Systemic Propranolol Acts Centrally to Reduce Conditioned Fear in Rats Without Impairing Extinction

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Background: Previous work has implicated noradrenergic β-receptors in the consolidation and reconsolidation of conditioned fear. Less is known, however, about their role in fear expression and extinction. The β-receptor blocker propranolol has been used clinically to reduce anxiety. With an auditory fear conditioning task in rats, we assessed the effects of systemic propranolol on the expression and extinction of two measures of conditioned fear: freezing and suppression of bar-pressing.

Methods: One day after receiving auditory fear conditioning, rats were injected with saline, propranolol, or peripheral β-receptor blocker sotalol (both 10 mg/kg, IP). Twenty minutes after injection, rats were given either 6 or 12 extinction trials and were tested for extinction retention the following day. The effect of propranolol on the firing rate of neurons in prelimbic (PL) prefrontal cortex was also assessed.

Results: Propranolol reduced freezing by more than 50%, an effect that was evident from the first extinction trial. Suppression was also significantly reduced. Despite this, propranolol had no effect on the acquisition or retention of extinction. Unlike propranolol, sotalol did not affect fear expression, although both drugs significantly reduced heart rate. This suggests that propranolol acts centrally to reduce fear. Consistent with this, propranolol reduced the firing rate of PL neurons.

Conclusion: Propranolol reduced the expression of conditioned fear, without interfering with extinction learning. Reduced fear with intact extinction suggests a possible use for propranolol in reducing anxiety during extinction-based exposure therapies, without interfering with long-term clinical response.

Key Words: Anxiety disorder, heart rate, noradrenergic, prefrontal, prelimbic, PTSD

Emotional memories are associated with enhanced noradrenergic signaling via β-receptors (1), and perturbations of the β-noradrenergic system might contribute to the persistence of disturbing emotional memories (2). In general, studies of the noradrenergic system in memory formation have focused on post-training consolidation (3) and reconsolidation processes (4), but less is known about how norepinephrine affects the expression and extinction of learned fear.

Propranolol, a centrally acting β-receptor antagonist, has been shown to reduce anxiety and fear. In humans, propranolol reduces acute stage fright (5), test anxiety (6), and contextual fear (7). In rats, propranolol dose-dependently decreases anxiety in an open field (8) and in a light-enhanced startle paradigm (9). Propranolol reduced the expression of conditioned startle responses in rats (10) but not conditioned freezing in mice (11). Additionally, there is evidence that both shock and conditioned fear stimuli evoke noradrenergic efflux throughout the cortex (12–14). The effect of propranolol on fear extinction has led to mixed results, showing no effect when given systemically (11) and impairment when infused into the medial prefrontal cortex (mPFC) (15).

These mixed findings prompted us to re-examine the effects of systemic propranolol on the expression and extinction of conditioned fear in an auditory fear conditioning task, with the same dosage as previous studies (10 mg/kg, IP). We administered propranolol to rats before extinction training and examined both freezing and bar-press suppression responses to a conditioned tone. The following day, we assessed retention of extinction. Our purpose was two-fold: 1) to assess the effects of propranolol on expression of conditioned fear, and 2) to assess the effects of propranolol on extinction memory. We also assessed the effect of systemic propranolol on the activity of neurons in the prelimbic region (PL) of mPFC, an area implicated in the expression of conditioned fear (16–18). Clarifying the effects of systemic propranolol on the expression and extinction of conditioned fear could have clinical significance, because extinction is the basis of exposure-based therapies for the treatment of anxiety disorders (19).

Methods and Materials

Subjects
A total of 131 male Sprague-Dawley rats (Hilltop Laboratory, Scottsdale, Pennsylvania) weighing approximately 300 g were housed and handled as previously described (20). Rats were maintained on a 12-hour light/dark cycle and fed standard laboratory rat chow in a restricted manner (18 g/day) until they reached 85% of their free-feeding weight. Rats had free access to water throughout the experiment. Rats were individually housed and transported daily from the animal facility to a holding room in our laboratory during experimental phases. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Puerto Rico, in compliance with National Institutes of Health (NIH) guidelines.

