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Involvement of Noradrenergic Neurotransmission in the Stress- but not Cocaine-Induced Reinstatement of Extinguished Cocaine-Induced Conditioned Place Preference in Mice: Role for β -2 Adrenergic Receptors

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Abstract: The responsiveness of central noradrenergic systems to stressors and cocaine poses norepinephrine as a potential common mechanism through which drug re-exposure and stressful stimuli promote relapse. This study investigated the role of noradrenergic systems in the reinstatement of extinguished cocaine-induced conditioned place preference by cocaine and stress in male C57BL/6 mice. Cocaine- (15 mg/kg, i.p.) induced conditioned place preference was extinguished by repeated exposure to the apparatus in the absence of drug and reestablished by a cocaine challenge (15 mg/kg), exposure to a stressor (6-min forced swim (FS); 20–25°C water), or administration of the α -2 adrenergic receptor (AR) antagonists yohimbine (2 mg/kg, i.p.) or BRL44408 (5, 10 mg/kg, i.p.). To investigate the role of ARs, mice were administered the nonselective β -AR antagonist, propranolol (5, 10 mg/kg, i.p.), the α -1 AR antagonist, prazosin (1, 2 mg/kg, i.p.), or the α -2 AR agonist, clonidine (0.03, 0.3 mg/kg, i.p.) before reinstatement testing. Clonidine, prazosin, and propranolol failed to block cocaine-induced reinstatement. The low (0.03 mg/kg) but not high (0.3 mg/kg) clonidine dose fully blocked FS-induced reinstatement but not reinstatement by yohimbine. Propranolol, but not prazosin, blocked reinstatement by both yohimbine and FS, suggesting the involvement of β -ARs. The β -2 AR antagonist ICI-118551 (1 mg/kg, i.p.), but not the β -1 AR antagonist betaxolol (10 mg/kg, i.p.), also blocked FS-induced reinstatement. These findings suggest that stress-induced reinstatement requires noradrenergic signaling through β -2 ARs and that cocaine-induced reinstatement does not require AR activation, even though stimulation of central noradrenergic neurotransmission is sufficient to reinstate.

Keywords: relapse, norepinephrine, propranolol, prazosin, clonidine, beta adrenergic receptors

Introduction

The sudden reemergence of cocaine craving even after protracted periods of abstinence and the resulting relapse of drug use represent formidable obstacles to the effective management of cocaine addiction. Anecdotal reports and clinical and preclinical research have identified stress and cocaine re-exposure as likely precipitating events for drug relapse (Shaham *et al*, 2000a, 2000b, 2003; Sinha, 2001; Stewart, 2003; Lu *et al*, 2003). Understanding the neurobiological mechanisms that contribute to the relapse of drug use in response to these stimuli should lead to the development of new and more effective approaches for treating addiction.

The preclinical study of relapse involves the use of reinstatement protocols in which the ability of various stimuli to reestablish extinguished drug-seeking behavior is examined (Shaham *et al*, 2003). For example, in rats and monkeys, cocaine administration or exposure to a variety of stressors will reinstate extinguished lever pressing that was previously reinforced by cocaine delivery (see Shaham *et al*, 2000a, 2000b, 2003; Sinha, 2001; Stewart, 2003; Lu *et al*, 2003 for reviews). Relapse can also be investigated using a conditioned place preference reinstatement approach in which the ability of stimuli to reestablish extinguished preference for a previously cocaine-paired environment is examined (Tzschentke, 2007). In mice, extinguished cocaine-induced conditioned place preference can be reinstated by administration of a priming injection of cocaine (Itzhak and Martin, 2002; Orsini *et al*, 2008) or by exposure to stressors, including social stress (Ribeiro Do Couto *et al*, 2006), uncontrollable electric footshock (Redila and Chavkin, 2008), or forced swim (Kreibich and Blendy, 2004). The use of mouse reinstatement models is important in that it should facilitate the application of genetic approaches not available in other species to further advance understanding of relapse substrates.

The responsiveness of central noradrenergic systems to both stressors (Abercrombie *et al*, 1988; Tanaka *et al*, 1991; Finlay *et al*, 1995) and cocaine (Reith *et al*, 1997; Li *et al*, 1996) poses norepinephrine (NE) as a potential common mechanism through which

drug re-exposure and stressful environmental stimuli promote relapse. Accordingly, previous studies have suggested that antagonizing adrenergic receptors (ARs; Leri *et al*, 2002; Zhang and Kosten, 2005) or suppressing noradrenergic neurotransmission (Erb *et al*, 2000; Platt *et al*, 2007) can attenuate cocaine seeking, whereas augmenting adrenergic neurotransmission through antagonism of α -2 adrenergic autoreceptors (Lee *et al*, 2004; Feltenstein and See, 2006; Fletcher *et al*, 2008; Brown *et al*, 2009) or blockade of reuptake (Platt *et al*, 2007; but see Schmidt and Pierce, 2006) can evoke cocaine seeking as measured using reinstatement protocols. Considering that in human cocaine addicts, drug craving is associated with a robust peripheral adrenergic response (Sinha *et al*, 2003) that seems to be augmented during early withdrawal (McDougle *et al*, 1994) and that repeated cocaine exposure produces long-lasting adaptations within central noradrenergic systems that may lead to enhanced noradrenergic responsiveness during stress (Belej *et al*, 1996; Macey *et al*, 2003; Baumann *et al*, 2004; Beveridge *et al*, 2005; Lanteri *et al*, 2008), central noradrenergic signaling may represent an important therapeutic target for the prevention of relapse (Weinshenker and Schroeder, 2007; Smith and Aston-Jones, 2008; Sofuoglu and Sewell, 2009).

This study investigated the role of noradrenergic systems in the reinstatement of extinguished cocaine-induced conditioned place preference by cocaine and stress in adult male C57 BL/6 mice. The effects of the α -1 AR antagonist, prazosin, the β -AR antagonists, propranolol, betaxolol, and ICI-118551, and the α -2 AR agonist, clonidine, on reinstatement by cocaine, forced swim (FS) stress, and/or the α -2 AR antagonist, yohimbine, were examined. Our findings suggest that noradrenergic activation of β -2 ARs is selectively involved in stress-induced reinstatement.

Materials and Methods

Subjects

A total of 250 male C57BL/6 mice (8–9 weeks old) were used (Harlan). Mice were housed singly in a temperature- and humidity-

controlled, AAALAC-accredited animal facility under a 12/12 h light/dark cycle (lights on at 0700 hours) and had access to food and water at all times, except when in the experimental chambers. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH.

Drugs

Cocaine HCl was acquired from the National Institute on Drug Abuse (NIDA) through the NIDA Drug Supply Program. Clonidine HCl, \pm propranolol HCl, betaxolol HCl, ICI-118551 HCl, and yohimbine HCl were purchased from Sigma-Aldrich. BRL 44408 maleate was acquired from Tocris Bioscience. Yohimbine, prazosin, and clonidine were dissolved in sterile water. Cocaine, propranolol, betaxolol, ICI-118551, and BRL 44408 were dissolved in saline (0.9% NaCl solution). All drug solutions were administered i.p. in a volume of 0.1 ml per 20 g body mass.

Equipment

Behavioral testing was conducted using four ENV-3013 mouse place preference chambers from Med-Associates, Inc. The stainless steel and polyvinyl chloride chambers consisted of three distinct compartments separated by 5 cm wide \times 5.9 cm high manual guillotine doors. The two 46.5 \times 12.7 \times 12.7 cm³ side compartments consisted of a white compartment with a 6.35 \times 6.35 mm² stainless steel mesh floor and a black compartment with a stainless steel grid rod floor consisting of 3.2 mm rods spaced 7.9 mm apart. The side compartments were attached by a gray-colored 7.2-cm-long center compartment with a smooth floor. The clear tops of the compartments were hinged to permit placement and removal of the mice. Ceiling lights were attached to each top. To balance unconditioned side preference, only the light in the black compartment was illuminated during training or testing. Automated data collection was accomplished using photobeams (six beams for the white and black test areas and two beams for the center gray area), which were evenly spaced across the length of the chamber and interfaced with a computer containing

Med-PC software. Using this automated photobeam system, entry into a side compartment was defined as consecutive breaks of the first two photocell beams in that compartment located adjacent to the door separating that compartment from the center compartment. Exiting of a side compartment (and entry into the center compartment) was indicated by occlusion of the beams in the center compartment.

Cocaine-Induced Conditioned Place Preference

Cocaine-induced conditioned place preference was established using a design in which one of the side compartments was randomly designated as the cocaine compartment and the other as the saline compartment. On the first day of the procedure, mice were placed into the center compartment of the chamber and provided free access to both side compartments for 30 min in the absence of saline or cocaine pretreatment to determine preconditioning preference. During the 8-day conditioning phase of the experiment, mice received cocaine (15 mg/kg, i.p.) and saline injections on alternating days, after which they were confined to the randomly designated treatment-appropriate compartment for 30 min. After the final conditioning session, mice were tested for the expression of cocaine-induced conditioned place preference by once again placing them into the center compartment of the chamber and providing them with free access to the side compartments for 30 min. Conditioned place preference was defined as the change in time spent (seconds) within the cocaine-paired compartment after conditioning when compared with the initial preconditioning session. The 15 mg/kg cocaine dose was selected on the basis of results of a preliminary study in which the ability of various cocaine doses to produce place preference was examined (Figure 1a).

