

2.4.1 MGH. On the basis of the animal results reported in previous annual reports and published in Pitman et al (2011), we decided to perform a double-blind, placebo-controlled study of pre-activation mifepristone's ability to reduce psychophysiologic responding during traumatic mental imagery in trauma-exposed human subjects. We analyzed data from 34 completed subjects who received PR mifepristone (n=14), NR mifepristone (n=10), or placebo (n=10), randomized and double-blind. The analysis revealed no effect of drug on the primary outcome measure, viz., posterior physiological probability of PTSD. The mean physiological posterior probabilities were as follows: **for PR mifepristone, M=0.41, SD=0.17; for NR mifepristone, M=0.38, SD=0.06; for placebo, M=0.37, SD=0.16**. The subjects recruited in all groups had probabilities that were below the normative cut-offs for PTSD, which largely motivated the addition of our collaborating site at Dallas VA Hospital in the hope of obtaining more suitable PTSD subjects.

In light of the above negative results, the study design at the MGH was changed as follows. In order for reconsolidation blockade to work, the memory first has to be destabilized. Because *antagonism* at NMDA receptors prevents this destabilization, we reasoned that a partial NMDA *agonist* d-cycloserine (DCS) could promote destabilization. Therefore, a single pre-activation dose of DCS was added to the currently administered dose of pre-activation mifepristone. All necessary IRB and FDA approvals were obtained. As of the end of the 04 year, 14 subjects have completed their first three study visits under the new design, and more are being recruited. The blind has not yet been broken, so there are not yet any results to report.

2.4.2. McGill University/Douglas Mental Health University Institute

2.4.2.1 Background for current study. We decided to undertake a double-blind, randomized, placebo-controlled trial of six sessions of trauma-reactivation under the influence of 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 et 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, pre-activation propranolol case series in 32 PTSD patients yielded promising results (Brunet et al, 2011-see Reportable Outcomes). Fourth, a meta-analysis conducted by our group (Lonergan et al. 2012-see Reportable Outcomes) also converged to suggest that propranolol is a robust consolidation and reconsolidation blocker in healthy participants. Results from these studies serve the basis for a double-blind, randomized, placebo-controlled trial that is now underway. The study is looking at the therapeutic effects of six once-a-week 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 (2) psychophysiologic responding to script-driven imagery depicting the person's traumatic event (post-treatment and at follow-up).

2.4.2.2. Progress to date. At the time of the last annual report, 60 patients had been screened and 24 patients had completed the treatment protocol. During the 04 year, an additional 28 patients were screened. Of those, 14 were randomized, and 9 have completed the protocol. An additional 3 subjects are in the midst of study participation. Thus, overall, there are to date 88 screened participants, 46 of whom were randomized (intent-to-treat sample), and 33 treatment completers. We have not yet broken the blind, so there are not yet any results to report.

2.4.3 Dallas VA Medical Center. We subcontracted to recruit and PTSD patients the veteran population for the above double-blind placebo-controlled study of pre-reactivation mifepristone's ability to reduce psychophysiologic responding during traumatic imagery in trauma-exposed human subjects. We obtained all necessary IRB and HRPO approvals for this study site and began recruiting subjects in February 2012. To date we have enrolled 9 subjects and each of these subjects has completed the first 3 sessions of the 4-session protocol. The blind has not yet broken, so there are not yet any results to report. We have had no adverse events, nor have we had any study withdrawals. Recruitment is ongoing and we foresee no barriers in achieving/exceeding our completion goal.

3. KEY RESEARCH ACCOMPLISHMENTS (past year)

3.1. Publication of original discovery that the post-reactivation administration of clonidine impairs reconsolidation of auditory fear memories in rats.

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 review in *PNAS*).

3.4. Completion of double-blind controlled trial of pre-reactivation mifepristone's ability to reduce psychophysiologic responding during traumatic imagery in PTSD subjects, unfortunately with negative results. Began new study adding DCS to mifepristone.

3.4. Continued progress in studying human subjects in a randomized, double-blind controlled trial of six sessions of post-reactivation propranolol for the treatment of PTSD.

4. REPORTABLE OUTCOMES (past year)

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.

Gamache K, Pitman RK, Nader K. Preclinical evaluation of reconsolidation blockade by clonidine as a potential novel treatment for posttraumatic stress Disorder. *Neuropsychopharmacology* 2012;37:2789-2796. *Note: Attached as appendix.*

Lonergan MH, Olivera-Figueroa LA, Pitman RK, Brunet A. Propranolol's effects on the consolidation and reconsolidation of long-term emotional memory in healthy participants: a meta-analysis. *Journal of Psychiatry and Neuroscience* 2012; Nov 26;37(6):1201-11. [Epub ahead of print].

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 four promising candidate drugs that are approved for human use, viz., propranolol, mifepristone, clonidine, and rapamycin. Randomized, placebo-controlled, double-blind clinical trials are underway to test some of these drugs (propranolol and mifepristone plus DCS).

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7. APPENDICES/SUPPORTING DATA

The following reprint is attached:

Gamache K, Pitman RK, Nader K. Preclinical evaluation of reconsolidation blockade by clonidine as a potential novel treatment for posttraumatic stress Disorder. *Neuropsychopharmacology* 2012;37:2789-2796.