Fear Conditioning
Fear conditioning was carried out in standard operant chambers (Coulbourn Instruments, Whitehall, Pennsylvania) located inside sound-attenuating boxes (Med Associates, Burlington, Vermont) in an isolated testing room. Further details regarding the apparatus have been previously described (20). Before fear conditioning, rats were trained in the operant chambers to press a bar for food pellets (Bioserve, Frenchtown, New Jersey) on a variable interval reinforcement schedule (VI-60). Bar-pressing
was used to maintain a constant level of activity against which freezing could reliably be measured. Rats pressed for food throughout all phases of the experiment. After bar-press training, rats were fear conditioned. On day 1, rats were presented with five nonreinforced tones (4 kHz; 78 dB; 30 sec; Habituation) followed by seven tones paired with footshock (55 mA, .5 sec; Conditioning). After conditioning, rats were assigned to two groups that were matched for levels of freezing across conditioning. On day 2, rats were injected with either saline or propranolol (10 mg/kg; IP) 20 min before extinction training. Separate groups of rats were given either complete extinction (12 nonreinforced tone presentations) or partial extinction (6 nonreinforced tone presentations). On day 3, extinction tones were given to test for retention of extinction memory. In all phases of the experiment, the intertrial interval was variable, with an average of 3 min. Forty-eight hours after testing (day 5), a subgroup of rats that had received partial extinction were given six additional extinction trials, followed by two unsigned shocks (55 mA). On day 6, rats were tested for reinstatement of conditioned fear. In a separate experiment, rats were administered sotalol (10 mg/kg; IP), a β-receptor antagonist that does not cross the blood-brain barrier (21), 20 min before 12 extinction trials to assess the role of central versus peripheral receptors in fear expression and extinction.

Open Field and Heart Rate Testing
To test the effects of propranolol on locomotor activity, rats were given injections of saline or propranolol (10 mg/kg, IP) 20 min before testing in an open field. Grid lines drawn on the floor of the arena (91.5 × 91.5 × 61 cm) divided it into a peripheral region (within 15.25 cm of the walls) and central region (61 × 61 cm) of approximately equal area. The number of line crosses and time spent in the central region were scored by an observer blind with respect to drug treatment.

Resting heart rate was acquired from anesthetized rats with an electrocardiogram (ECG) monitor (EC 60, Silogic International Limited, Mitcham, Surrey, United Kingdom). Rats were anesthetized with ketamine (80 mg/kg), xylazine (5 mg/kg; IP) and connected to the ECG. Baseline heart rates were recorded, after which rats received an injection of either propranolol (10 mg/kg; IP) or sotalol (10 mg/kg; IP). Ten minutes after injection, heart rate was again recorded. During each session, heart rates were sampled every 10 sec for 1 min and averaged.

Behavioral Analysis
Throughout all phases of the fear conditioning experiments, we used two measures of conditioned fear: percent of time spent freezing (22) and suppression of bar-pressing (20,23). Freezing is defined as the absence of all movement except respiration and was quantified from digital videos during each tone presentation with commercial software (FreezeScan, Clever Systems, Reston, Virginia). Rats failing to show > 20% freezing (averaged over conditioning trials 4–7) were excluded, which resulted in the exclusion of 14 of 90 rats. Bar-press suppression ratios were determined by comparing pressing during the tone to pressing before the tone as follows: suppression ratio = (pretone rate − tone rate)/(pretone rate + tone rate). A value of 0 indicates no suppression of bar-pressing (no fear), whereas a value of 1 indicates complete suppression (high fear). We also analyzed the rate of bar-pressing before the first extinction trial to observe any differences in motivation to bar-press for food. Freezing, bar-press suppression, activity in the open field, and bar-press rate were analyzed with Student t test or repeated-measures analysis of variance. Heart rate changes were analyzed with a confidence interval of the mean and Student t test.