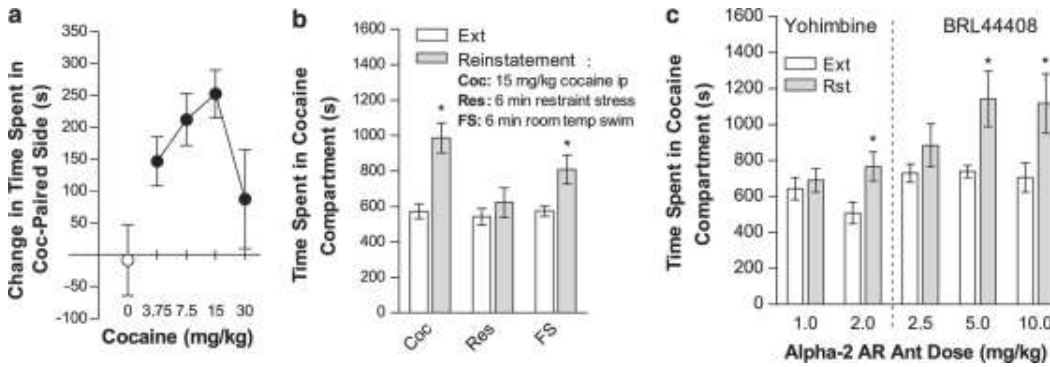


Figure 1 Induction and reinstatement of cocaine-induced conditioned place preference. Cocaine dose-dependently induced conditioned place preference with peak effects at the 15 mg/kg (i.p.) dose. Data in (a) represent the mean change in time spent (sec±SE) in the cocaine-paired compartment between the pre- and post-conditioning test sessions in mice that were conditioned with various cocaine doses (0–30 mg/kg, i.p.; $n=10-20$ /group). On the basis of these data, the 15 mg/kg cocaine dose was selected as the conditioning and reinstatement dose for each experiment. (b and c) Show the reinstatement of extinguished cocaine-induced conditioned place preference by cocaine (Coc; 10 mg/kg, i.p.; $n=23$; b), physical restraint (Res; $n=26$; b), forced swim (FS; $n=16$; b), and by the α -2 AR antagonists yohimbine (Yoh; 1 and 2 mg/kg, i.p.; $n=23$; c) and BRL 44408 (BRL; 2.5, 5, and 10 mg/kg, i.p.; $n=6-8$; c). Data represent the time spent in the Coc compartment (sec±SE) during the 30-min reinstatement (Rst) session (filled bars) or during the preceding extinction session (open bars). Coc, FS, 2 mg/kg Yoh, and 5 and 10 mg/kg BRL but not Res, 1 mg/kg Yoh or 2.5 mg/kg BRL, produced significant reinstatement ($*p<0.05$ vs Ext).

Extinction

After conditioning, daily extinction training was conducted. During the extinction sessions, mice were placed into the center compartment and once again provided free access to the side compartments for 30 min. Mice underwent daily extinction training until the preference for the cocaine-paired compartment during the session (ie, change in time spent in the cocaine side relative to preconditioning values) was reduced by at least 50% compared with the post-conditioning test session, at which time reinstatement testing was conducted.

Reinstatement Testing

The reinstatement sessions were identical to the extinction sessions, except that mice received exposure to stressors and/or drug

injections before the session. Reinstatement was defined according to the time spent in the compartment previously paired with cocaine. Mice were tested more than once for reinstatement. Generally, each mouse was tested with each of the three reinstating stimuli (cocaine, forced swim (FS), and yohimbine) alone and in combination with one drug pretreatment and its respective vehicle in counter-balanced order to avoid potential sequence effects. Reinstatement sessions were separated by additional extinction sessions. Mice were required to reach the extinction criterion once again before the next reinstatement test was conducted. Initially, separate groups of mice were tested for the ability of various stressors and drugs, when delivered in the absence of pretreatments, to produce reinstatement.

Drug-Induced Reinstatement

Cocaine- and yohimbine-induced reinstatement testing was conducted by injecting mice with cocaine (15 mg/kg, i.p.) or yohimbine (1 or 2 mg/kg, i.p.) either alone or 30 min after pretreatment with clonidine (0.03 and 0.3 mg/kg), propranolol (5 and 10 mg/kg), prazosin (1 and 2 mg/kg), betaxolol (10 mg/kg), or ICI-118551 (10 mg/kg), or their respective vehicles and placing mice into the center compartment 5 min later with free access to both sides of the compartment for 30 min. Reinstatement by clonidine, propranolol, prazosin, betaxolol, and ICI-118551 alone was also tested after administration 30 min before the reinstatement session, a time point that matched the pretreatment time when these drugs were administered before cocaine, yohimbine, or FS during testing. To confirm that antagonism of α -2 AR by yohimbine may have contributed to reinstatement, a separate group of mice was tested for reinstatement in response to the highly selective α -2 AR antagonist, BRL 44408 (2.5, 5, and 10 mg/kg, i.p.).

Stress-Induced Reinstatement

FS-induced reinstatement was examined by placing mice into a 30 cm high \times 20 cm deep cylindrical polypropylene container filled with water (20–25°C) for 6 min, 30 min after pretreatment with clonidine (0.03 and 0.3 mg/kg), propranolol (5 and 10 mg/kg), prazosin (1 and

2 mg/kg), or their respective vehicles. After FS, mice were placed back into their home cages for 3–4 min before introducing them into the center compartment of the place conditioning chamber with free access to both of the side compartments for reinstatement testing, as described above. Initially, some mice were also tested for reinstatement following 5 min of physical restraint using a mouse restrainer cone (5.7 cm diameter tapered to 1.2 cm diameter; 11 cm length). These mice were also returned to their home cages after restraint for 3–4 min before placement into the apparatus for reinstatement testing. As propranolol blocked FS-induced reinstatement, we also tested the mice for the effects of the selective β -1 AR antagonist, betaxolol (10 mg/kg, i.p.), and the selective β -2 AR antagonist, ICI-118551 (1 mg/kg, i.p.), on reinstatement after FS.

Locomotor Testing

To assist with dose selection, the effects of various doses of clonidine, prazosin, and propranolol on locomotor activity were tested in 26 mice using an automated AccuScan activity system (AccuScan Instruments, Inc., Columbus, OH) consisting of a frame containing photocells (eight per cage), in which clear Plexiglas 29.5 cm long \times 19 cm wide \times 12.7 cm high cages were placed. Activity was measured as total photobeam breaks. During the week before the locomotor testing, mice were habituated to the test environment during two 2-h sessions. On the test day, mice were placed into the chambers for 2 h before the drug administration, after which was measured over a 2-h period. The effects of clonidine (0, 0.03, 0.3, and 1 mg/kg, i.p.), prazosin (0, 1, 2, and 4 mg/kg, i.p.), and propranolol (0, 5, 10, and 20 mg/kg, i.p.) were tested in separate groups of mice. Only doses that failed to produce statistically significant reductions in activity were tested for effects on reinstatement.

Statistical Analyses

Place preference during testing, extinction, and reinstatement was defined as the time spent (sec) in the designated cocaine compartment. The abilities of individual stimuli to reinstate place preference in the absence of drug pretreatments were examined

statistically using two-tailed Student's *t*-tests comparing the time spent in the cocaine compartment during the previous extinction session with that during the reinstatement session. The significance of the effects of drug pretreatments on reinstatement by each of the stimuli was determined using two-way repeated measures reinstatement \times drug pretreatment ANOVA followed by *post-hoc* testing using two-tailed Student's *t*-tests. The significance of dose-related drug effects on locomotor activity was determined using one-way (drug dose) ANOVA followed by *post-hoc* testing using the Dunnett's test. For all analyses, significance was defined as $p < 0.05$.

RESULTS

Establishment and Reinstatement of Cocaine-Induced Conditioned Place Preference

Dose-response analysis showed that the 15 mg/kg cocaine dose was optimal for producing conditioned place preference ($n = 10-20$ mice per cocaine/veh dose; Figure 1a). Although not shown, significant cocaine-induced conditioned place preference, defined as an increase in time spent in the cocaine-paired compartment, was observed in each of the experiments in which reinstatement was later examined ($p < 0.05$ for each experiment). The overall average number of extinction sessions before the first reinstatement test session was 8.757. Overall, 28 mice (out of 224 that underwent conditioning) were not tested for reinstatement, because they either did not show cocaine-induced conditioned place preference or failed to reach the extinction criterion.

The ability of cocaine and stress to reinstate extinguished cocaine-induced place preference is shown in Figure 1b. Administration of 15 mg/kg cocaine ($t_{22} = 4.949$; $n = 23$) or FS ($t_{15} = 3.128$; $n = 16$), but not restraint ($n = 26$), significantly increased the time spent in the cocaine compartment following extinction ($p < 0.01$).

To determine whether stimulating central noradrenergic neurotransmission through antagonism of α -2 AR is sufficient to induce reinstatement, mice were tested for the ability of yohimbine and the

more selective α -2A AR antagonist, BRL 44408, to reinstate extinguished place preference (Figure 1c). For yohimbine, a two-way reinstatement condition (repeated measure) \times yohimbine dose (1 vs 2 mg/kg) ANOVA showed a significant overall effect of reinstatement condition ($F_{1,33}=5.252$; $p<0.05$) but not yohimbine dose, and a significant reinstatement condition \times dose interaction ($F_{1,33}=4.089$; $p=0.05$). The 2 mg/kg ($t_{22}=3.371$; $n=23$), but not 1 mg/kg ($n=23$), yohimbine dose produced significant reinstatement ($p<0.01$). Although yohimbine can increase central noradrenergic neurotransmission through antagonism of α -2 ARs, it also binds to several other receptors, including the 5-HT_{1A} receptor, where it functions as an antagonist and may produce effects independently of the noradrenergic system (Sanger and Schoemaker, 1992; Winter and Rabin, 1992). To confirm that α -2 AR antagonism is sufficient to induce reinstatement, we also tested some mice for reinstatement after administration of the selective α -2A AR antagonist BRL 44408, which has a 50-fold higher affinity for α -2A AR relative to 5-HT_{1A} receptors (Dwyer *et al*, 2010). A two-way repeated-measures reinstatement condition \times BRL 44408 dose (2.5, 5.0, 10.0 mg/kg) ANOVA showed a significant overall effect of reinstatement condition ($F_{1,6}=13.557$; $p=0.01$) but not BRL 44408 dose, and a significant reinstatement condition \times dose interaction ($F_{2,12}=4.222$; $p<0.05$). Similar to yohimbine, BRL 44408 dose-dependently reinstated, with significant effects observed at the 5 ($t_6=2.776$; $p<0.05$) and 10 ($t_7=2.538$; $p<0.05$), but not at the 2.5 mg/kg BRL 44408 doses.