Preclinical Evaluation of Reconsolidation Blockade by Clonidine as a Potential Novel Treatment for Posttraumatic Stress Disorder

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Exposure to traumatic events can lead to posttraumatic stress disorder (PTSD). Current PTSD treatments typically only produce partial improvement. Hence, there is a need for preclinical research to identify new candidate drugs and to develop novel therapeutic approaches. Animal studies have indicated that fear memories can be weakened by blocking restabilization after retrieval, a process known as reconsolidation. Furthermore, evidence suggests that there are important alterations of the noradrenergic system in PTSD, and hence it may be of interest to study drugs that target this pathway. Here, we investigated the efficacy of clonidine, an α_2 -adrenoreceptor agonist, to block reconsolidation in an animal model of persistent traumatic memories. Using an auditory fear conditioning paradigm in rats, we tested the efficacy of clonidine to weaken fear memory retention when administered systemically after retrieval. We evaluated dosage, number of treatments, and specificity in reconsolidation blockade. We found that postretrieval administration of clonidine disrupts fear-related memories in a dose-dependent manner and that two treatments are sufficient for maximal memory impairment. Furthermore, we determined that this effect is long lasting and specific to reconsolidation processes as shown by the selectivity to affect reactivated memories and the absence of spontaneous recovery and of postreactivation short-term memory impairment. Our results demonstrate the efficacy of systemic administration of clonidine following retrieval to persistently disrupt fear memory retention through reconsolidation blockade. This study provides important preclinical parameters for future therapeutic strategies involving clonidine to block reconsolidation as a novel treatment for PTSD symptoms.

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Keywords: clonidine; memory; reconsolidation; fear conditioning; α_2 -adrenoreceptor agonist; posttraumatic stress disorder

INTRODUCTION

In a substantial minority of individuals, experiencing a traumatic event can lead to posttraumatic stress disorder (PTSD). This condition is characterized by several symptoms including irritability, hypervigilance, avoidance behaviors, intrusive memories, and frequent re-experiencing of the traumatic event through nightmares and flashbacks. PTSD affects 10–20% of people who have experienced a traumatic event. It has a lifetime prevalence of 6.8% in the United States (Kessler *et al*, 2005). Current therapeutic strategies include psychotherapy and pharmacological treatments; however, only 60% of patients will be responsive to these treatments (Davidson *et al*, 2006; Onder *et al*, 2006) and only 20–30% will achieve full remission (Berger *et al*, 2009). Consequently,

there is a significant need to develop novel pharmacological approaches to reduce symptoms of PTSD.

A proposed therapeutic strategy involves the modification of memory reconsolidation processes. In order for a new memory to be retained, it has to be stabilized through a mechanism referred to as consolidation. When such a memory is retrieved (recalled), it becomes unstable again for a short period of time, at which point it is susceptible to modifications (Nader and Hardt, 2009). The memory is then restabilized (reconsolidated) in its modified state. In PTSD, flashbacks, nightmares, and recollection of intrusive memories allow the traumatic memory trace to be retrieved and then reconsolidated (Charney, 2004). Impairing reconsolidation of such memories may lead to their weakening and may consequently diminish PTSD symptoms.

In animal models, pharmacological interventions exploit the vulnerable state of a memory after recall in order to impair reconsolidation. Even though there is no animal model that recreates PTSD entirely, fear conditioning is known to model the fear that accompanies reminders of the traumatic event (Pitman *et al*, 1999; Siegmund and Wotjak,

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2006). Studies have shown that fear memories can be weakened by blocking the restabilization process with different drugs, such as protein synthesis inhibitors (Nader *et al*, 2000), *N*-methyl-*D*-aspartate (Ben Mamou *et al*, 2006), or adrenergic receptor antagonists (Przybylski *et al*, 1999; Debiec and Ledoux, 2004) and inhibitors of the mammalian target of rapamycin (Blundell *et al*, 2008; Jobim *et al*, 2012). A disadvantage of these pharmacological agents is that most of them are toxic, administered intracranially, and not approved for humans. In order to more easily extrapolate work in animal models to clinical trials, investigated drugs should be safe for human use.

Evidence suggests that among other physiological alterations, there is increased noradrenergic activity in PTSD patients (Southwick *et al*, 1997, 1999; Boehnlein and Kinzie, 2007). Furthermore, it has been proposed that this hyperactivity is associated with hyperarousal and re-experiencing symptoms present in PTSD (Southwick *et al*, 1997; Boehnlein and Kinzie, 2007). Consequently, drugs that specifically target noradrenergic system hyperactivity and are safe for human use may be of clinical interest. One of those candidate drugs is the α_2 -adrenoreceptor agonist clonidine. The effect of clonidine on memory has been shown to be mediated through the α_2 -adrenoreceptor subtype (Galeotti *et al*, 2004). These receptors are located both pre- and post-synaptically. Clonidine is thought to act mainly at the presynaptic level by activating the α_2 -autoreceptor (Southwick *et al*, 1999; Wilens, 2006), which leads to inhibition of voltage-gated calcium channels and inhibition of norepinephrine release (Southwick *et al*, 1999; Gilsbach and Hein, 2011). Clinically, clonidine is used to induce sedation, analgesia, and hypotension (MacMillan *et al*, 1996; Lakhani *et al*, 1997), as well as in the treatment of attention-deficit/hyperactivity disorder (Wilens, 2006). Additionally, a few open-label studies have shown beneficial effects of clonidine in treating some PTSD symptoms (Kinzie and Leung, 1989; Harmon and Riggs, 1996; Ziegenhorn *et al*, 2009), but none of these studies used clonidine specifically in combination with traumatic memory retrieval. In animal models, the use of clonidine has been found to produce memory impairments in step-down (Genkova-Papasova and Lazarova-Bakurova, 1988; Genkova-Papazova *et al*, 1997), shuttle box (Hawkins and Monti, 1979; Homayoun *et al*, 2003), and passive avoidance tasks (Galeotti *et al*, 2004); however, the use of clonidine to block memory reconsolidation has yet to be investigated.