Multichannel Unit Recording
A separate group of rats were surgically implanted with recording electrodes that consisted of drivable bundles of 16 microwires (22 μm, Stablohm 675; California Fine Wire, Grover Beach, California) as previously described (24). Electrodes were aimed at the PL, located 2.9 mm anterior, .6 mm lateral, and 4.0 mm ventral from bregma. After surgery, rats were allowed 6 days to recover. Rats were then acclimated to recording procedures in the same chambers as in the behavioral experiments, and electrodes were driven in increments of 31–44 μm until single units were isolated with principle components analysis and template matching (Offline Sorter; Plexon, Dallas, Texas). Once cells in PL were well-isolated, we assessed the effects of injections of saline or propranolol (10 mg/kg, IP) on spontaneous activity while rats were in the operant chamber pressing for food. Ten-minute sessions of spontaneous activity were recorded at four time-points: 10 min before and 30 min after saline injection, and 10 min before and 30 min after propranolol injection. Firing rates before and after injections were compared with a Wilcoxon matched-pairs test. After recording the four sessions at a given location, the electrode drive was advanced in 80–125 μm steps until new cells were found, and the experiment was repeated. Spike trains were analyzed with NeuroExplorer (NEX Technologies, Littleton, Massachusetts) to obtain firing rate and bursting. Bursts were defined as three or more successive spikes in which the first interspike interval was <25 msec and subsequent intervals were <50 msec (24). At the conclusion of the experiment, lesions were made at the tip of the recording wires by passing an anodal current of 25 μA for 18 sec. Rats were then perfused with 10% buffered formalin, and the brains were removed to mark the microlesions with a blue reaction of 6% ferrocyanide while fixing the tissue in 30% sucrose/10% buffered formalin. Locations of lesions were reconstructed onto coronal drawings adapted from Paxinos and Watson (25) from 40-μm Nissl-stained sections.

Results
Propranolol Reduces Fear Expression Without Affecting Extinction Memory
We first examined the effects of propranolol given before extinction training on cued fear expression and subsequent extinction learning. Rats were injected with saline (Sal) (n = 10) or propranolol (Prop) (n = 12) 20 min before extinction training. Reduced freezing under propranolol was evident from the first extinction training trial [Sal, 31%; Prop, 11%; t(20) = 2.55; p = .02], indicating reduced fear expression (see Figure 1). Across the extinction session, propranolol-treated rats maintained significantly lower levels of both freezing [F(1,20) = 12.49, p = .002] and bar-press suppression [F(1,20) = 6.42, p = .02] compared with saline-treated rats. The smaller effect in suppression versus freezing was likely due to ceiling levels of suppression in control subjects. Despite this reduction in fear expression, propranolol-treated rats exhibited normal extinction learning, as evidenced by a progressive decrease in bar-press suppression throughout the extinction session. The following day, when tested drug-free, saline- and propranolol-treated rats showed similar low levels of freezing [Sal, 7%; Prop, 3%; t(20) = 1.27; p = .22] and bar-press suppression [Sal, .41; Prop, .37; t(20) = .28; p = .78], indicating
that although propranolol reduced fear expression during extinction training it did not impair extinction learning.

Although the previous experiment showed that propranolol did not impair extinction retention, both saline- and propranolol-treated rats showed little freezing during the extinction recall test. With floor levels of freezing, it is not possible to detect whether propranolol-treated rats exhibited less fear than control subjects. Enhanced recall of extinction might be expected if, for example, propranolol interfered with reconsolidation of the fear memory on day 2 (4). To address this issue, we repeated the experiment with partial extinction training (6 nonreinforced tone presentations), resulting in moderate levels of freezing during the drug-free test, thereby allowing us to detect any enhancement of extinction (Figure 2). As in the previous experiment, rats were injected with saline or propranolol 20 min before extinction training (Sal, \(n = 15\); Prop, \(n = 14\)). Again, propranolol significantly reduced freezing during the first extinction trial [Sal, 47%; Prop, 24%; \(t(27) = 2.12, p = .04\)] and reduced both freezing [\(F(1,27) = 6.54, p = .02\)] and bar-press suppression [\(F(1,27) = 13.16, p = .001\)] throughout extinction training. The following day, however, propranolol-treated rats showed the same level of freezing [Sal, 31%; Prop, 23%; \(t(27) = .66, p = .52\)] and suppression [Sal, 74%; Prop, 68%; \(t(27) = .53, p = .60\)] as control subjects. Furthermore, when given unsignaled footshocks, propranolol-treated rats showed levels of fear reinstatement similar to control subjects in both freezing [Sal, 26%; Prop, 30%; \(t(27) = −.44, p = .66\)] and suppression [Sal, 82%; Prop, 77%; \(t(27) = .36, p = .71\)] measurements. Thus, propranolol neither facilitated extinction nor blocked reconsolidation of fear under these conditions.