Locomotor Testing

Propranolol, prazosin, and clonidine each produced dose-dependent reductions in locomotor activity (Figure 2). One-way prazosin dose ANOVA examining the effects of 1, 2, and 4 mg/kg prazosin on locomotor activity showed a significant overall effect ($F_{1,36}=3.300$; $p<0.05$). *Post-hoc* testing using the Dunnett's *t*-test showed that the 4 mg/kg dose, but not the other prazosin doses, significantly reduced locomotor activity compared with vehicle-treated controls ($p<0.01$). Similarly, one-way propranolol dose ANOVA examining the effects of 5, 10, and 20 mg/kg propranolol on locomotor activity showed a significant overall effect ($F_{1,36}=3.244$; $p<0.05$). *Post-*

hoc testing using the Dunnett's *t*-test showed that the 20 mg/kg dose, but not the other propranolol doses, significantly reduced locomotor activity compared with vehicle-treated controls ($p < 0.05$). One-way clonidine dose ANOVA examining the effects of 0.03, 0.3, and 1 mg/kg clonidine on locomotor activity failed to show a significant overall effect of clonidine, despite a near-significant trend ($F_{3,33} = 2.693$; $p = 0.06$). *Post-hoc* testing using the Dunnett's *t*-test showed that the 1 mg/kg dose, but not the other clonidine doses, significantly reduced locomotor activity compared with vehicle-treated controls ($p < 0.05$). As 4 mg/kg prazosin, 20 mg/kg propranolol, and 1 mg/kg clonidine produced significant reductions in locomotor activity, we chose not to test the effects of these doses on reinstatement.

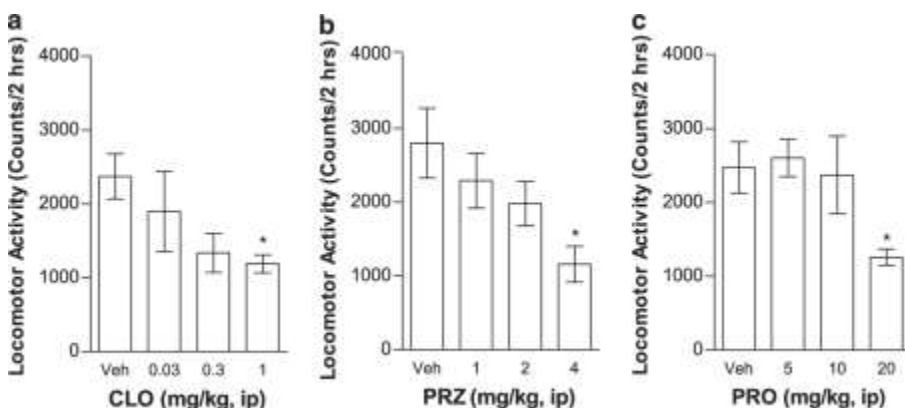


Figure 2 Effects of clonidine (CLO; a), prazosin (PRZ; b), and propranolol (PRO; c) or their respective vehicles (Veh) on locomotor activity. To facilitate dose selection, mice ($n = 6-10$ per dose) were tested for the effects of CLO, PRZ, and PRO on locomotor activity. Data represent locomotor counts (total photobeam breaks) measured during a 2-h session that immediately followed drug administration. Each drug dose-dependently attenuated locomotor activity ($*p < 0.05$; significant reduction vs Veh).

Reinstatement by Prazosin, Propranolol, Clonidine, Betaxolol, and ICI-118551

To ensure that the α -1 AR antagonist prazosin, the β -AR antagonists propranolol, betaxolol and ICI-118551, and the α -2 AR agonist clonidine did not, by themselves, alter preference for the cocaine compartment after extinction, we initially examined the ability of these drugs, when administered alone, to reinstate extinguished

conditioned place preference at the doses to be used (Table 1). Whereas prazosin (1 mg/kg; $n=12$ or 2 mg/kg; $n=14$), propranolol (5 mg/kg; $n=8$ or 10 mg/kg; $n=10$), betaxolol (10 mg/kg; $n=12$), or ICI-118551 (5 mg/kg; $n=12$) failed to alter the amount of time spent in the cocaine-paired compartment after extinction, clonidine, at the higher (0.3 mg/kg; $t_{12}=2.491$, $p<0.05$; $n=13$) but at not the lower (0.03 mg/kg; $n=13$) dose, produced significant reinstatement ($p<0.05$).

Table 1 Effects of Prazosin (Prz), Propranolol (Pro), Clonidine (Clo), Betaxolol (Bet), and ICI-118551 (ICI) on Time Spent in the Cocaine Compartment Following the Extinction of Cocaine-Induced Conditioned Place Preference ($n = 8-14$ per drug)

Drug (dose)	Ext	Reinstatement
Prz (1.0 mg/kg)	614.84 (± 47.81)	592.86 (± 54.40)
Prz (2.0 mg/kg)	583.29 (± 24.59)	673.34 (± 85.93)
Pro (5.0 mg/kg)	617.53 (± 58.31)	582.35 (± 85.03)
Pro (10.0 mg/kg)	640.60 (± 80.72)	602.77 (± 91.61)
Clo (0.03 mg/kg)	583.55 (± 70.28)	613.27 (± 75.18)
Clo (0.3 mg/kg)	595.80 (± 34.81)	913.11 (± 131.27)*
Bet (10.0 mg/kg)	668.42 (± 43.11)	659.47 (± 63.01)
ICI (1.0 mg/kg)	622.45 (± 36.01)	607.18 (± 87.56)

Data represent the time spent in the cocaine compartment (sec \pm SE) during the reinstatement session after drug administration (reinstatement) or during the preceding extinction session (Ext). Only the high (ie, 0.3 mg/kg) clonidine dose reinstated extinguished conditioned place preference (* $p < 0.05$ vs Ext; $n = 13$).

Effects of Clonidine on Reinstatement

As augmentation of central noradrenergic neurotransmission by administration of yohimbine or the selective α -2A AR antagonist, BRL-44408, induced reinstatement, we hypothesized that noradrenergic neurotransmission during stress or after cocaine exposure may contribute to stress- and cocaine-induced drug seeking. To test this hypothesis, mice were tested for FS- or cocaine-induced reinstatement after administration of clonidine, an α -2 AR agonist that suppresses

evoked increases in NE (Florin *et al*, 1994; Tjurmina *et al*, 1999) presumably by the activation of inhibitory autoreceptors on noradrenergic nerve terminals.

Forced Swim

The effects of clonidine (0.03 and 0.3 mg/kg, i.p.) on FS-induced reinstatement are shown in Figure 3. Clonidine suppressed FS-induced reinstatement, but only at the 0.03 mg/kg dose. A total of 27 mice were used to test for the effects of 0.03 mg/kg clonidine dose on FS-induced reinstatement (Figure 3a). Two-way repeated-measures FS × clonidine treatment condition ANOVA showed a significant overall effect of FS ($F_{1,26}=6.199$; $p<0.05$) but not clonidine treatment condition on time spent in the cocaine compartment and a significant clonidine × FS interaction ($F_{1,26}=12.264$; $p<0.01$). *Post-hoc* examination of reinstatement in each group revealed that FS significantly reinstated place preference in vehicle-treated but not clonidine-treated mice ($t_{26}=3.230$; $p<0.05$) and that time spent in the cocaine compartment was significantly reduced by clonidine after FS ($t_{26}=3.012$; $p<0.05$). Nine mice were used to test the effects of the 0.3 mg/kg clonidine dose on FS-induced reinstatement (Figure 3b). In contrast to the 0.03 mg/kg clonidine dose, 0.3 mg/kg clonidine failed to block FS-induced reinstatement. Two-way repeated measures FS × clonidine treatment condition ANOVA showed a significant overall effect of FS ($F_{1,8}=6.122$; $p<0.05$), but not of clonidine and no FS × clonidine interaction.

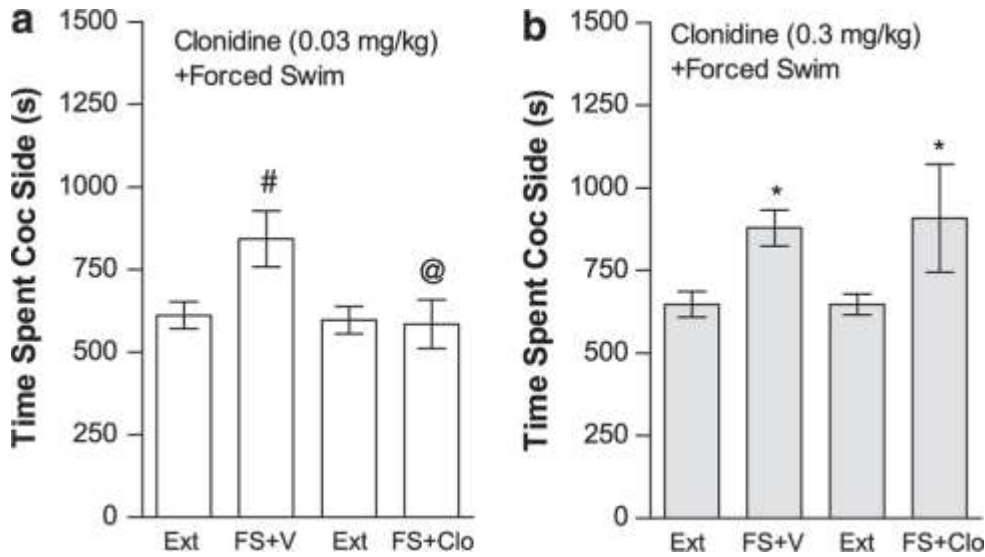


Figure 3 The α -2 AR agonist clonidine dose-dependently blocks stress-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment (sec \pm SE) during the final extinction session prior to reinstatement testing (Ext) and during reinstatement testing after forced swim (FS) after pretreatment with vehicle (V) or clonidine (Clo). A significant interaction between FS and Clo was found in mice tested at the 0.03 mg/kg (i.p.) Clo dose (a; $n=27$; $p>0.05$). Post-hoc testing showed that FS increased time spent in the Coc compartment after treatment with V ($\#p<0.05$) but not 0.03 mg/kg Clo. Further, time spent in the Coc compartment was significantly reduced after 0.03 mg/kg Clo compared with V ($@p<0.05$). The effect of 0.3 mg/kg (i.p.) Clo ($n=9$) on FS-induced reinstatement is shown in b. A significant main effect of FS ($*p<0.05$) but no FS \times Clo interaction was found at this dose.