The present study aims to examine the use of clonidine as a potential novel treatment for PTSD by testing its effects on the reconsolidation of a fear memory in rats. We investigated key parameters necessary to develop clinical studies involving reconsolidation blockade with clonidine. We determined the most effective dose through a dose-response curve, established the optimal number of treatments, and verified that the observed effects were reconsolidation specific.

MATERIALS AND METHODS

Animals

Equal numbers of male and female Sprague-Dawley rats weighing between 250 and 350 g (Harlan Laboratories,

Indianapolis, IN) were co-housed with *ad libitum* access to food and water. Rats were maintained on a 12 h light/dark cycle. All experiments were performed during the light (day) phase. All procedures were approved by McGill Animal Care Committee and complied with the Canadian Council for Animal Care guidelines.

Drugs

Clonidine hydrochloride (Sigma-Aldrich, Canada) was dissolved in sterile saline (0.9% NaCl) to the final concentration (50, 100, or 200 $\mu\text{g}/\text{kg}$) and administered intraperitoneally at a volume of 1 ml/kg (Galeotti *et al*, 2004).

Behavioral Procedure

Rats underwent auditory fear conditioning, reactivation, and testing in the same experimental chamber to further resemble, in our animal model, a PTSD-like intrusive memory in which cue and context are usually not easily separated. The conditioning chamber consisted of a brightly lit plexiglass box (25 \times 29 \times 29 cm) with stainless steel-grid floor that was enclosed within a sound-attenuating box (Coulbourn Instruments, Whitehall, PA).

Experiment 1. Rats were first habituated to the chamber for 5 min on 2 consecutive days. The following day (day 1), rats were conditioned. Conditioning involved 2 min of acclimation to the chamber after which rats received a single pairing of a tone (30 s, 5 kHz, 75 dB) coterminating with a foot shock (1 s, 0.75 mA). Rats remained in the chamber an additional minute before being returned to their home cages. On day 2, the fear memory was reactivated by placing the animals in the experimental chamber and presenting the tone without the shock. Rats were then removed from the context and clonidine (50, 100, or 200 $\mu\text{g}/\text{kg}$) or its vehicle was administered immediately. On days 3 and 10, animals were tested for postreactivation long-term memory (PR-LTM) with the presentation of a single tone.

Experiment 2. Nonreactivated controls were habituated and trained as in experiment 1, but rats did not receive the reactivation and instead remained in the animal colony where they received the clonidine treatment on day 2.

Experiment 3. As a postreactivation short-term memory (PR-STM) control, animals were habituated, trained, reactivated, and given postreactivation clonidine as in experiment 1. They were tested 4 h after the reactivation session on day 2, and again 24 h later.

Experiment 4. Rats underwent the same procedure as in experiment 1 and received clonidine (200 $\mu\text{g}/\text{kg}$) or vehicle following reactivation. After the test on day 10, rats were allowed 2 days of rest before undergoing habituation, new conditioning, and testing in a different experimental chamber. The conditioning chamber consisted of a dimly lit plexiglass and steel box (25 \times 29 \times 29 cm) with one curved white plastic wall and one black and white striped wall, enclosed within a sound-attenuating box (Med Associates, VT). A smaller steel-grid floor was used in this design and peppermint-scented water was also vaporized

inside the box to create a different scent than before. Rats were first habituated to the chamber for 5 min on 2 consecutive days. The following day (day 14), rats were newly conditioned. After 3 and a half minutes of acclimation to the chamber, rats received a single pairing of a different frequency tone (20 s, 3 kHz, 85 dB) coterminating with a foot shock (1 s, 1.1 mA). Rats remained in the chamber an additional 2 min before being returned to their home cages.

Experiment 5. Rats were habituated, trained, and reactivated as described in experiment 1. However, rats underwent reactivation on days 2, 3, and 4, each time followed by an injection of clonidine (100 µg/kg) or its vehicle. Rats were tested on days 5 and 12 using the same procedure as above.

Behavior was recorded using FreezeView software (Actimetrics). Freezing, defined as immobilization with the exception of respiration (Blanchard and Blanchard, 1969), was the conditioned response taken as a measure of fear memory retention. Scores are presented as the percentage of time spent freezing during the total duration of the tone.

Statistical Analysis

A repeated-measures analysis of variance (ANOVA) followed by Fisher's *post hoc* analysis was used to compare groups across days. Significance was set as $p < 0.05$.

