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Prazosin during fear conditioning facilitates subsequent extinction in male C57BI/6N mice

Elizabeth K. Lucas^{1,2}, Wan-Chen Wu¹, Ciorana Roman-Ortiz¹, and Roger L. Clem^{1,3,*}

¹Fishberg Department of Neuroscience and the Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029

²Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, NC 27606

³Department of Psychiatry, Icahn School of Medicine at Mount Sinai, New York, NY 10029

Abstract

RATIONALE: Recovery from a traumatic experience requires extinction of cue-based fear responses, a process that is impaired in post-traumatic stress disorder. While studies suggest a link between fear behavioral flexibility and noradrenaline signaling, the role of specific receptors and brain regions in these effects is unclear.

OBJECTIVES: Here we examine the role of prazosin, an $\alpha 1$ -adrenergic receptor ($\alpha 1$ -AR) antagonist, in auditory fear conditioning and extinction.

METHODS: C57BI/6N mice were subjected to auditory fear conditioning and extinction in combination with systemic (0.1-2 mg/kg) or local microinjections (3 or 6 mM) of the $\alpha 1$ -AR antagonist prazosin into the prelimbic division of medial prefrontal cortex or basolateral amygdala. Conditioned fear and anxiety-like behaviors were compared with vehicle-injected control animals.

RESULTS: Mice that received systemic prazosin prior to fear conditioning exhibited similar initial levels of cue-elicited freezing compared to vehicle controls on the following day. However, at all doses tested fear that was acquired during prazosin treatment was more readily extinguished, whereas anxiety-like behavior on the day of extinction was unaffected. A similar pattern of results was observed when prazosin was microinjected into the basolateral amygdala but not the prelimbic cortex. In contrast to pre-conditioning injections, prazosin administration prior to extinction had no effect on freezing.

CONCLUSIONS: Our results indicate that $\alpha 1$ -AR activity during aversive conditioning is dispensable for memory acquisition, but renders conditioned fear more impervious to extinction. This suggests that behavioral flexibility is constrained by noradrenaline at the time of initial learning via activation of a specific AR isoform.

Keywords

ADRA1A; terazosin; exposure therapy

*Corresponding author: Roger L. Clem, PhD, Associate Professor of Neuroscience and Psychiatry, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, Box 1065, New York, NY 10029, roger.clem@mssm.edu, Phone: 212-824-8976, Fax: 212-849-2621.

Introduction.

The extinction of debilitating fear reactions triggered by discrete, contextual, and situational reminders of traumatic events is thought to be an important component of recovery from conditions like post-traumatic stress disorder (PTSD; Milad and Quirk 2012). Unfortunately, extinction efficacy varies among individuals, and diagnosis with PTSD is associated with profound extinction impairments (Jovanovic and Norrholm 2011; Maren and Holmes 2016; Zuj et al. 2016). One causal factor that has been implicated in extinction resistant fear in both rodents and humans is elevated noradrenergic signaling (Gazarini et al. 2014; Lin et al. 2016; Rodrigues et al. 2009; Soeter and Kindt 2011; 2012). Therefore, while more frequently considered within the context of hyperarousal and sleep disturbance, a positive correlation between PTSD and serum noradrenaline levels may also be relevant to the etiology of impaired extinction (Geraciotti et al. 2001; Liberzon et al. 1999; Pervanidou and Chrousos 2012; Pitman 1989; Yehuda et al. 1998). However, our understanding of how noradrenaline signaling acts via specific receptors and brain regions to influence behavioral flexibility remains incomplete.