Cocaine

The effects of clonidine (0.03 or 0.3 mg/kg, i.p.) on cocaine-induced reinstatement are shown in Figure 4. Neither clonidine dose blocked cocaine-induced reinstatement. The effect of 0.03 mg/kg clonidine was tested in 25 mice (Figure 4a). Two-way cocaine \times 0.03 mg/kg clonidine treatment condition ANOVA showed a significant overall effect of cocaine priming ($F_{1,24}=22.807$; $p<0.001$) on time spent in the cocaine compartment. However, no significant main effect of clonidine treatment condition or interaction between clonidine treatment and cocaine-induced reinstatement was found. In all, 13 mice were tested for the effect of a 10-fold higher clonidine dose (0.3 mg/kg, i.p.) on cocaine-induced reinstatement (Figure 4b). Not only did this higher clonidine dose fail to block cocaine-induced

reinstatement, but it also tended to augment reinstatement in response to cocaine. Similar to the lower clonidine dose, two-way cocaine \times clonidine treatment condition ANOVA showed a significant overall effect of cocaine priming ($F_{1,12}=9.319$; $p=0.01$) on time spent in the cocaine compartment, with no main effect of clonidine treatment condition and no interaction between clonidine treatment and cocaine-induced reinstatement.

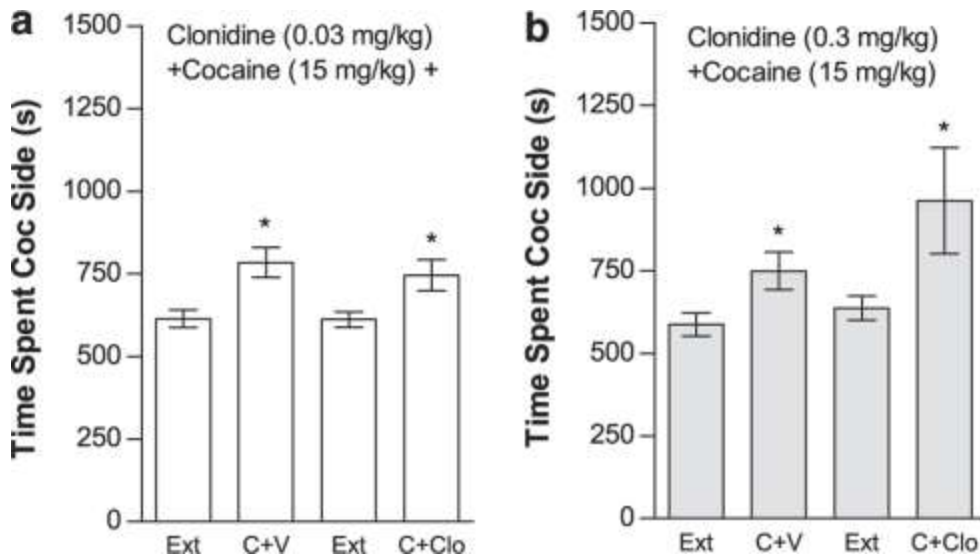


Figure 4 The α -2 AR agonist clonidine does not block cocaine-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment ($\text{sec} \pm \text{SE}$) during the final extinction session before reinstatement testing (Ext) and during reinstatement testing after administration of cocaine (C; 15 mg/kg, i.p.) after pretreatment with vehicle (V) or 0.03 mg/kg clonidine (i.p.) (Clo; $n=25$; a) or with V or 0.3 mg/kg Clo ($n=13$; b). In both cases, significant main effects of C ($*p<0.05$) but no significant C \times Clo interactions were found.

Yohimbine

The effects of clonidine on yohimbine-induced reinstatement are shown in Figure 5. As yohimbine likely reinstates extinguished cocaine seeking by blockade of α -2 ARs, we anticipated that yohimbine-induced reinstatement would be attenuated by clonidine. Surprisingly, neither the low (ie, 0.03 mg/kg) nor the high (ie, 0.3 mg/kg) clonidine dose blocked yohimbine-induced reinstatement. A total of 40 mice were tested for the effects of 0.03 mg/kg clonidine on yohimbine-induced reinstatement (Figure 5a). Two-way repeated-measures

ANOVA showed a significant overall effect of yohimbine on time spent in the cocaine compartment ($F_{1,39}=13.306$) but failed to show a significant main effect of 0.03 mg/kg clonidine treatment condition or a significant interaction between clonidine treatment and yohimbine-induced reinstatement. We reasoned that our inability to fully block yohimbine-induced reinstatement with 0.03 mg/kg clonidine may have been dose-related because of the competition for the same receptor site. However, when we tested mice with 0.3 mg/kg clonidine ($n=38$), there was little evidence for a reduction of yohimbine-induced reinstatement at this dose (Figure 5b). Two-way repeated-measures yohimbine-induced reinstatement \times 0.3 mg/kg clonidine treatment condition ANOVA showed a significant overall effect of yohimbine ($F_{1,37}=8.507$; $p<0.01$), but no overall effect of clonidine pretreatment and no yohimbine reinstatement \times clonidine pretreatment interaction.

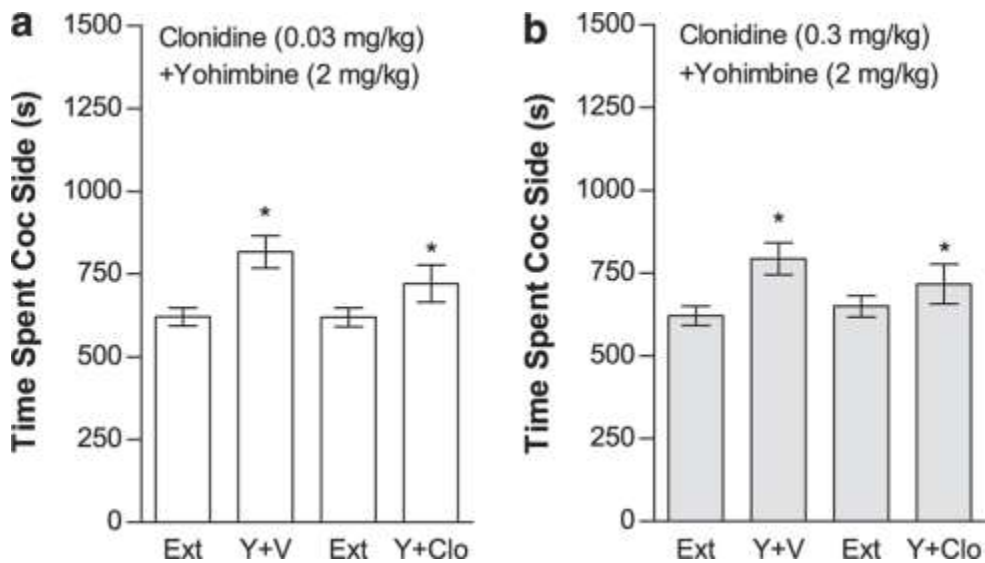


Figure 5 The α -2 AR agonist clonidine does not block yohimbine-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment ($\text{sec}\pm\text{SE}$) during the final extinction session prior to reinstatement testing (Ext) and during reinstatement testing after administration of yohimbine (Y; 2 mg/kg, i.p.) after pretreatment with vehicle (V) or 0.03 mg/kg clonidine (i.p.) (Clo; $n=40$; a) or with V or 0.3 mg/kg Clo ($n=38$; b). In both cases, significant main effects of Y (* $p<0.05$) but no significant Y \times Clo interactions were found.

Effects of Prazosin on Reinstatement

To examine the role of α -1 ARs in reinstatement by various stimuli, mice were pretreated with the α -1 AR antagonist prazosin (2 and 4 mg/kg) before FS or administration of cocaine or yohimbine. In all cases, prazosin failed to alter reinstatement.

Forced Swim

The effects of prazosin (1 or 2 mg/kg, i.p.) on FS-induced reinstatement are shown in Figure 6. Neither prazosin dose blocked reinstatement after FS. The effect of 1 mg/kg prazosin was tested in five mice (Figure 6a). Two-way repeated-measures FS \times 1 mg/kg prazosin treatment condition ANOVA showed a significant overall effect of FS ($F_{1,4}=12.251$; $p<0.05$) on time spent in the cocaine compartment. However, no significant main effect of prazosin treatment condition or interaction between prazosin and FS-induced reinstatement was found. In all 14 mice were tested for the effect of 2 mg/kg prazosin on FS-induced reinstatement (Figure 6b). Similar to the lower prazosin dose, two-way repeated-measures FS \times 2 mg/kg prazosin treatment condition ANOVA showed a significant overall effect of FS ($F_{1,13}=5.368$; $p<0.05$) on time spent in the cocaine compartment, with no main effect of prazosin treatment condition and no interaction between prazosin and FS.