RESULTS

For all experiments, no significant sex main effect or interaction was observed for freezing to the tone. A repeated-measures ANOVA across days revealed no difference in freezing scores between males and females for any experiment. This lack of sex differences allowed us to combine the freezing scores for males and females for each experiment.

Pre-tone freezing was also analyzed with a repeated-measures ANOVA across days and no significant main effect of treatment or interaction was observed for any of the experiments. A main effect of sex was observed on pre-tone freezing only for experiments 1 and 3, where there was a lower pre-tone freezing response in the females. In light of these isolated results, the lack of treatment effect on pre-tone freezing, and because our measure of memory retention was tone-related freezing, pre-tone freezing was not further investigated.

Experiment 1: Postreactivation Administration of Clonidine Impairs Reconsolidation of Auditory Fear Memories in a Dose-Dependent Manner

We evaluated whether clonidine is effective at disrupting fear memory reconsolidation when administered systemically at 50, 100, or 200 µg/kg. We conditioned the animals on day 1 and reactivated them the following day by exposing them again to the conditioning chamber and the tone. After reactivation, animals received an injection of clonidine or its vehicle and were tested for memory retention a day later. To establish if the effects of clonidine were long lasting, rats were also tested again on day 10 (Figure 1a). Clonidine was effective at blocking memory reconsolidation at all tested doses, and its effect was long lasting as the memory impairment was still observed a week after the treatment (Figure 1b–d). A repeated-measures ANOVA revealed a main effect of treatment ($F(1, 34) = 6.08$, $p < 0.05$ for 50 µg/kg; $F(1, 48) = 10.61$, $p < 0.01$ for 100 µg/kg; $F(1, 37) = 7.99$, $p < 0.01$ for 200 µg/kg) and day ($F(2, 68) = 9.09$, $p < 0.001$ for 50 µg/kg; $F(2, 96) = 36.04$, $p < 0.001$ for 100 µg/kg; $F(2, 74) = 22.05$, $p < 0.0001$ for 200 µg/kg). A significant treatment × day interaction was observed for 100 µg/kg ($F(2, 96) = 4.66$, $p < 0.05$) and 200 µg/kg ($F(2, 74) = 5.71$, $p < 0.01$). Subsequent Fisher's *post hoc* tests indicated significant differences between the clonidine-treated group and the controls at both memory retention

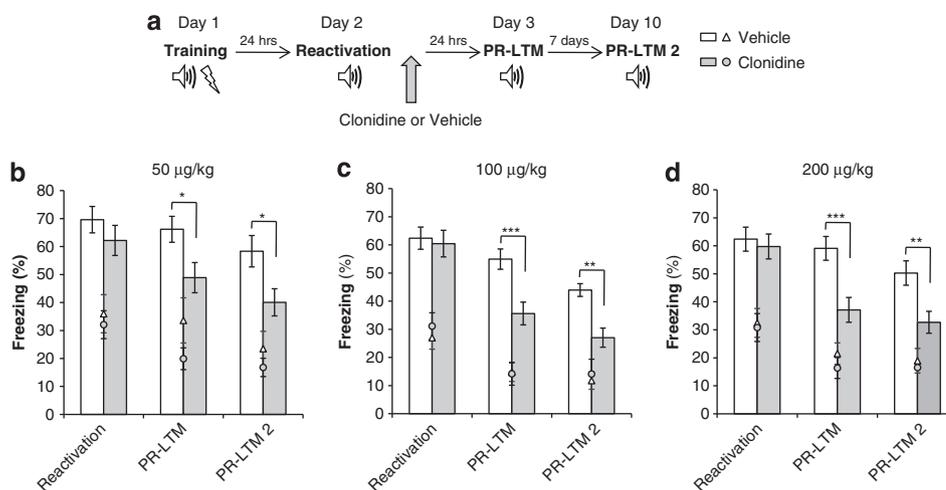


Figure 1 Postreactivation administration of clonidine impairs reconsolidation of auditory fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine or its vehicle immediately after a reactivation session, and were tested for postreactivation long-term memory 1 day (PR-LTM) and 1 week later (PR-LTM 2). A dose of (b) 50 µg/kg ($n = 20$), (c) 100 µg/kg ($n = 25$) and (d) 200 µg/kg ($n = 20$) was effective at impairing memory reconsolidation compared with the vehicle group (respectively, $n = 16$, $n = 25$, and $n = 20$) as shown by an impaired conditioned response (freezing) at both time points. Bars represent mean \pm SEM freezing to the tone. Markers represent the mean \pm SEM freezing before the onset of the tone. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

tests (for 50 $\mu\text{g}/\text{kg}$, $p < 0.05$ for both tests; for 100 $\mu\text{g}/\text{kg}$, $p < 0.001$ for PR-LTM and $p < 0.01$ for PR-LTM 2; for 200 $\mu\text{g}/\text{kg}$, $p < 0.001$ for PR-LTM and $p < 0.01$ for PR-LTM 2). In addition, significant freezing decreases were observed within the clonidine group between reactivation and both PR-LTM performances (for 50 $\mu\text{g}/\text{kg}$, $p < 0.05$; for 100 $\mu\text{g}/\text{kg}$, $p < 0.001$; for 200 $\mu\text{g}/\text{kg}$, $p < 0.001$). Taken together, the present data suggest that clonidine disrupted fear memory reconsolidation in a dose-dependent manner. Clonidine reached its maximum effect at 100 $\mu\text{g}/\text{kg}$, as increasing the dose further did not lead to a greater impairment of the conditioned response in the treated group.