Although its synthesis is limited to relatively sparse cellular populations, noradrenaline is released throughout the brain and exerts a range of physiological effects via three distinct adrenergic receptor (AR) classes: $\alpha 1$, $\alpha 2$ and β . To date most investigations of noradrenaline in fear conditioning have focused on limbic system β -ARs, which recruit intracellular Gs signaling and are required for fear memory acquisition (Bush et al. 2010; Grillon et al. 2004). However, interest in Gq-coupled $\alpha 1$ -ARs has gained momentum since the selective $\alpha 1$ -AR antagonist prazosin began to show promise in alleviating recurrent nightmares and other symptoms of PTSD (George et al. 2016; Singh et al. 2016). Despite the increasing prevalence of prazosin therapy in this condition, little is known about the role of $\alpha 1$ -ARs in fear memory regulation. Therefore, we conducted systemic and brain-region-specific pharmacological manipulations in mice to determine the effects of prazosin on cue-evoked defensive behavior after fear conditioning. We demonstrate that acquisition of fear conditioning is not affected by systemic $\alpha 1$ -AR antagonism with prazosin. However, fear responses acquired during prazosin treatment are more susceptible to extinction. Targeted cannulation experiments indicate that the extinction-enhancing effects of prazosin are mediated by the basolateral amygdala. Our data indicate that noradrenaline acting through $\alpha 1$ -ARs in the basolateral amygdala sets the stage for behavioral inflexibility in fear conditioning.

Methods and Methods.

Animals.

All experiments were approved in advance by the Institutional Care and use Committee of the Icahn School of Medicine at Mount Sinai. Three-week old male C57Bl/6N mice were ordered from Charles River (Strain Code 027) and allowed to acclimate in our animal facility for at least one week prior to behavior experiments or surgical procedures. Mice were housed 2-5 per cage with access to food and water *ad libitum* on a 12 hour light-dark cycle (lights on at 0700 hours). All experiments were carried out during the light phase.

Surgery.

Mice were deeply anesthetized with isoflurane and mounted in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) for intracranial cannula implantation. Coordinates were obtained from the mouse brain atlas (Franklin & Paxinos, 2007) with anteroposterior (AP), mediolateral (ML), and dorsoventral (DV) positions referenced from Bregma. A midline incision was made to reveal Bregma, the skull was cleaned with hydrogen peroxide, and small holes were drilled through the skull at the designated stereotaxic coordinates. Single guide cannula (PlasticsOne, Roanoke, VA, USA) were bilaterally implanted above the basolateral amygdala (mm from Bregma: AP -1.4 , ML ± 3.3 , DV -3.7), and bilateral guide cannula (PlasticsOne) were implanted above the prelimbic cortex (mm from Bregma: AP $+1.8$, ML ± 0.6 , DV -1.2). Guide cannula were secured to the skull using anchor screws and dental cement. Matching dummy cannula were inserted into the guide cannula and secured with a dust cap to ensure guide cannula patency. Post-operative analgesia was provided with banamine (2.5 mg/kg). Mice recovered in their home cages for one week before behavioral testing.

After behavioral testing, cannula placement was verified by infusion of 0.2 μ l fast green (0.1% in saline; Sigma-Aldrich, Burlington, MA, USA) at a rate of 0.1 μ l per minute. After infusion, the internal cannula was left in place for 5 minutes. Mice were then deeply anesthetized with isoflurane prior to decapitation, and brains were rapidly removed, frozen on pulverized dry ice, and stored at -80°C . Brains were cryostat sectioned at 25 μ m, mounted onto charged slides, and allowed to dry overnight before freezing at -80°C . For histology, sections were thawed, rehydrated in graded ethanol, stained with cresyl violet, dehydrated in graded ethanol, and coverslipped with non-aqueous mounting media. Histology confirmed bilateral hits for 19/23 BLA cannula and 18/24 mPFC cannula. Representative images were captured on a Revolve4 microscope (Echo, San Diego, CA, USA) at 4x magnification with an iPadPro camera. Behavioral results from mice with mistargeted cannula were excluded from statistical analysis.

Drug Administration.

Prazosin hydrochloride (Tocris; Minneapolis, MN, USA) was reconstituted in 100% DMSO stored at 4°C for no more than 1 month. Freshly prepared working concentrations of prazosin were diluted in normal saline heated to 37°C and injected/infused 1 hour prior to behavioral manipulation. For IP injections, working concentrations were prepared from a 10 mg/ml stock with 10% final DMSO concentration and 100 μ l injection volume. For cannula infusion, working concentrations were prepared from a 50 mg/ml stock with 5% final DMSO concentration. A 0.2 μ l volume was infused at a rate of 0.1 μ l per minute through internal cannula that extended 1.0 mm below basolateral amygdala guide cannula and 0.5 mm below prelimbic cortex guide cannula. After infusion, the internal cannula was left in place for 5 minutes to allow adequate absorption of the drug.