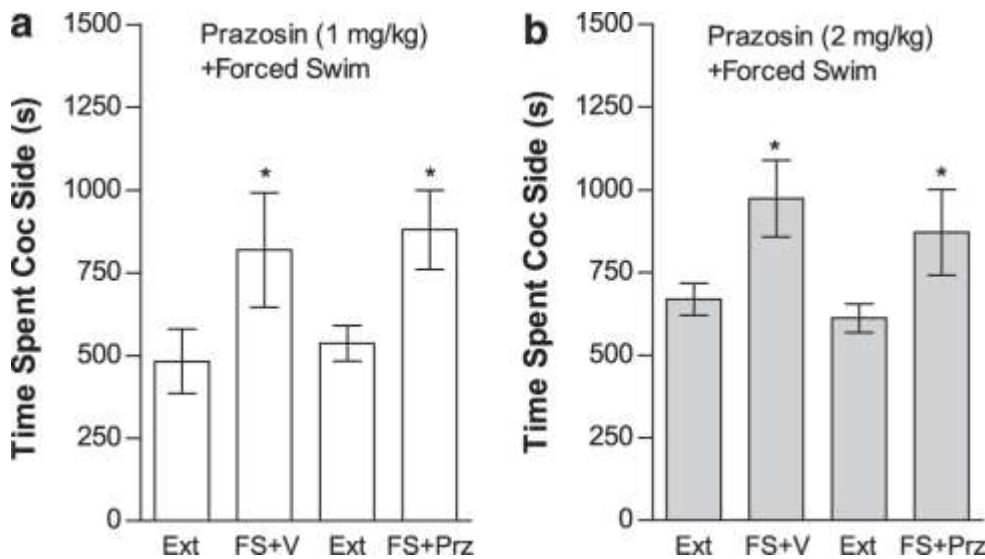


Figure 6 The selective α -1 AR antagonist prazosin does not block stress-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment (sec \pm SE) during the final extinction session prior to reinstatement testing (Ext) and during reinstatement testing after forced swim (FS) after pretreatment with vehicle (V) or 1 mg/kg (i.p.) prazosin (Prz; $n=5$; a) or pretreatment with V or 2 mg/kg (i.p.) Prz ($n=14$; b). In both cases, significant main effects of FS ($*p<0.05$) but no FS \times Prz interactions were found.

Cocaine

The effects of prazosin on cocaine-induced reinstatement are shown in Figure 7. Neither prazosin dose blocked cocaine-induced reinstatement. The effect of 1 mg/kg prazosin was tested in a total of 11 mice (Figure 7a). Two-way cocaine \times 1 mg/kg prazosin treatment condition ANOVA showed a significant overall effect of cocaine ($F_{1,10}=9.000$; $p<0.05$) on time spent in the cocaine compartment. However, no significant main effect of prazosin treatment condition or interaction between prazosin and cocaine-induced reinstatement was found. A total of 13 mice were tested for the effect of 2 mg/kg prazosin on cocaine-induced reinstatement (Figure 7b). Similar to the lower prazosin dose, two-way cocaine \times 2 mg/kg prazosin treatment condition ANOVA showed a significant overall effect of cocaine ($F_{1,12}=17.241$; $p<0.01$) on time spent in the cocaine compartment, with no main effect of prazosin treatment condition and no interaction between prazosin treatment and cocaine-induced reinstatement.

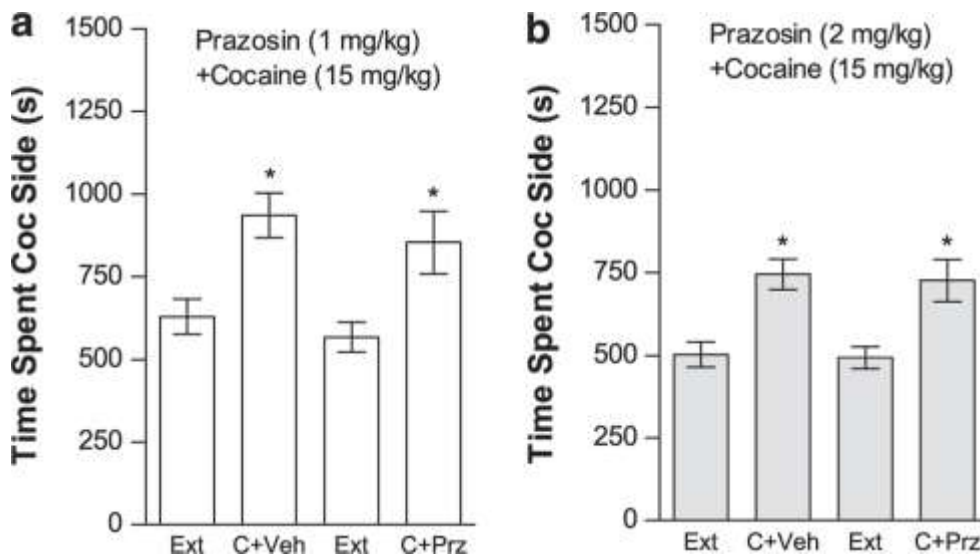


Figure 7 The selective α -1 AR antagonist prazosin does not block cocaine-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment ($\text{sec} \pm \text{SE}$) during the final extinction session before reinstatement testing (Ext) and during reinstatement testing after administration of cocaine (C; 15 mg/kg, i.p.) after pretreatment with vehicle (V) or 1 mg/kg (i.p.) prazosin (Prz; $n=11$; a) or pretreatment with V or 2 mg/kg (i.p.) Prz ($n=13$; b). In both cases, significant main effects of C ($*p < 0.05$) but no C \times Prz interactions were found.

Yohimbine

The effects of prazosin on cocaine-induced reinstatement are shown in Figure 8. Neither prazosin dose blocked yohimbine-induced reinstatement. The effect of 1 mg/kg prazosin was tested in six mice (Figure 8a). Two-way repeated measures yohimbine \times 1 mg/kg prazosin treatment condition ANOVA showed a significant overall effect of yohimbine ($F_{1,5}=13.114$; $p < 0.05$) on time spent in the cocaine compartment. However, no significant main effect of prazosin treatment condition or interaction between prazosin and yohimbine-induced reinstatement was found. In all 15 mice were tested for the effect of 2 mg/kg prazosin on yohimbine-induced reinstatement (Figure 8b). Similar to the lower prazosin dose, two-way repeated-measures yohimbine \times 2 mg/kg prazosin treatment condition ANOVA showed a significant overall effect of yohimbine ($F_{1,14}=11.558$; $p < 0.01$) on time spent in the cocaine compartment, with no main effect of prazosin treatment condition and no interaction between prazosin and yohimbine.

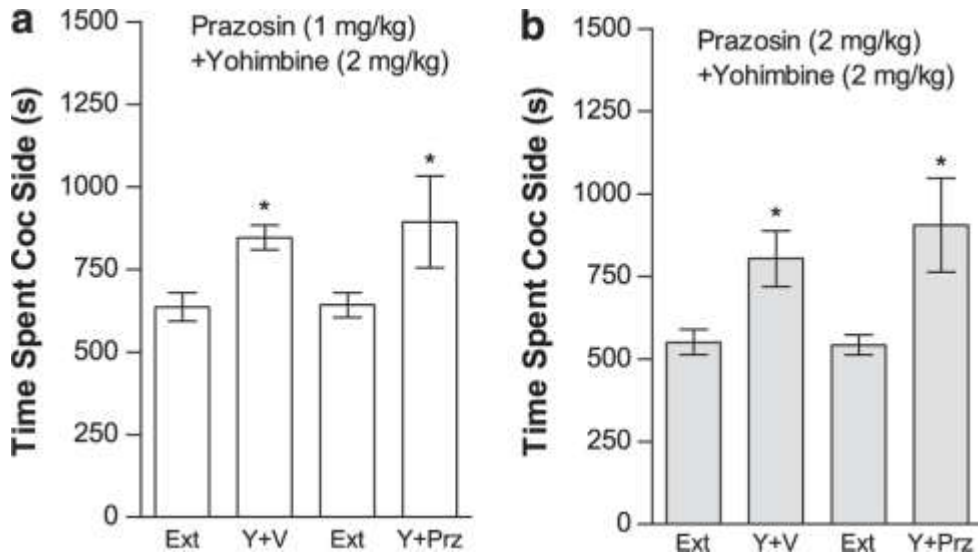


Figure 8

The selective α -1 AR antagonist prazosin does not block yohimbine-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment ($\text{sec} \pm \text{SE}$) during the final extinction session prior to reinstatement testing (Ext) and during reinstatement testing after administration of yohimbine (Y; 2 mg/kg, i.p.) after pretreatment with vehicle (V) or 1 mg/kg (i.p.) prazosin (Prz; $n=6$; a) or pretreatment with V or 2 mg/kg (i.p.) Prz ($n=15$; b). In both cases, significant main effects of Y ($*p < 0.05$) but no Y \times Prz interactions were found.

Effects of Propranolol on Reinstatement

To examine the role of β -ARs in reinstatement by various stimuli, mice were pretreated with the nonselective β -AR antagonist, propranolol, before FS or administration of cocaine or yohimbine. Propranolol blocked reinstatement by FS and yohimbine, but not cocaine. Further, FS-induced reinstatement was more sensitive to blockade by propranolol than yohimbine-induced reinstatement.

Forced Swim

The effects of propranolol (5 or 10 mg/kg, i.p.) on FS-induced reinstatement are shown in Figure 9. Both propranolol doses blocked FS-induced reinstatement. The effect of 5 mg/kg propranolol was tested in seven mice (Figure 9b). Two-way repeated-measures FS \times 5 mg/kg propranolol treatment condition ANOVA failed to show a significant overall effect of propranolol or FS on time spent in the

cocaine compartment. However, a significant propranolol \times FS interaction was observed ($F_{1,6}=8.598$; $p<0.05$). *Post-hoc* examination of reinstatement in each group revealed that FS significantly reinstated place preference in vehicle- but not 5 mg/kg propranolol-treated mice ($t_6=4.32$; $p<0.05$). In all 16 mice were tested for the effects of 10 mg/kg propranolol on FS-induced reinstatement (Figure 9b). Two-way repeated-measures FS \times 10 mg/kg propranolol treatment ANOVA showed a significant main effect of propranolol ($F_{1,15}=7.018$; $p<0.05$) but not FS and a significant interaction between 10 mg/kg propranolol treatment and FS ($F_{1,15}=5.116$; $p<0.05$). *Post-hoc* examination of reinstatement in each group revealed that FS significantly reinstated place preference in vehicle-treated but not 10 mg/kg propranolol-treated mice ($t_{15}=2.729$; $p<0.05$) and that time spent in the cocaine compartment was significantly reduced by propranolol after FS ($t_{15}=2.411$; $p<0.05$) but not under basal conditions.