Experiment 2: Reconsolidation Blockade by Clonidine Is Selective to Reactivated Fear Memories

We assessed whether the effect of clonidine on reconsolidation was dependent on memory reactivation. We injected clonidine at a dose of 100 $\mu\text{g}/\text{kg}$ 24 h after training without exposing the animals to the conditioning chamber and tone. Rats were tested for memory retention on days 3 and 10 (Figure 2a). No significant effect of clonidine (repeated-measures ANOVA, $F(1, 22) = 0.002$, $p > 0.05$) was observed in the absence of reactivation, as compared with the vehicle-injected group 1 day and 1 week after receiving the treatment (Figure 2b). In addition, a repeated-measures ANOVA showed no significant effect of day ($F(1, 22) = 1.34$, $p > 0.05$) and no treatment \times day interaction ($F(1, 22) = 0.39$, $p > 0.05$). Thus, clonidine disrupts reconsolidation of an auditory fear memory only when administered following reactivation of that memory.

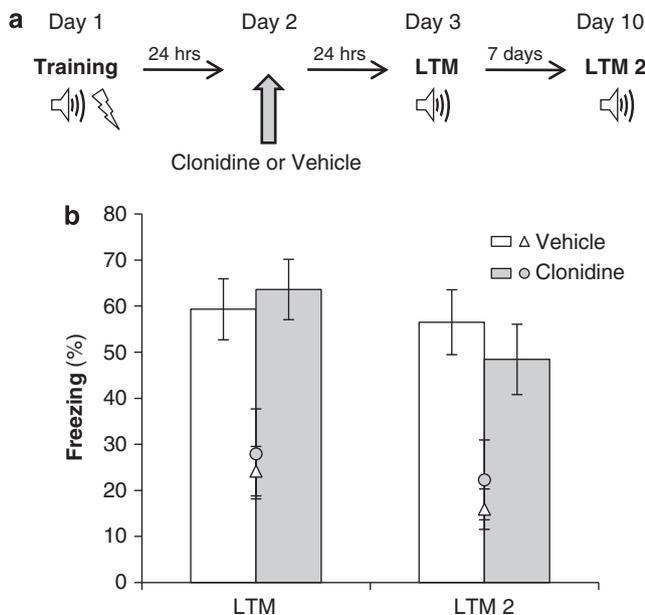


Figure 2 Clonidine does not impair retention of nonreactivated fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine (100 $\mu\text{g}/\text{kg}$) or its vehicle without a memory reactivation session and were tested for long-term memory retention 1 day (LTM) and 1 week later (LTM 2). (b) Clonidine-treated rats ($n = 12$) showed a similar conditioned response (freezing) to the vehicle group ($n = 9$) when tested 24 h or 1 week after injection. Bars represent mean \pm SEM freezing to the tone. Markers represent the mean \pm SEM freezing before the onset of the tone.

Experiment 3: Postreactivation Administration of Clonidine Does Not Impair Short-Term Fear Memories

To rule out the possibility that nonspecific effects of postreactivation clonidine create temporary dysfunctions of the memory system, we trained and reactivated rats as described before. After reactivation, animals received 100 $\mu\text{g}/\text{kg}$ of clonidine or vehicle and were tested for memory retention 4 and 24 h later (Figure 3a). If the memory impairment seen at PR-LTM is due to reconsolidation blockade, then animals should show an intact conditioned response 4 h after reactivation (PR-STM) but reduced freezing behavior 24 h later (PR-LTM). A repeated-measures ANOVA showed a significant main effect of treatment ($F(1, 19) = 5.49$, $p < 0.05$) and day ($F(2, 38) = 10.9$, $p < 0.001$), but no treatment \times day interaction ($F(2, 38) = 2.21$, $p > 0.05$; Figure 3b). Nevertheless, Fisher's *post hoc* test revealed a similar conditioned response for the clonidine-treated rats as compared with the vehicle group at PR-STM ($p > 0.05$) but showed a significant decrease in freezing for the clonidine group at PR-LTM as compared with PR-STM ($p < 0.001$) and to controls at PR-LTM ($p < 0.001$). Hence, the results confirm that postretrieval clonidine selectivity disrupts reconsolidation of long-term memories.

Experiment 4: Reconsolidation Blockade by Clonidine Does Not Impair the Ability to Learn New Fear Memories

To evaluate whether postreactivation clonidine could induce permanent learning impairments, we conditioned animals to fear a different tone using a different auditory

