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# Yohimbine Depresses Excitatory Transmission in BNST and Impairs Extinction of Cocaine Place Preference Through Orexin-Dependent, Norepinephrine-Independent Processes

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The alpha2 adrenergic receptor ($\alpha_2$-AR) antagonist yohimbine is a widely used tool for the study of anxiogenesis and stress-induced drug-seeking behavior. We previously demonstrated that yohimbine paradoxically depresses excitatory transmission in the bed nucleus of the stria terminalis (BNST), a region critical to the integration of stress and reward pathways, and produces an impairment of extinction of cocaine-conditioned place preference (cocaine-CPP) independent of $\alpha_2$-AR signaling. Recent studies show yohimbine-induced drug-seeking behavior is attenuated by orexin receptor 1 ($OX_1R$) antagonists. Moreover, yohimbine-induced cocaine-seeking behavior is BNST-dependent. Here, we investigated yohimbine-orexin interactions. Our results demonstrate yohimbine-induced depression of excitatory transmission in the BNST is unaffected by alpha1-AR and corticotropin-releasing factor receptor-1 ($CRFR_1$) antagonists, but is (1) blocked by OxR antagonists and (2) absent in brain slices from orexin knockout mice. Although the actions of yohimbine were not mimicked by the norepinephrine transporter blocker reboxetine, they were by exogenously applied orexin A. We find that, as with yohimbine, orexin A depression of excitatory transmission in BNST is $OX_1R$–dependent. Finally, we find these *ex vivo* effects are paralleled *in vivo*, as yohimbine-induced impairment of cocaine-CPP extinction is blocked by a systemically administered $OX_1R$ antagonist. These data highlight a new mechanism for orexin on excitatory anxiety circuits and demonstrate that some of the actions of yohimbine may be directly dependent upon orexin signaling and independent of norepinephrine and CRF in the BNST.
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## INTRODUCTION

The widely used pharmacological stressor yohimbine induces relapse to drug- and alcohol-seeking behavior (Shaham *et al*, 2000; Shalev *et al*, 2010). In abstinent drug-dependent subjects, yohimbine increases anxiety (McDougle *et al*, 1994) and drug craving (Stine *et al*, 2002). Yohimbine is thought to act primarily via its inhibition of presynaptic noradrenergic alpha2 adrenergic receptor ($\alpha_2$-ARs), thereby increasing norepinephrine levels (Abercrombie *et al*, 1988; Aghajanian and VanderMaelen, 1982; Starke, 2001). In addition to research using yohimbine as a pharmacological stressor to induce illicit drug- and alcohol-reinstatement (Shaham *et al*, 2000; Shalev *et al*, 2010), recent studies demonstrate yohimbine impairs extinction of cocaine-conditioned place preference (cocaine-CPP) and self-administration (Davis *et al*, 2008; Kupferschmidt *et al*, 2009; Marinelli *et al*, 2007).

The bed nucleus of the stria terminalis (BNST), a key site for the integration of stress and reward processing, is a critical region mediating the effects of stressors on cocaine-seeking behavior (Buffalari and See, 2010; Erb *et al*, 2001; Leri *et al*, 2002). In particular, the BNST is critical for yohimbine-induced reinstatement of drug-seeking behavior (Brown *et al*, 2009; Buffalari and See, 2010). Yohimbine depresses

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excitatory transmission in the adult dorsal BNST (Davis *et al*, 2008). This finding is paradoxical given that $\alpha_2$-AR agonists produce similar actions (Davis *et al*, 2008; Shields *et al*, 2009). Further, the effects of yohimbine on both excitatory transmission and extinction of cocaine-CPP are intact in $\alpha_{2A}$-AR knockout mice and not fully mimicked by the $\alpha_2$-AR antagonist atipamezole, suggesting that they are mediated by targets other than $\alpha_2$-ARs (Davis *et al*, 2008).