Fear Conditioning and Extinction.

Mice were handled for 3 consecutive days prior to fear conditioning. Fear conditioning and extinction learning were conducted with an NIR Video Fear Conditioning System (MedAssociates; St. Albans, VT, USA) as previously described (Lucas et al. 2014). On the

first day, mice underwent fear conditioning consisting of 6 pairings of an auditory tone (2 kHz, 80 dB, 20 s) with foot shock (1 mA, 2 s) in which the tone and foot shock co-terminated. An acclimation period of 200 s in training arena preceded the onset of cues, and pairings were separated by an 80 s inter-trial interval. The training arena was cleaned with 70% ethanol between sessions. On the second and third days, mice underwent extinction and re-extinction that consisted of 20 tone presentations in an altered context cleaned with isopropanol between sessions. Data were analyzed with VideoFreeze (MedAssociates), and freezing bouts ≥ 1 second were included in analyses.

Elevated plus maze.

For the prazosin dose response experiment, the elevated plus maze test was used to assess anxiety-like behavior 3 hours prior to extinction training on the second day. Mice were placed in the center of the elevated plus maze (Model ENV-560A, Med Associates), and exploration of the maze was tracked with Ethovision software (Noldus, Leesburg, VA, USA) over a 5 minute period. The maze was cleaned with Virox Disinfectant (Oakville, ON, USA) between sessions.

Statistical Analyses.

All data were analyzed with Prism Graphpad (La Jolla, CA, USA). Normal distribution and homogeneity of variance were tested before proceeding to the appropriate parametric or non-parametric tests. Baseline freezing was analyzed with two-tailed unpaired t-tests (2 groups) or one-way ANOVA followed by Tukey's post-test (≥ 3 groups). CS-evoked freezing during fear conditioning and extinction was analyzed with two-way repeated-measures ANOVA followed by Holm-Bonferroni post-test in the case of significant interaction. All freezing data are presented as mean \pm SEM. Elevated plus maze data, which violated homogeneity of variance, was analyzed with Kruskal-Wallis and presented as box-and-whisker plots. For all tests, familywise $\alpha = 0.05$.

Results.

Fear acquired during $\alpha 1$ -AR blockade is more readily extinguished.

To examine effects of systemic $\alpha 1$ -AR blockade during fear conditioning, IP injections of the selective $\alpha 1$ -AR antagonist prazosin (2 mg/kg) or vehicle (10% DMSO) were administered to mice 1 hour prior to training (Fig. 1 A). Prazosin administration had no effect on fear memory acquisition or retrieval (Extinction 1, CS 1-4) but significantly attenuated freezing during extinction days 1 (main effect of treatment, $F_{(1,22)} = 6.21$, $p = 0.02$) and 2 (main effect of treatment, $F_{(1,22)} = 6.19$, $p = 0.02$; Fig. 1B). Similar results were obtained from male mice at 8-12 weeks of age (Figure S1). Because a ceiling effect might have obscured a role for $\alpha 1$ -ARs in fear memory acquisition and retrieval, we also performed systemic prazosin administration prior to low-intensity training and found that memory retrieval was still unaffected by prazosin (Figure S2).

Due to the short half-life of prazosin (2-3 hours), enhanced extinction in prazosin-treated mice is unlikely to be accounted for by the carryover of unmetabolized drug. However, to evaluate the direct effect of $\alpha 1$ -AR blockade on extinction learning, prazosin (2 mg/kg) or

vehicle (10% DMSO) was administered to mice 1 hour prior to each extinction session (Figure 1C). Contrary to prazosin treatment during fear acquisition, prazosin administered during extinction learning had no effect on freezing (main effect of treatment: Extinction 1, $F_{(1,14)} = 0.40$, $p = 0.53$; Extinction 2, $F_{(1,14)} = 2.34$, $p = 0.15$; Fig. 1D). These data suggest that prazosin indirectly facilitates extinction by interfering with a process that occurs during initial fear acquisition.

Low doses of prazosin accelerate extinction without impacting anxiety.