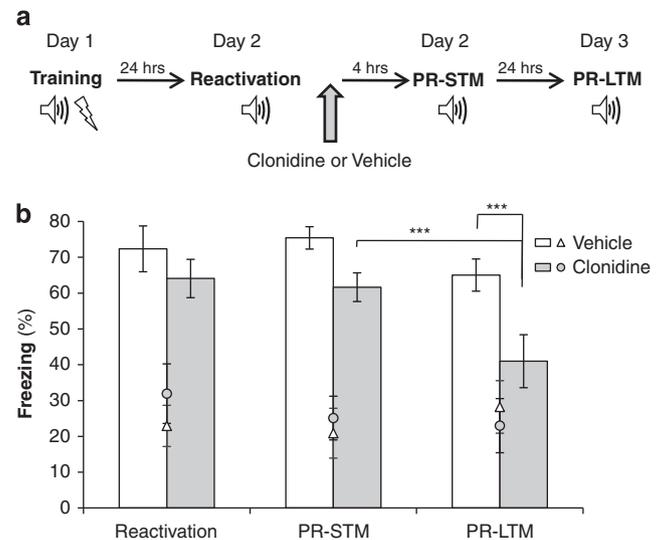


Figure 3 Postreactivation administration of clonidine does not impair short-term fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine (100 $\mu\text{g}/\text{kg}$) or its vehicle immediately after a reactivation session and were tested 4 h later for postreactivation short-term memory (PR-STM) and 1 day later for postreactivation long-term memory (PR-LTM). (b) Clonidine-treated rats ($n = 12$) showed a similar conditioned response (freezing) to the vehicle group ($n = 9$) when tested 4 h after reactivation, but reduced freezing behavior 1 day after injection. Bars represent mean \pm SEM freezing to the tone. Markers represent the mean \pm SEM freezing before the onset of the tone. Statistical significance: *** $p < 0.001$.

fear protocol. After receiving a postreactivation injection of clonidine (200 $\mu\text{g}/\text{kg}$) or vehicle, and a memory retention test 1 and 7 days later, rats were trained again and tested for memory of the new tone (Figure 4a). The highest dose was chosen for this experiment to ensure that if no impairments were observed, it could not be attributed to the use of a low concentration. 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 when tested for memory of the new tone. A repeated-measures ANOVA revealed no significant main effect of treatment ($F(1,22)=0.002$, $p>0.05$), day ($F(1,22)=2.17$, $p>0.05$), and no treatment \times day interaction ($F(1,22)=0.7$, $p>0.05$; Figure 4b). As both groups exhibited similar levels of conditioned response on the two test days, our data indicate that administering clonidine after reactivation does not induce a long-lasting, generalized fear learning impairment.

Experiment 5: Two Postretrieval Treatments of Clonidine Are Sufficient to Induce Maximal Disruption of Fear Memories

To assess whether a greater memory impairment could be achieved using a dose of 100 $\mu\text{g}/\text{kg}$, we trained animals as described before but we reactivated them 3 times over 3 days. Following each reactivation session, rats received an injection of clonidine or its vehicle. Rats were also tested 24 h after the last treatment and 1 week later (Figure 5a). A repeated-measures ANOVA revealed a significant main effect of treatment ($F(4,116)=9.91$, $p<0.01$) and day ($F(4,116)=26.04$, $p<0.001$), and a treatment \times day interaction ($F(4,116)=2.70$, $p<0.05$). Fisher's *post hoc* test found a significant decrease in conditioned response for the clonidine-treated group between reactivations 1 and 2 ($F(4,116)=26.04$, $p<0.001$) and reactivations 2 and 3 ($F(4,116)=26.04$, $p<0.05$; Figure 5b). Although the third treatment

showed a trend toward additional freezing reduction, it did not have a significant additive effect. The *post hoc* analysis also revealed a significant difference between the treated rats and the controls at days 2, 3, 4, and 12 (all $p<0.01$). Altogether, the results indicate that reconsolidation blockade by clonidine was effective after one treatment and reached its maximum effect after two treatments.

DISCUSSION

This study demonstrates the effectiveness of clonidine in persistently impairing fear memory retention through reconsolidation blockade in male and female rats. We suggest that the combination of memory reactivation sessions followed by clonidine administration represent a potentially novel therapeutic approach to reduce symptoms in PTSD patients.

Dosage and Number of Treatments

All tested doses of clonidine showed effectiveness in reducing postreactivation fear memory retention in a long-lasting and dose-dependent manner. The dose of 100 $\mu\text{g}/\text{kg}$ was determined to be optimally effective because it resulted in a greater memory impairment from reactivation to the PR-LTM test than did the 50 $\mu\text{g}/\text{kg}$ dose. However, the dose of 200 $\mu\text{g}/\text{kg}$ did not induce a larger reduction in freezing than the 100 $\mu\text{g}/\text{kg}$ dose, which suggests that the dose-response curve reaches a plateau, and increasing the dose further will not lead to a more substantial decrease in conditioned responding. On the other hand, we did find that the fear memory could be disrupted further with repeated treatments. Indeed, we established that two reactivation sessions followed by a 100 $\mu\text{g}/\text{kg}$ clonidine administration were sufficient to induce maximal memory disruption.