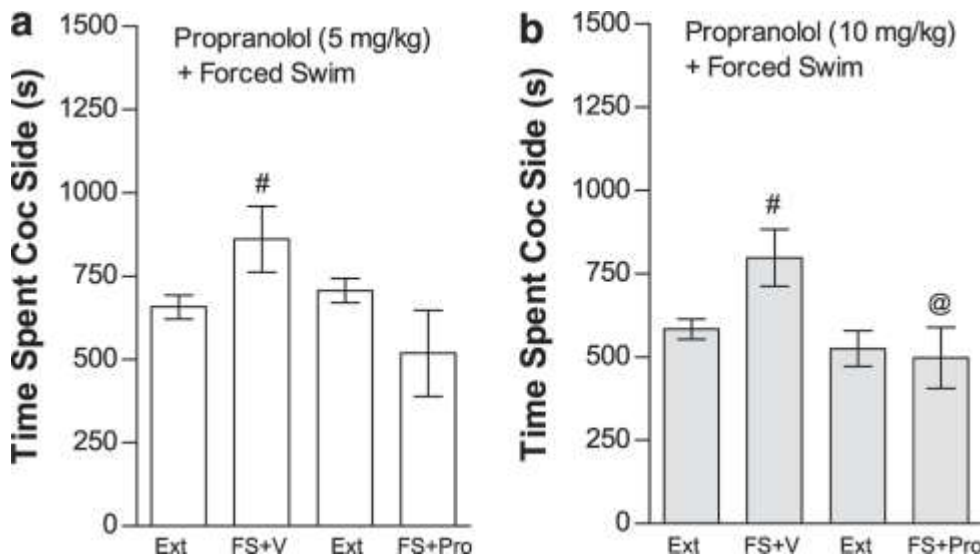


Figure 9 The nonselective β -AR antagonist propranolol blocks stress-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment (sec \pm SE) during the final extinction session previous reinstatement testing (Ext) and during reinstatement testing after forced swim (FS) after pretreatment with vehicle (V) or propranolol (Pro). Significant interactions between FS and Pro were found in mice tested at the 5 mg/kg (i.p.; a; $n=7$) and 10 mg/kg (i.p.; b; $n=16$) Pro doses ($p<0.05$). In each case, post-hoc testing showed that FS increased time spent in the Coc compartment after treatment with V (# $p<0.05$) but not Pro. Further, time spent in the Coc compartment was significantly reduced following Pro compared to V at the 10 mg/kg, but not 5 mg/kg, Pro dose (@ $p<0.05$).

Cocaine

The effects of propranolol (5 or 10 mg/kg, i.p.) on cocaine-induced reinstatement are shown in Figure 10. Neither propranolol dose blocked cocaine-induced reinstatement. The effect of 5 mg/kg propranolol was tested in nine mice (Figure 10a). Two-way cocaine \times 5 mg/kg propranolol treatment condition ANOVA showed a significant overall effect of cocaine ($F_{1,8}=9.731$; $p<0.05$) on time spent in the cocaine compartment. However, no significant main effect of propranolol treatment condition or interaction between propranolol and cocaine-induced reinstatement was found. In all 11 mice were tested for the effect of 10 mg/kg propranolol on cocaine-induced reinstatement (Figure 10b). Similar to the lower propranolol dose, two-way cocaine \times 10 mg/kg propranolol treatment condition ANOVA showed a significant overall effect of cocaine ($F_{1,10}=13.624$; $p<0.01$) on time spent in the cocaine compartment, with no main effect of propranolol treatment condition and no interaction between propranolol treatment and cocaine-induced reinstatement.

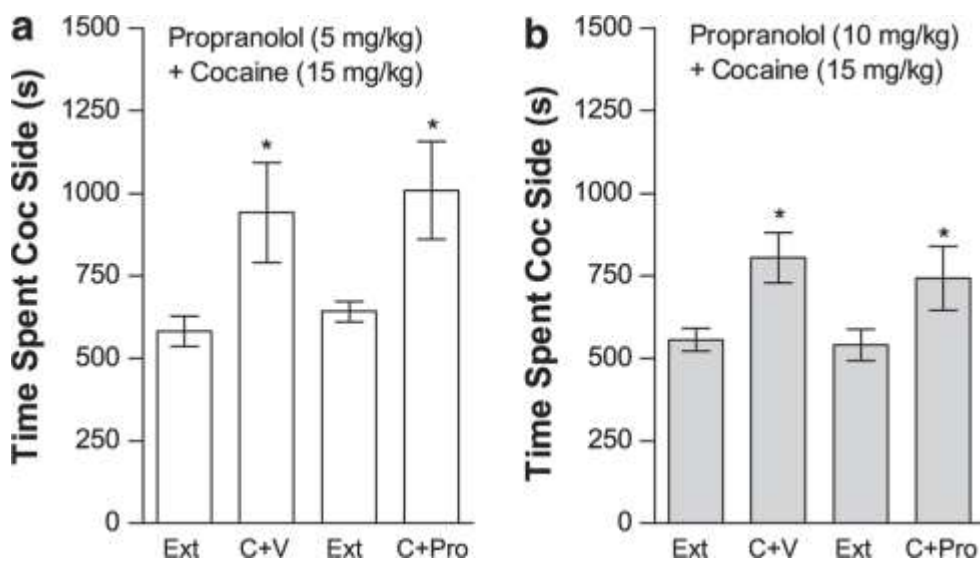


Figure 10 The non-selective β -AR antagonist propranolol does not block cocaine-induced reinstatement. Data represent the time spent in the cocaine (Coc) compartment (sec \pm SE) during the final extinction session before the reinstatement testing (Ext) and during reinstatement testing after administration of cocaine (C; 15 mg/kg, i.p.) following pretreatment with vehicle (V) or 5 mg/kg (i.p.) propranolol (Pro; $n=9$; a) or pretreatment with V or 10 mg/kg (i.p.) Pro ($n=11$; b). In both cases significant main effects of C ($*p<0.05$) but no C \times Pro interactions were found.

Yohimbine

The effects of propranolol (5 or 10 mg/kg, i.p.) on yohimbine-induced reinstatement are shown in Figure 11. Propranolol blocked yohimbine-induced reinstatement at the 10 mg/kg, but not at the 5 mg/kg dose. The effect of 5 mg/kg propranolol was tested in eight mice (Figure 11a). Two-way repeated-measures yohimbine \times 5 mg/kg propranolol treatment condition ANOVA showed a significant overall effect of yohimbine ($F_{1,7}=7.865$; $p<0.05$) on time spent in the cocaine compartment. However, no significant main effect of 5 mg/kg propranolol treatment condition or interaction between 5 mg/kg propranolol and yohimbine-induced reinstatement was found. The effect of 10 mg/kg propranolol on yohimbine-induced reinstatement was tested in 10 mice (Figure 11b). In contrast to the lower propranolol dose, 10 mg/kg propranolol prevented reinstatement after yohimbine administration. Two-way repeated-measures yohimbine reinstatement \times 10 mg/kg propranolol treatment condition ANOVA failed to show significant main effects of yohimbine or propranolol ($p=0.06$) However, a significant interaction between 10 mg/kg propranolol treatment \times yohimbine-induced reinstatement was found ($F_{1,9}=10.563$; $p=0.01$). *Post-hoc* analyses showed that yohimbine significantly increased the time spent in the cocaine compartment in vehicle-pretreated (paired *t*-test; two-tailed $t_9=2.334$; $p<0.05$) but not propranolol-pretreated mice, and that time spent in the cocaine compartment after yohimbine was significantly reduced in propranolol-pretreated mice compared with vehicle controls ($t_9=2.728$; $p<0.05$).

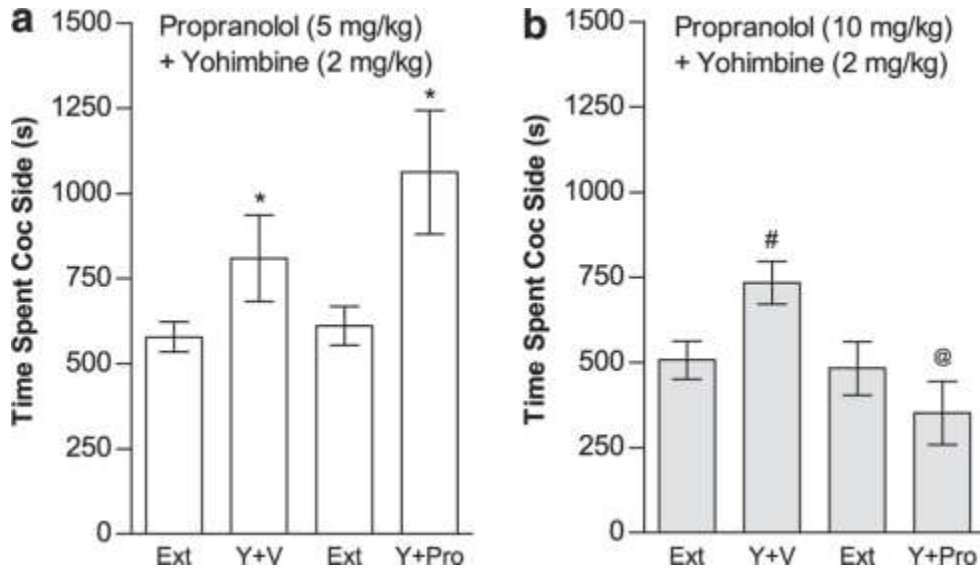


Figure 11 The nonselective β -AR antagonist propranolol dose-dependently blocks yohimbine-induced reinstatement. Data in (a) represent the time spent in the cocaine (Coc) compartment ($\text{sec} \pm \text{SE}$) during the final extinction session before the reinstatement testing (Ext) and during reinstatement testing after administration of yohimbine (Y; 2 mg/kg, i.p.) after pretreatment with vehicle (V) or 5 mg/kg (i.p.) propranolol (Pro; $n=8$). A significant main effect of Y ($*p<0.05$) but no Y \times Pro interaction was found at this dose. The effect of 10 mg/kg (i.p.) Pro ($n=10$) on Y-induced reinstatement is shown in (b). A significant interaction between Y and Pro was found at this dose ($p<0.05$). *Post-hoc* testing showed that Y increased time spent in the Coc compartment after treatment with V ($\#p<0.05$) but not ICI. Further, time spent in the Coc compartment was significantly reduced following 10 mg/kg Pro compared to V ($@p<0.05$).