In humans, the therapeutic dose of prazosin ranges from 3-20 mg per day (George et al. 2016; Singh et al. 2016). To determine the dose response range of prazosin in facilitating extinction, IP injections of 0.1, 0.5, or 2 mg/kg prazosin or vehicle (10% DMSO) were administered 1 hour prior to fear conditioning (Fig. 2A). Consistent with a sedative effect of α 1-AR blockade, mice that were administered prazosin exhibited increased immobility during baseline ($F_{(3,29)} = 4.72$, $p = 0.008$) and the first two tone-shock pairings, but not subsequent trials, of fear conditioning (main effect of treatment, $F_{(3,29)} = 4.95$, $p = 0.007$; treatment x trial interaction, $F_{(15,145)} = 4.20$, $p < 0.0001$). To determine whether enhanced extinction learning after prazosin administration could be attributable to an altered anxiety state (Fig. 1B), anxiety-like behavior was assessed with the elevated plus maze 3 hours prior to extinction day 1. Prazosin administration during fear acquisition did not affect time spent in the open arms of the elevated plus maze at any dose ($p = 0.81$), indicating that differences in anxiety states could not have accounted for enhanced extinction in prazosin-treated animals. At all tested doses, prazosin attenuated freezing during extinction days 1 (main effect of treatment, $F_{(3,29)} = 4.88$, $p = 0.007$) and 2 (main effect of treatment, $F_{(3,29)} = 3.62$, $p = 0.02$; Fig. 2B). These results suggest that prazosin doses within the human therapeutic range (0.1 mg/kg) are sufficient to render fear memories more susceptible to extinction.

α 1-AR blockade in the basolateral amygdala recapitulates the fear-attenuating properties of systemic prazosin.

Since prazosin could have poor blood-brain barrier permeability (Taylor et al. 1977), it is possible that above effects of α 1-AR blockade are attributable to peripheral rather than central mechanisms. To examine this possibility, guide cannula were chronically implanted in either the prelimbic cortex or the basolateral amygdala (Fig. S3), brain structures in which noradrenaline signaling has been previously implicated in fear conditioning (Bush et al. 2010; Fitzgerald et al. 2015). Prazosin (3 mM or 6 mM) or vehicle (5% DMSO) was bilaterally infused into the prelimbic cortex or basolateral amygdala 1 hour prior to fear conditioning (Fig. 3A). While α 1-AR blockade in the prelimbic cortex had no effect on fear conditioning or extinction day 1 (Fig. 3B-C), 6 mM prazosin infused into the basolateral amygdala led to enhancement of extinction day 2 (main effect of treatment; $F_{(2,17)} = 8.93$, $p = 0.002$; Fig. 3D-E). These results suggest that the basolateral amygdala is a critical locus of α 1-AR-mediated effects.

Discussion.

The noradrenergic substrates that regulate fear behavioral flexibility represent potentially valuable therapeutic targets. Here we found that the rate of extinction learning in mice is

constrained by α 1-AR activity. However, the time frame when α 1-AR blockade influenced the outcome of extinction training was not during extinction itself but instead during initial fear conditioning. Our results further indicate that the basolateral amygdala, but not the prelimbic cortex, is an important locus for these effects. This implies that the conditions for extinction impairment can to some extent be formed during a traumatic event as a result of noradrenaline release in the amygdala. Because our study did not include female mice, however, it will be important for future studies to examine whether these effects are sex-dependent.

In contrast to the β -AR antagonist propranolol (Bush et al. 2010), and similar to the α 1-AR antagonist terazosin (Lazzaro et al. 2010), prazosin did not impede fear acquisition, since there were no group differences in freezing after high (Fig. 1,2) or low-intensity training (Fig. S2). However, contrary to our findings, Lazzaro and colleagues observed increased tone-evoked freezing following fear conditioning that coincided with systemic and amygdala-specific terazosin injections in rats (Lazzaro et al. 2010). A number of factors could have contributed to this discrepancy, including species differences (rats versus mice), pharmacological differences between terazosin and prazosin, or dose selection. Because this study did not examine extinction, we are unable to evaluate whether terazosin treatments might nevertheless improve extinction in rats despite initially higher freezing levels during memory retrieval.