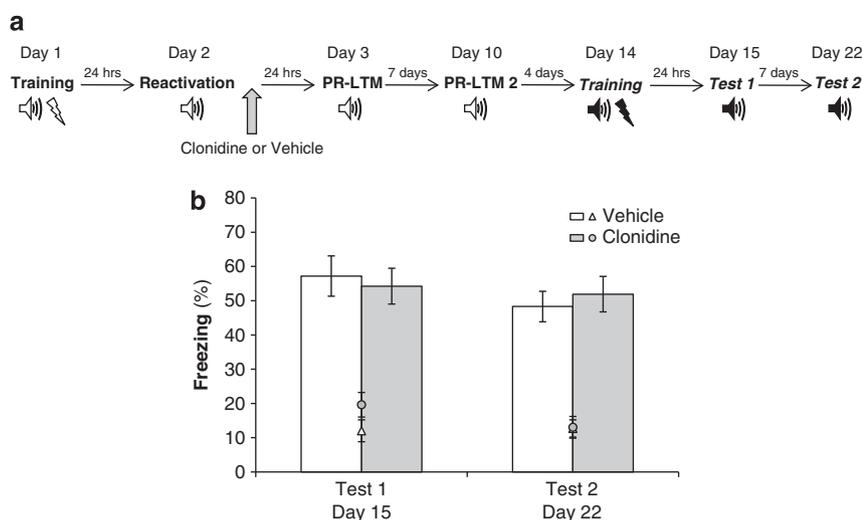


Figure 4 Postreactivation administration of clonidine does not impair the ability to learn new fear memories. (a) Schematic of the experimental design. After receiving a postreactivation injection of clonidine (200 $\mu\text{g}/\text{kg}$) or vehicle, and being tested for memory retention 1 day (PR-LTM) and 1 week later (PR-LTM 2), rats were conditioned to fear a different tone using a different auditory fear protocol. (b) Rats that previously received clonidine ($n=12$) showed intact fear behavior (freezing) compared with the vehicle-treated animals ($n=12$) when tested 1 day (test 1) or 1 week later (test 2). Bars represent mean \pm SEM freezing to the tone. Markers represent the mean \pm SEM freezing before the onset of the tone.

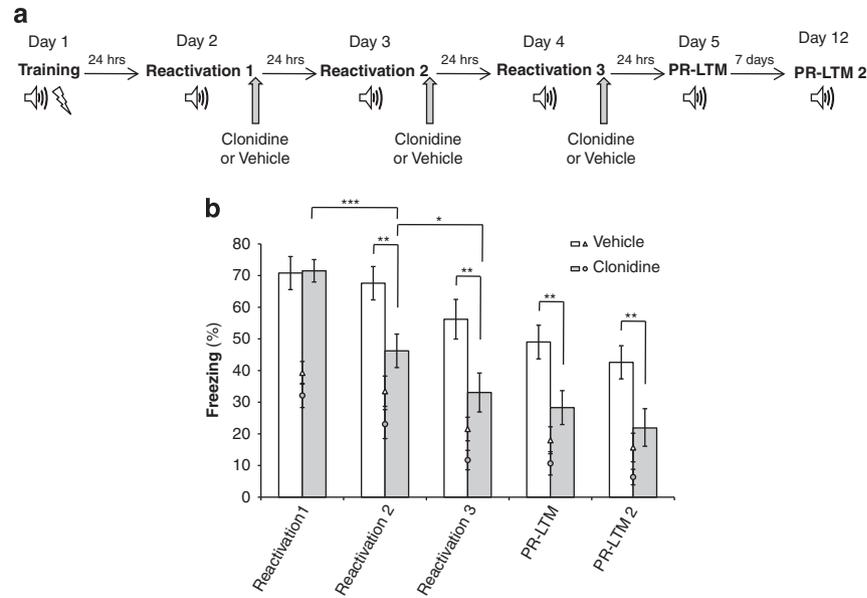


Figure 5 Two postreactivation clonidine treatments are sufficient to maximally impair fear memory retention. (a) Schematic of the experimental design. Rats received a systemic injection of clonidine (100 $\mu\text{g}/\text{kg}$) or its vehicle immediately after a reactivation session for 3 consecutive days and were tested for postreactivation long-term memory 1 day (PR-LTM 1) and 1 week later (PR-LTM 2). (b) Clonidine-treated rats ($n = 16$) showed an impaired conditioned response (freezing) as compared with the vehicle group ($n = 15$) at each test session. Memory disruption was observed after the first clonidine treatment and reached its maximum after two treatments at day 3. Bars represent mean \pm SEM freezing to the tone. Markers represent the mean \pm SEM freezing before the onset of the tone. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Our results are consistent with studies showing that clonidine has detrimental effects on memory. In animals, clonidine has been found to produce memory impairments in several learning paradigms ranging from shuttle box (Hawkins and Monti, 1979; Homayoun *et al*, 2003) to avoidance tasks (Galeotti *et al*, 2004; Genkova-Papasova and Lazarova-Bakurova, 1988; Genkova-Papazova *et al*, 1997) and to cue detection (Smith and Aston-Jones, 2011; Brown *et al*, 2012). Some studies in humans have also reported memory impairments associated with clonidine administration in healthy subjects (Riekkinen *et al*, 1999; Hall *et al*, 2001) and in Alzheimer's disease patients (Jakala *et al*, 1999a, b).

It is well known that α_2 -adrenoreceptor agonists can induce sedation (Lakhlani *et al*, 1997; MacDonald *et al*, 1997). However, the possibility that a sedative effect of clonidine influenced the behavioral results in our study can be ruled out as we tested the animals 24 h and again 7 days after injection, the time points well beyond the 30–120 min half-life of clonidine in rats (Conway and Jarrott, 1982).