Effects of Betaxolol and ICI 118,551 on FS-Induced Reinstatement

As FS-induced reinstatement seemed to involve β -AR but not α -1 ARs, mice were tested with the β -1 AR selective receptor antagonist, betaxolol (10 mg/kg, i.p.), or the β -2 AR selective antagonist, ICI-118551 (5 mg/kg, i.p.), in order to determine the role of β receptor subtypes in reinstatement by FS (Figure 12). ICI-118551 (Figure 12b) but not betaxolol (Figure 12a) blocked FS-induced reinstatement. The effect of betaxolol was tested in 11 mice. Two-way repeated-measures ANOVA showed a significant main effect of FS on time spent in the cocaine compartment ($F_{1,10}=9.762$; $p<0.05$) but no effect of betaxolol or FS \times betaxolol reinstatement. In all, 11 mice were tested for the

effect of ICI-118551 on FS-induced reinstatement. Two-way repeated-measures ANOVA showed no main effects of ICI-118551 or FS on time spent in the cocaine compartment. However, a significant ICI-118551 \times FS interaction was observed ($F_{1,10}=9.364$; $p<0.05$). *Post-hoc* analysis showed that FS produced significant reinstatement in vehicle, but not ICI-118551, treated mice ($t_{10}=2.874$; $p<0.05$).

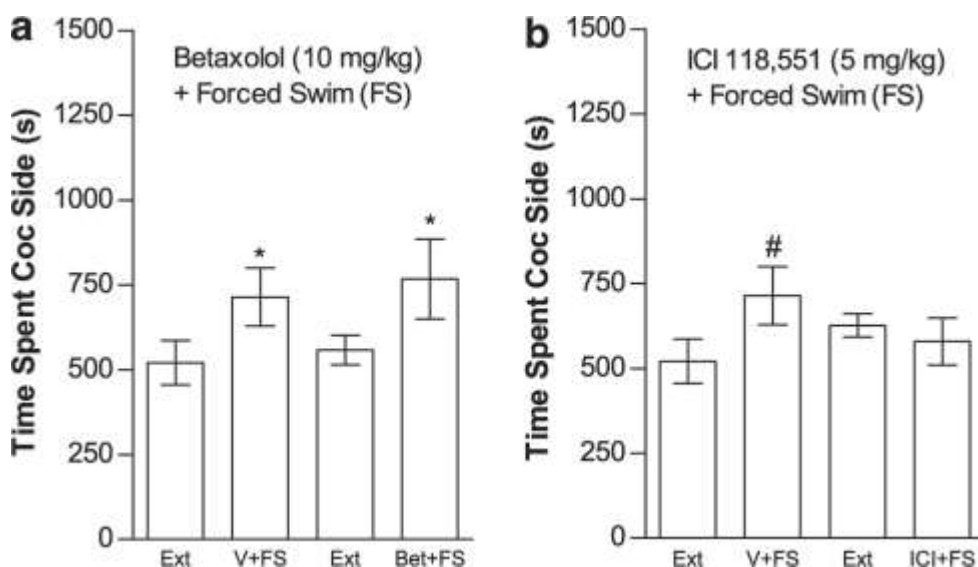


Figure 12 The β -2 AR selective antagonist ICI-118551 but not the β -1 AR selective antagonist betaxolol blocks stress-induced reinstatement. Data in (a) represent the time spent in the cocaine (Coc) compartment (sec \pm SE) during the final extinction session prior to reinstatement testing (Ext) and during reinstatement testing after forced swim (FS) following pretreatment with vehicle (V) or betaxolol (Bet; 10 mg/kg, i.p.; $n=11$). A significant main effect of FS ($*p<0.05$) but no FS \times Bet interaction was found. The effect of ICI-118551 (ICI; 1 mg/kg, i.p.; $n=11$) on FS-induced reinstatement is shown in (b). A significant interaction between FS and ICI was found ($p<0.05$). *Post-hoc* testing showed that FS increased time spent in the cocaine compartment after treatment with V ($\#p<0.05$) but not ICI.

Discussion

Drug re-exposure and stress are commonly cited as precipitating factors for relapse in recovering cocaine addicts. Although both stimuli can trigger drug use, they do not necessarily do so through activation of completely overlapping neurobiological pathways. The present study poses the noradrenergic system as a likely mediator of relapse in response to stressors but not cocaine. First, activation of

central noradrenergic systems through disinhibition of noradrenergic signaling by administration of the α -2 AR antagonist yohimbine or the more selective α -2A AR antagonist BRL 44408 (Dwyer *et al*, 2010) is sufficient to induce reinstatement. Second, suppression of noradrenergic neurotransmission by the administration of the α -2 AR agonist clonidine suppresses reinstatement by FS but not cocaine. Third, reinstatement by FS and yohimbine, but not cocaine, is blocked by the nonselective β -AR antagonist propranolol but not by the α -1 AR antagonist, prazosin. In the case of FS, reinstatement is prevented by the selective β -2 AR antagonist ICI-118551, but not the selective β -1 AR antagonist, betaxolol. Altogether, these findings suggest that elevated norepinephrine (NE) during periods of stress, but not after cocaine re-exposure, likely induces drug seeking through activation of β -2 ARs as measured in this mouse model of relapse.

The apparent role for NE in stressor-induced relapse is also supported by previous studies. Icv NE administration reinstates cocaine seeking after SA (Brown *et al*, 2009), as does disinhibition of noradrenergic signaling through α -2 adrenergic autoreceptor blockade (Lee *et al*, 2004; Feltenstein and See, 2006; Fletcher *et al*, 2008; Brown *et al*, 2009) and, in some cases, blockade of NE reuptake (Platt *et al*, 2007, but see Schmidt and Pierce, 2006). Conversely, suppression of stressor-induced NE release through administration of α -2 AR agonists such as clonidine (Tjurmina *et al*, 1999) attenuates stress-induced drug seeking after SA of cocaine (Erb *et al*, 2000), heroin (Shaham *et al*, 2000a, 2000b), alcohol (Le *et al*, 2005; Dzung Lê *et al*, 2009), or a heroin-cocaine mixture (Highfield *et al*, 2001) in rats, stress-induced reinstatement of morphine-induced place preference in rats (Wang *et al*, 2001) and drug craving induced by stress imagery in heroin-dependent human subjects (Sinha *et al*, 2007).

Although stress-induced reinstatement was blocked using propranolol and clonidine, these pretreatments failed to alter reinstatement in response to cocaine, even though clonidine has been shown to prevent cocaine-induced increases in brain extracellular NE levels (Florin *et al*, 1994). Considering that cocaine, like other NET blockers that have been shown to induce reinstatement (Platt *et al*,

2007), markedly elevates central NE levels (Reith *et al*, 1997; Li *et al*, 1996), and that elevation of central NE is sufficient to induce reinstatement (Brown *et al*, 2009), our findings suggest that cocaine-responsive processes activated either concurrently with or downstream from noradrenergic systems are sufficient for relapse in the absence of a NE response. This assertion is consistent with a number of reports suggesting that many neurobiological systems underlying stress-induced cocaine seeking (eg, CRF and κ -opioid) do not completely overlap with those underlying reinstatement in response to cocaine delivery (Erb *et al*, 1998; Shaham *et al*, 1998; Redila and Chavkin, 2008) and is supported by studies showing that suppression of noradrenergic neurotransmission through α -2 AR activation (Erb *et al*, 2000) or antagonism of central β -ARs (Leri *et al*, 2002) blocks stress- but not cocaine-induced reinstatement in rats, as well as by findings that mixed α - and β -adrenergic receptor antagonist drugs do not attenuate the subjective responses to smoked cocaine, despite reducing peripheral adrenergic responses (Sofuoglu *et al*, 2000a, 2000b). However, it should be noted that other studies have indicated that noradrenergic neurotransmission may be involved in cocaine-induced reinstatement in monkeys (Platt *et al*, 2007) and rats (Zhang and Kosten, 2005).

Our finding that propranolol blocks stressor-induced reinstatement is supported by those of Leri *et al* (2002), who found that administration of a combination of β -1 and β -2 AR antagonists directly into brain regions implicated in stressor-induced cocaine seeking blocks footshock-induced reinstatement after self-administration in rats. Propranolol has also been found to block reinstatement resulting from NET blockade after self-administration in monkeys (Platt *et al*, 2007). Further, as β -AR are G_s G-protein coupled receptors, the findings are consistent with a report that forced swim- but not cocaine-induced reinstatement of conditioned place preference is dependent on cAMP and CREB activation in mice (Kreibich and Blendy, 2004). Interestingly, β -blockers have been reported to attenuate anxiety-like effects resulting from cocaine delivery (Schank *et al*, 2008; elevated plus maze) or cocaine withdrawal (Harris and Aston-Jones, 1993; defensive burying). Thus, to the extent that these anxiogenic effects contribute to drug craving and relapse during

withdrawal and/or continued cocaine use, our findings provide further evidence of potential utility of β -blockers for the management of cocaine addiction. Despite these findings, clinical trials examining the ability of propranolol to promote drug abstinence in cocaine-dependent individuals have found only modest improvement (Kampman *et al*, 2001, 2006) potentially because of the inability of propranolol to attenuate cocaine-induced craving and rewarding effects. Consistent with our own findings, it has been reported that propranolol fails to decrease cocaine-induced reinstatement in rats (Leri *et al*, 2002) and monkeys (Platt *et al*, 2007) and that drugs with β -AR antagonist properties fail to alter cocaine's subjective effects (Sofuoglu *et al*, 2000a, 2000b). In fact, it has been reported that propranolol can actually augment cocaine's locomotor (Harris *et al*, 1996 but see Vanderschuren *et al*, 2003), discriminative stimulus (Kleven and Koek, 1997; but see Spealman, 1995), and reinforcing effects (Harris *et al*, 1996). It may be that, because of the selective attenuation of stress-induced cocaine seeking, targeting β -receptors may be more useful as an adjunct approach and/or only in subpopulations of cocaine-dependent individuals whose use is stress driven. In support, Kampman *et al* (2001, 2006) found that propranolol significantly increased abstinence time only in cocaine-dependent individuals experiencing pronounced withdrawal symptoms, which for cocaine dependence include intense anxiety (Gawin and Kleber, 1986).