Intriguingly, facilitation of extinction by prazosin is highly reminiscent of the relief by immediate post-training propranolol injections of the so-called immediate extinction deficit, a phenomenon in which the extinction of newly-acquired fear memory (less than 1 day old) is profoundly impaired (Fitzgerald et al. 2015; Giustino et al. 2017). Maren and colleagues demonstrated that this deficit is rescued by systemic as well as amygdala-specific propranolol injections (Giustino et al. 2017). Despite these similarities, a critical difference in the outcome of propranolol and prazosin treatments is that the benefits of propranolol appear to be limited to the initial hours after fear conditioning, as propranol administration prior to delayed extinction impairs extinction learning (Fitzgerald et al. 2015). When viewed in this light, the circuit changes underlying more persistent effects of α 1-ARs might represent more useful targets for alleviating extinction impairments in PTSD patients, who do not typically seek treatment in the immediate aftermath of trauma.

What cellular mechanisms might account for extinction resistance following α 1-AR stimulation? Aversive foot shocks trigger a massive release of noradrenaline into the basolateral amygdala (Quirarte et al. 1998), which plays an important role in both the acquisition and extinction of fear memory (Rodrigues et al. 2009). Therefore, a clear possibility is that α 1-ARs support persistent changes in excitability or synaptic signaling that interfere with subsequent extinction-related plasticity. These changes may ultimately impair the reorganization of ensemble activity during extinction and the recruitment of extinction-correlated conditioned stimulus responses (Grewe et al. 2017; Herry et al. 2008). Although noradrenaline acts on both α - and β -ARs in the basolateral amygdala, these receptors elicit distinct intracellular responses and may selectively modulate specific cell types. Stimulation of α 1-ARs increases inhibitory synaptic transmission in several brain regions including the basolateral amygdala (Braga et al. 2004; Han et al. 2002; Hillman et al.

2009; Kaneko et al. 2008; Luo et al. 2015; Skelly et al. 2017). Interestingly, this effect is reportedly lost in slice preparations of the basolateral amygdala following fear conditioning (Skelly et al. 2017). This suggests that molecular events linked to prior learning, and perhaps directly arising from $\alpha 1$ -AR signaling, can occlude the modulation of inhibitory neuronal populations by $\alpha 1$ -ARs. Given the involvement of amygdala GABAergic plasticity in fear (Lucas et al. 2016) and extinction (Davis et al. 2017; Trouche et al. 2013) learning, these effects may provide a clue as to the mechanism by which pre-training prazosin treatments can improve behavioral flexibility.

Long relied on for the treatment of hypertension, prazosin has more recently gained favor as an “off-label” therapy for PTSD-related nightmares, sleep disturbance, and hyperarousal (George et al. 2016; Singh et al. 2016). While treatment for PTSD is typically initiated long after the experience of stress, our results suggest that prazosin may also confer therapeutic benefits when used as a prophylactic against the development of extinction-resistant fear during initial trauma or re-traumatization. It is also possible that $\alpha 1$ -AR activity contributes to an ongoing dysregulation of other aspects of fear learning in these psychiatric populations. For example, in a recent study of normal human subjects we demonstrated that therapeutic doses of prazosin improve the learned discrimination of safe and threatening visual stimuli in a fear conditioning paradigm (Homan et al. 2017). Other work indicates that prazosin can disrupt the reconsolidation of cued fear memories in rats (Do Monte et al., 2013). Future investigations of $\alpha 1$ -AR signaling might therefore help establish novel applications for prazosin as well as identify new mechanistic bases for intervention after trauma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