Propranolol-Induced Fear Reductions Are Not Due to Nonspecific Behavioral Effects, and Are Mediated Centrally

To examine nonspecific effects of propranolol that might account for the observed reduction in fear expression, we evaluated its effects on locomotion and anxiety in an open field (Sal, \(n = 8\); Prop, \(n = 8\)) as well as on motivation to bar-press for food (Sal, \(n = 25\); Prop, \(n = 26\); Figure 3). Propranolol had no effect on spontaneous locomotion as measured by the number of line crosses in the open field [Sal, 77 crosses; Prop, 73 crosses; \(t(14) = .41, p = .69\)]. In addition, anxiety was not affected, because both groups spent a similar percentage of time in the center of the open field [Sal, 6.0%; Prop, 6.7%; \(t(14) = −.35, p = .73\)]. Propranolol also had no effect on the rate of bar-pressing for food reward (Sal, 25 presses/min; Prop, 25 presses/min; \(t(14) = .01, p = .99\)).

Figure 1. Propranolol reduces fear expression but does not impair extinction learning. Systemic injections of propranolol (arrow) before complete extinction on day 2 led to a significant decrease in fear expression as measured by (A) percent freezing and (B) bar-press suppression. On day 3 both groups recalled extinction similarly. Data shown in blocks of two trials. Cond, conditioning.

Figure 2. Propranolol does not facilitate extinction learning or erase fear memory. Injection of propranolol (arrow) before partial extinction on day 2 led to a significant decrease in fear expression as measured by (A) percent freezing and (B) bar-press suppression. There were no differences between groups in further extinction sessions on day 3 and day 5. Two unpaired shocks on day 5 reinstated fear on day 6 equally in both groups. Data shown in blocks of two trials. Cond, conditioning; Ext, extinction; Reinst, reinstatement; D2, day 2.

Figure 3. Propranolol does not alter spontaneous locomotion (left), anxiety in an open field (center), or rate of bar-pressing for food reward (right).
food before the first extinction trial [Sal, 16 presses/min; Prop, 17 presses/min; \(t(49) = -.39; p = .70\)]. Collectively, these results indicate that the reduction in freezing observed after propranolol administration was not due to nonspecific effects such as changes in anxiety levels, locomotor behavior, or motivation to bar-press.

Because propranolol acts both centrally and peripherally, it is possible that the reduction in fear was caused by reduced feedback from the peripheral nervous system (e.g., cardiovascular responses) (26). To assess whether reduced fear expression by propranolol is centrally or peripherally mediated, we repeated the experiment with the noradrenergic receptor antagonist sotalol (Sot), which does not cross the blood-brain barrier (21,27). We injected rats with saline or sotalol (10 mg/kg) 20 min before receiving 12 extinction trials (Sal, \(n = 11\); Sot, \(n = 14\);

Figure 4). Sotalol-treated rats did not differ from the saline-treated rats in levels of freezing during the first extinction trial [Sal, 28%; Sot, 31%; \(t(23) = -.33; p = .74\)] or during the rest of the extinction session as indicated by freezing \([F(1,23) = .37; p = .54]\) and bar-press suppression \([F(1,23) = .71; p = .41]\). The following day, sotalol- and saline-treated rats showed similar extinction retrieval, as measured by freezing [Sal, 6.9%; Sot, 4.9%; \(t(23) = .25; p = .80\)] and bar-press suppression [Sal, .54; Sot, .44; \(t(23) = .72; p = .48\)]. Thus when confined to the periphery, \(\beta\) blockers do not reduce conditioned fear expression.

To confirm that both sotalol and propranolol had similar peripheral actions, we monitored heart rate in a separate group of anesthetized rats (Prop, \(n = 10\); Sot, \(n = 10\); Figure 4C).

Propranolol Reduces Firing Rate of PL Neurons

We have recently shown that activity in the PL is necessary for the expression of conditioned fear (16,18). Thus, we examined the
effect of propranolol on spontaneous activity of individual PL neurons (Figure 5). Spontaneous activity was recorded before and after injection of saline or propranolol (10 mg/kg). A total of 22 neurons from five rats were maintained across all four 10-min recording sessions (Pre-Sal, Post-Sal, Pre-Prop, Post-Prop). Propranolol significantly reduced the spontaneous firing rate of PL neurons, from 5.2 Hz to 3.2 Hz (Wilcoxon matched-pairs test: Z = 2.94, p = .003). There was no effect on high-frequency bursting (t(21) = .41, p = .68). The response of individual neurons to injections of saline and propranolol are shown in scatter plots in Figure 5C. Unlike saline, propranolol reduced the firing rate of the majority of neurons (Fisher exact test: p = .013). Taken together, these results suggest that reduced fear expression by propranolol could be due to a decrease in PL excitability.