In the present study, stressor-induced reinstatement seemed to involve β -2 AR, presumably within the CNS, but not β -1 AR. FS-induced reinstatement was blocked by the selective β -2 AR antagonist ICI-118551 but not the β -1 AR antagonist betaxolol. This finding was somewhat surprising, considering that it has been previously reported that betaxolol blocked anxiety-like behavior during withdrawal from chronic cocaine administration (Rudoy and Van Bockstaele, 2007). The role of β -2 AR in stress- and anxiety-related processes is unclear. However, β -2 AR are found throughout the rat brain, including in the BNST (Rainbow *et al*, 1985) and the amygdala (Tiong and Richardson, 1990), sites in which nonselective antagonism of β -AR has been shown to attenuate stressor-induced cocaine seeking (Leri *et al*, 2002) and in which β -2 AR have been implicated in affective, but not sensory or somatic, elements of pain (Deyama *et al*, 2008) or opiate withdrawal

(Watanabe *et al*, 2003). Confirmation that central but not peripheral β -2 AR activation is required for stress-induced reinstatement will require further investigation.

In contrast to propranolol, the α -1 AR antagonist prazosin (1 or 2 mg/kg) failed to alter reinstatement by any of the stimuli tested. The finding that prazosin did not alter cocaine-induced reinstatement was somewhat surprising considering that prazosin has been reported to attenuate cocaine-induced reinstatement after self-administration in rats (Zhang and Kosten, 2005) but not monkeys (Platt *et al*, 2007). This disparity in prazosin effects on cocaine-evoked reinstatement across studies could be attributable to a number of factors, including species, general approach/experimental parameters, and dose. A role for α -1 ARs in a number of cocaine effects has been identified, including cocaine's reinforcing (Drouin *et al*, 2002; Wee *et al*, 2008 but see Woolverton, 1987), sensitizing (Jiménez-Rivera *et al*, 2006; Zhang and Kosten, 2007), locomotor stimulating (Drouin *et al*, 2002; Snoddy and Tessel, 1985 but see Vanderschuren *et al*, 2003), and discriminative stimulus (Spealman, 1995) effects.

Our finding that yohimbine administration reinstates extinguished place preference in mice is consistent with previous reports that yohimbine reinstates extinguished cocaine seeking after SA in rats (Feltenstein and See, 2006; Fletcher *et al*, 2008; Brown *et al*, 2009) and monkeys (Lee *et al*, 2004) and impairs the extinction of cocaine-induced conditioned place preference in mice (Davis *et al*, 2008). As we were able to block yohimbine-induced reinstatement with propranolol, we presumed that yohimbine's effects on cocaine seeking were attributable to disinhibition of noradrenergic activity resulting from antagonism of α -2 adrenergic autoreceptors. Consistent with this, it has been reported that the highly selective α -2 adrenergic receptor antagonist RS-79948 can induce reinstatement in monkeys (Lee *et al*, 2004) and that RS-79948- and yohimbine-induced reinstatement are blocked by the α -2 adrenergic receptor agonist, clonidine (Lee *et al*, 2004). A similar clonidine-inhibited β -AR-dependent mechanism has been reported to be involved in the reinstatement of extinguished cocaine seeking by the selective NET inhibitor, nisoxetine in squirrel monkeys (Platt *et al*, 2007).

Surprisingly however, in the present study, clonidine failed to block yohimbine-induced reinstatement. These findings are consistent with a recent report that clonidine failed to prevent yohimbine-induced reinstatement of cocaine seeking after SA in rats (Brown *et al*, 2009).

It is unclear why clonidine is ineffective in blocking yohimbine-induced reinstatement of cocaine seeking. However, there are two possibilities. First, yohimbine could be inducing reinstatement through a non- α -2 AR mechanism. In support, Davis *et al* (2008) reported that the impairment of extinction of cocaine-induced conditioned place preference and suppression of glutamate-induced electrophysiological responses in brain slices containing the bed nucleus of the stria terminalis by yohimbine were unaltered in α -2A adrenergic receptor deficient mice and were not mimicked by the more selective α -2A AR antagonist, atipamezole. Yohimbine has been reported to function as an agonist at 5-HT_{1A} receptors (Winter and Rabin, 1992; Millan *et al*, 2000). This 5-HT_{1A} receptor agonist effect may be relevant to drug seeking, as yohimbine-induced reinstatement of extinguished alcohol seeking in rats is attenuated by the 5-HT_{1A} receptor antagonist, WAY 100635, as well as clonidine, but is minimally reduced by lesions of ascending noradrenergic systems (Dzung Lê *et al*, 2009). To further examine the role for α -2 AR antagonism in reinstatement, we also tested mice with the selective α -2A antagonist BRL 44408, a compound that has a 50-fold higher affinity for α -2A AR relative to 5-HT-1A receptors (Dwyer *et al*, 2010). Like yohimbine, this more selective α -2A AR antagonist also produced robust reinstatement, suggesting that α -2 AR antagonism alone is likely sufficient for reinstatement. Notably, if indeed yohimbine is working through a mechanism that is independent of α -2 AR, our data suggest that this mechanism still involves β -AR.

Secondly, clonidine itself is somewhat nonselective. Lower clonidine doses likely primarily target presynaptic α -2 ARs, thereby competitively reversing yohimbine effects and generally suppressing noradrenergic neurotransmission through α -2 AR-mediated auto-inhibitory actions. Accordingly, at the lower (ie, 0.03 mg/kg) dose, clonidine prevented reinstatement by FS. In addition to attenuating NE release, higher clonidine doses also activate lower affinity postsynaptic

α -2 ARs and/or bind to imidazoline I1 receptor sites (Bricca *et al*, 1989; Eglen *et al*, 1998), thus mimicking components of the noradrenergic response despite indirectly reducing activation of α -1 and β -AR or producing nonnoradrenergic effects. On the basis of our finding that the higher (ie, 0.3 mg/kg) clonidine dose not only failed to attenuate yohimbine and FS-induced reinstatement but induced reinstatement on its own, we hypothesize that, similar to β -AR, these high-dose targets may contribute to reinstatement during stress. This finding is consistent with another report that high doses of clonidine can induce conditioned place preference in rats (Cervo *et al*, 1993). Future studies using putative imidazoline receptor ligands as well as other α -2 AR agonists with relatively low affinity for imidazoline receptors (eg, guanabenz) are needed to determine the relative contribution of these receptor populations to reinstatement. Overall, this dose-dependent regulation of differential receptor populations with varying effects on drug seeking, along with the tendency of yohimbine to interact with multiple receptor targets, likely contributed to the observed variation in clonidine's ability to block yohimbine-induced reinstatement in this study and others.

NE-dependent stress-induced reinstatement seems to involve projections from the lateral tegmental region of the brainstem to the bed nucleus of the stria terminalis (BNST) and/or the central nucleus of the amygdala (CeA) by the ventral noradrenergic bundle and activation β -ARs. In rats, stress- but not drug-induced reinstatement is attenuated by administration of clonidine into the lateral tegmentum, but not the LC (Shaham *et al*, 2000a, 2000b; heroin) or into the BNST (Wang *et al*, 2001; cocaine) or by delivery of a combination of β -1 and β -2 adrenergic receptor antagonists into the BNST or CeA (Leri *et al*, 2002; cocaine). It has been postulated that NE released into these regions regulates the release of corticotropin releasing factor (CRF) which in turn can indirectly (by actions in the BNST; Erb *et al*, 2001) or directly (by actions in the ventral tegmental area; Wang *et al*, 2005) activate mesocorticolimbic activity, thus producing drug-seeking behavior. The apparent lack of involvement of CRF (Shaham *et al*, 1998; Erb *et al*, 1998) and NE (Erb *et al*, 2000, Leri *et al*, 2002, and present findings; but see Platt *et al*, 2007, Zhang and Kosten, 2005) in cocaine-induced reinstatement suggests that despite convergence

within the mesocorticolimbic system, the neurobiological pathways underlying drug and stress induced relapse are largely distinct.

β -AR, specifically β -2 AR, have also been implicated in the post-retrieval reconsolidation of cocaine-induced conditioned place preference in mice. Systemic propranolol (Bernardi *et al*, 2006; Fricks-Gleason and Marshall, 2008) and intrabasolateral amygdalar ICI-118551 (Bernardi *et al*, 2009) injections interfere specifically with the reconsolidation of conditioned place preference after retrieval without enhancing extinction learning. As, in our study, mice did not receive propranolol or ICI-118551 until after place preference had already been extinguished and as drug administration occurred immediately before to exposure of the reinstating stimuli, it is unlikely that effects on reconsolidation contributed to the observed attenuation of reinstatement. Nonetheless, the potential ability of β -2 AR antagonists to attenuate stress-evoked relapse and interfere with reconsolidation of drug-associated memories, establishes β -2 ARs as a target of interest for the treatment of addiction.

In summary, these findings further suggest that central noradrenergic systems contribute to drug-seeking behavior during times of stress. Considering that repeated cocaine exposure is associated with progressive changes in brain noradrenergic systems (Belej *et al*, 1996; Macey *et al*, 2003; Baumann *et al*, 2004; Beveridge *et al*, 2005; Horne *et al*, 2008; Lanteri *et al*, 2008) and that early abstinence from cocaine in dependent individuals is associated with an exaggerated noradrenergic response (McDougle *et al*, 1994), targeting noradrenergic neurotransmission, particularly β -2 AR, may be particularly beneficial for the management of relapse in cocaine-dependent individuals whose drug use is stress-related.

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