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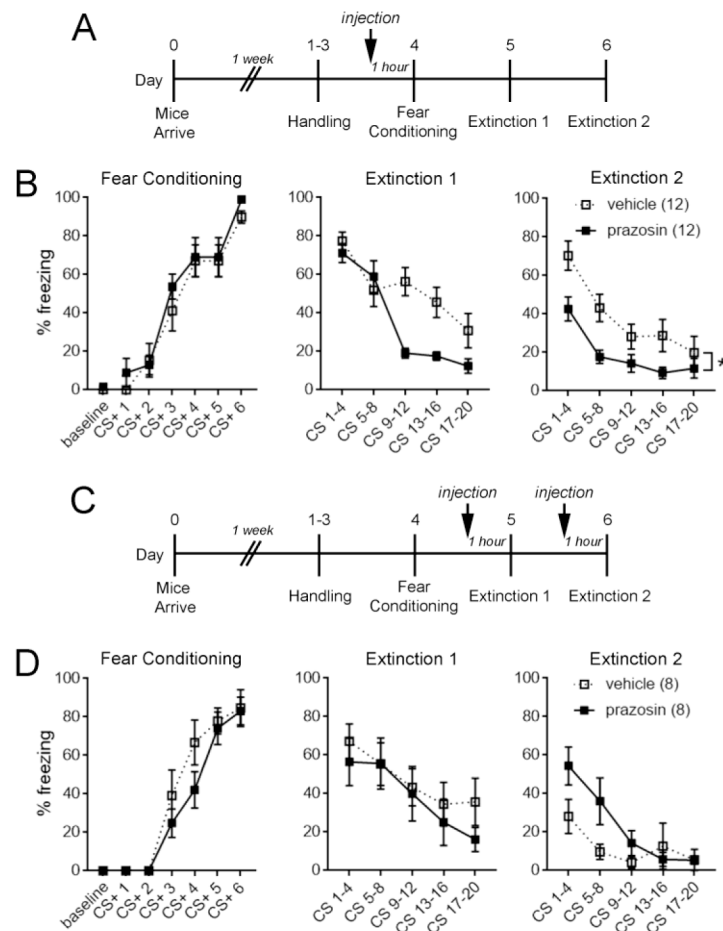


Figure 1. Fear acquired during α 1-AR blockade is more readily extinguished.

A. Experimental timeline for B. **B.** Systemic injection of prazosin (2 mg/kg) prior to fear conditioning significantly reduced freezing behavior during extinction days 1 and 2 while having no effect on fear memory acquisition or retrieval (Extinction 1, CS 1–4). **C.** Experimental timeline for D. **D.** Systemic injection of prazosin prior to extinction days 1 and 2 did not reduce cue-evoked freezing behavior. Two-way repeated-measures ANOVA, * $p < 0.05$ for main effect of treatment. n/group indicated in parentheses in legend. CS, conditioned stimulus.

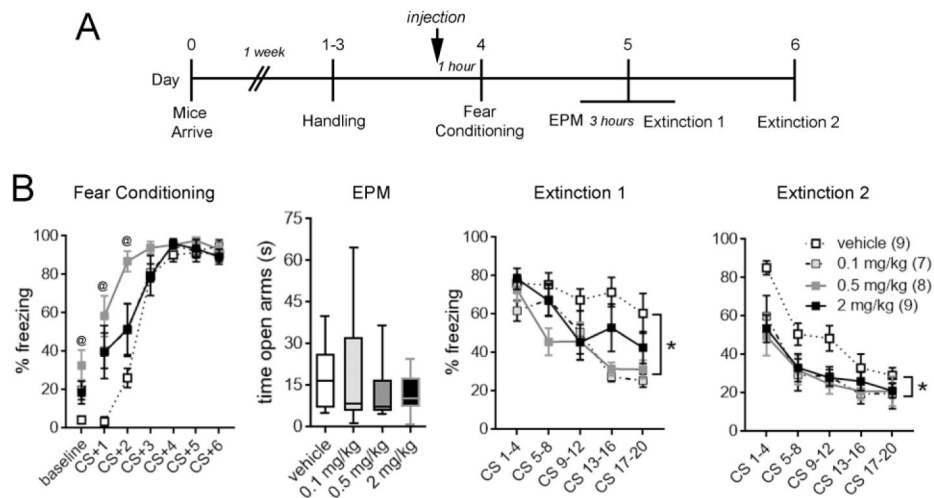


Figure 2. Low doses of prazosin accelerate extinction without impacting anxiety.