**Discussion**

We examined the effects of the noradrenergic β-receptor antagonist propranolol on the expression and extinction of cued fear conditioning. Propranolol significantly reduced fear expression, as measured by freezing and bar-press suppression. Extinction learning, however, was unaffected by propranolol, as evidenced by normal acquisition and recall of extinction. Propranolol-induced reductions of fear could not be attributed to effects on locomotion, motivation to press for food, or anxiety. Propranolol’s effects seem to be mediated centrally, because the peripheral β-adrenergic antagonist sotalol had no effect on fear expression. Consistent with this, propranolol reduced the activity of neurons in PL.

Propranolol-induced reduction in the expression of cued fear generally agrees with prior findings in other conditioning procedures. The same dose of propranolol reduced expression of fear-potentiated startle (10) and tone-induced freezing (28) in rats as well as contextual freezing in mice (29). Cain et al. (11) observed accelerated extinction under propranolol, but because freezing to the first extinction tone was not reduced, they concluded that propranolol did not impair expression of conditioned fear. In contrast, we observed a significant reduction in freezing from the first extinction tone onward, consistent with decreased expression of fear. Thus, differences in species used or experimental parameters could account for the variability in the effects of propranolol on fear expression.

Despite previous reports that central infusions of propranolol can impair extinction (15,30), we observed no impairment of extinction consolidation after systemic injections of propranolol, in agreement with Cain et al. (11). Furthermore, with partial extinction training, we observed that propranolol did not facilitate extinction consolidation. Thus, at the dose used here, pre-extinction propranolol did not alter extinction learning or retention. The apparent discrepancy with local infusion studies could be due to differences in the concentration of propranolol that reaches structures such as the prefrontal cortex with systemic versus localized administration.

Although our study was not designed to identify the site of action of propranolol in the brain, we observed a significant reduction in the spontaneous firing rate of PL neurons after systemic propranolol injections. Reduced excitability in PL would be expected to reduce tone-evoked responses. Several lines of evidence implicate PL in expression of conditioned fear. Pharmacological inactivation of PL reduces tone-evoked freezing (17,18), and electrical stimulation of PL has the opposite effect (16). In addition, the tone responsiveness of PL neurons increases during auditory fear conditioning (31,32). Thus, propranolol might act by blocking norepinephrine-induced increases in PL activity during high-fear states. Propranolol could also reduce the activity of afferents to PL, such as the basolateral amygdala (33). Either way, reduced activity of PL neurons is consistent with the hypothesis that propranolol reduces activity in fear expression circuits.

The ability of propranolol to reduce fear expression without interfering with extinction learning suggests that propranolol might be useful as an adjunct to exposure-based cognitive-behavioral therapy for anxiety disorders. The stress associated with repeated exposure to fear-inducing stimuli is thought to contribute to dropout rates in these therapies (34). Reducing excessive stress during the exposure session with propranolol could make these therapies more tolerable, especially for patients with high-fear reactions. Although propranolol does not facilitate extinction of fear like other adjuncts (35), neither does it impair extinction. Thus, the reduction in fear would not interfere with the clinical effectiveness of the therapy. This differs from benzodiazepines, which can reduce fear expression but lead to a return of fear via a state-dependent learning effect (36).

In humans, propranolol has traditionally been used to reduce performance anxiety in musicians (37), but its effects on other types of anxiety have been mixed. Propranolol successfully reduced anxiety in dental phobic patients (38) and avoidance behavior in panic disorder patients (39). Other studies, however, showed no effect of propranolol on subjective anxiety in phobic subjects (40) or expression of cued fear conditioning (7). It is interesting to note that propranolol improved cognitive ability under stressful conditions (6,41), suggesting potential use in therapies that combine exposure and cognitive therapies.

Our rodent findings suggest that propranolol might be useful if given before exposure to traumatic stimuli to reduce the expression of fear responses during extinction-based therapies. Given the conflicting reports in the rodent and human literature, however, additional studies are needed to determine whether propranolol reduces learned fear in healthy humans and anxiety patients while leaving extinction learning intact.

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Note added in proof: In agreement with our present findings, a recent study in rats showed that propranolol (but not peripheral blocker nadolol) blocked behavioral fear responses to predator odor (42).


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