Reconsolidation Specificity

We have shown that postretrieval administration of clonidine is effective in reducing fear-related memory retention. In order to confirm whether reconsolidation is the mechanism underlying the effect, we examined key elements that define the reconsolidation process. First, our results demonstrate that the effect of clonidine is selective to the reactivated memory, as no memory impairment was observed when clonidine was administered without prior reactivation. Furthermore, when animals were tested a week after treatment, we did not observe any spontaneous recovery of the conditioned response. Spontaneous recovery is a phenomenon found with extinguished memories, but

not after reconsolidation blockade (Duvarci and Nader, 2004). As reconsolidation is a time-dependent process that is known to affect long-term but not short-term memory (Nader *et al*, 2000; Nader and Hardt, 2009), we also tested the animals 4 h after reactivation. The results revealed an intact conditioned response at that time point but impaired behavior the next day. This demonstrates that clonidine affects postreactivation long-term memory, but not short-term memory. Given that this test was performed only 4 h after clonidine administration, one could argue that the sedative effects of clonidine altered the results at this shorter interval after drug administration. However, the treated rats displayed low levels of freezing during the pre-tone period, indicating an ability to move; thus, the intact freezing levels observed at PR-LTM after clonidine administration are unlikely to be attributable to motor impairments due to sedation in these animals. In addition, it is reasonable to believe that the drug was no longer present in the rats' systems at the time of testing because clonidine has a short half-life (30–120 min; Conway and Jarrott, 1982).

Evaluation of the above-mentioned criteria all rule in favor of the implication of reconsolidation processes in the present study. Our results are consistent with several studies investigating reconsolidation blockers either systemically (Debiec and Ledoux, 2004; Blundell *et al*, 2008; Taubenfeld *et al*, 2009; Pitman *et al*, 2011) or intracranially (Nader *et al*, 2000; Debiec and Ledoux, 2004; Ben Mamou *et al*, 2006; Jin *et al*, 2007). Indeed, it is accepted in the literature that the lack of spontaneous recovery, the selectivity to reactivated memories, and the presence of intact short-term memory are criteria that define the reconsolidation process. Taken together, our results suggest that the effect of clonidine on memory is mediated by reconsolidation blockade.

Clinical Relevance

Currently, there are no specific pharmacological approaches to treat PTSD symptoms. Therefore, there is a need for preclinical research to identify new candidate drugs and to develop novel therapeutic interventions. The present study has implications for the potential clinical use of reconsolidation blockade by clonidine. First, we determined that the dose of 100 µg/kg optimally disrupts fear memory retention in both male and female rats. Conversion from the animal dose to a human equivalent dose in mg/kg may be obtained by applying a formula that takes the body surface area into account. With this calculation, our animal dosage of 100 µg/kg translates into a dose of 1.135 mg for a 70-kg person (Reagan-Shaw *et al*, 2008). Such a dose is well within the safe range for daily human use that has a maximum of 2.4 mg (Physician's Desk Reference; <http://www.pdr.net>). Nevertheless, as clonidine is known to induce hypotension, patients being treated with clonidine should be medically monitored. We also found that clonidine-induced memory impairments are selective to the reactivated memory. Thus, we can hypothesize that using clonidine in combination with traumatic memory reactivation will decrease the intensity of that memory without disrupting other unrelated memories. Additionally, we observed that postreactivation clonidine does not affect learning of new fear memories, implying that patients would be able to experience and remember new events normally. These are all valuable aspects for clinical use, as optimal treatments should be specific and not interfere with other processes (Steckler and Risbrough, 2011).

Clonidine has been found to improve symptoms such as hyperarousal (Harmon and Riggs, 1996; Donnelly, 2003), impulsivity (Donnelly, 2003) (Viola *et al*, 1997), and nightmares (Kinzie and Leung, 1989; Kinzie *et al*, 1994) when administered chronically to patients. However, some experienced a return of symptoms upon termination of treatment (Porter and Bell, 1999), and the possibility that the beneficial effects would decrease over time remains. A significant advantage of reconsolidation blockade by clonidine in treating PTSD symptoms would be that it does not require chronic administration of the drug, as based upon our animal findings the maximal effect would probably be obtained within a few sessions. Consequently, this would make lasting side effects unlikely. Furthermore, we showed that memory disruption following postretrieval clonidine is long lasting; thus, it is reasonable to hope that combining memory reactivation with clonidine administration could permanently weaken PTSD symptoms such as intrusive memories without the possibility of relapse.

Although fear conditioning models the enhanced fear response upon recollection of the traumatic event, this is only one of the many pathophysiological and behavioral characteristics of PTSD. Nightmares, avoidance, and hyperarousal are common, and alterations of several neurotransmitter systems have also been observed. Further investigations will be necessary to verify whether clonidine can improve other aspects of this complex pathology in an animal model.

In conclusion, results of this study demonstrate that systemic administration of clonidine after retrieval persistently weakens fear memories through reconsolidation blockade. We show that this effect is maximal after two

treatments, is present in both male and female rats, is selective to the reconsolidation time window and to reactivated memories, and does not affect further fear learning. These preclinical findings indicate potential to further develop clinical approaches using clonidine as a reconsolidation blocker in the treatment of PTSD symptoms.

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DISCLOSURE

The authors declare no conflict of interest.

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