A. Experimental timeline. **B.** Systemic administration of prazosin prior to fear conditioning enhanced freezing during baseline and the first and second, but not subsequent trials of tone-shock pairings. Baseline, one-way ANOVA followed by Tukey's post-test. CS-evoked freezing, two-way repeated-measures ANOVA, treatment x trial interaction followed by Holm-Bonferroni post-test. @ < 0.05 vehicle versus prazosin groups. Prazosin did not affect time spent in the open arms of an elevated plus maze (EPM; Kruskal-Wallis, $p = 0.81$) or freezing during fear memory retrieval (Extinction 1, CS 1-4) at any dose. However, prazosin at doses as low as 0.1 mg/kg significantly attenuated freezing during extinction days 1 and 2. Two-way repeated-measures ANOVA, * $p < 0.05$ for main effect of treatment. n/group indicated in parentheses in legend. CS, conditioned stimulus.

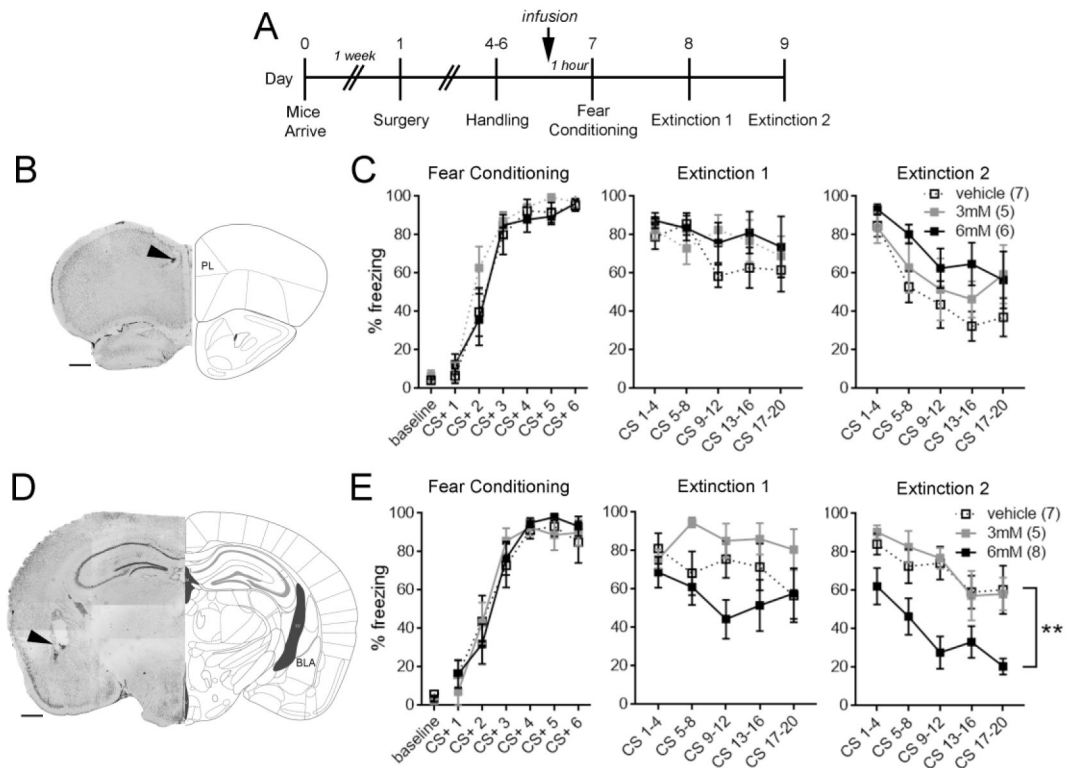


Figure 3. α_1 -AR blockade in the basolateral amygdala is sufficient to increase susceptibility to extinction.

A. Experimental timeline. **B.** Representative image of cannula placement in the prelimbic (PL) cortex. **C.** Prazosin infusion into the prelimbic cortex did not affect fear conditioning or extinction. **D.** Representative image of cannula placement in the basolateral amygdala (BLA). **E.** Basolateral amygdala prazosin infusion at 6mM significantly attenuated freezing during extinction day 2 while having no impact on fear memory acquisition or retrieval (Extinction 1, CS 1-4). Two-way repeated-measures ANOVA, ** $p < 0.005$ for main effect of treatment. n /group indicated in parentheses in legend. CS, conditioned stimulus. Scale bars = 500 μ M.