Yohimbine-induced reinstatement of cocaine-seeking behavior requires orexin 1 receptor ($OX_1R$) activity (Buffalari and See, 2010; Zhou *et al*, 2011). The orexin system was first described in the late 1990s, when the orexin neuropeptides were shown to be important in feeding and arousal-related behaviors (de Lecea *et al*, 1998; Sakurai *et al*, 1998). Since then, studies have demonstrated an important role for the orexin system in anxiety and reward-related behaviors (Boutrel *et al*, 2005; Dayas *et al*, 2008; Espana *et al*, 2010; Harris *et al*, 2005; Johnson *et al*, 2010; Lawrence *et al*, 2006; Richards *et al*, 2008; Zhou *et al*, 2011). The BNST receives dense orexinergic inputs (Baldo *et al*, 2003) and has a high density of $OX_1R$ mRNA and protein expression (Hervieu *et al*, 2001; Marcus *et al*, 2001). Further, anxiety-like behaviors in rodents are dependent on BNST $OX_1R$ signaling (Johnson *et al*, 2010).

Here, we investigated the role of OxRs in the actions of yohimbine on excitatory transmission in the BNST, and on extinction of cocaine-CPP. These studies reveal that yohimbine recruits an Ox1R-dependent depression of excitatory transmission in BNST, and that a systemically administered $OX_1R$ antagonist blocks yohimbine-mediated impairment of extinction of cocaine-CPP. Taken together, our results suggest yohimbine acts in a norephinephrine-independent manner to recruit orexin signaling and impact excitatory transmission in the adult BNST, demonstrating a novel role for orexin A as an inhibitory neuropeptide on excitatory input in anxiety and reward circuits. Finally, we identify a role for orexin in mediating the effects of the stressor yohimbine on cocaine extinction.

## SUBJECTS AND METHODS

### Subjects

Experiments on C57BL/6J mice were conducted using males aged 8–12 weeks obtained from the Jackson Laboratory (Bar Harbor, ME). Male $\alpha_{2A}$-AR KO mice were generated as previously described (Altman *et al*, 1999) and backcrossed onto a C57BL/6J genetic background for a minimum of eight generations. Mice were housed on a 12-h light/dark cycle in groups of two to five with *ad libitum* access to food and water. Testing commenced at least 1 week after acclimation to the facilities. All procedures were approved by the Vanderbilt University Animal Care and Use Committees and were in accordance with the Animal Welfare Act. The number of mice used is reported in the figure legends.

### Mouse Drug Treatment

All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg body weight. For the cocaine-CPP experiments: vehicle (0.9% sterile saline) and cocaine (20 mg/kg; i.p.) in vehicle were administered immediately before placement in the CPP apparatus.

For the cocaine-CPP extinction experiments: yohimbine in distilled $H_2O$ (5 mg/kg; i.p.; 30 min prior) or vehicle (30 min prior) or SB-334867 (15 mg/kg; i.p. 1 h prior) in 1% (w/v) (2-hyroxypropyl)-$\beta$-cyclodextrin/10% dimethyl sulfoxide (DMSO) in sterile water (termed DMSO-vehicle; 1 h prior) were administered for extinction sessions 1–5. SB-334867 has been found to reach peak plasma and brain concentrations at 30 min post injection and maintains good exposure for up to 4 h (Porter *et al*, 2001). The dose of 5 mg/kg yohimbine was selected based on previous reports that this dose increases plasma corticosterone levels (Banihashemi and Rinaman, 2006). Moreover, 5 mg/kg but not 1 mg/kg yohimbine induces Fos expression in the lateral BNST (Myers *et al*, 2005). Finally, other studies in C57Bl/6J mice (Cain *et al*, 2004; Hefner *et al*, 2008), as well as a previous study from our lab (Davis *et al*, 2008) have found the 5 mg/kg dose effective at altering fear and anxiety-related behaviors.

### Cocaine-CPP and Extinction

The CPP and extinction design used here was described in detail previously (Davis *et al*, 2008). Briefly, the two chamber place preference apparatus consists of a box insert that slides into the open-field chamber (Med Associates; St Albans, VT). The paradigm consisted of a pre-test (day 1) and post-test (day 8) in which mice were given an intraperitoneal, (i.p.) saline injection and allowed unrestricted access to the contextually distinct mesh and bar-floor compartments. During conditioning, mice were given an i.p. saline injection and restricted to the mesh-floor compartment (days 2, 4, and 6) and an i.p. cocaine injection (20 mg/kg) and restricted to the bar-floor compartment (days 3, 5, and 7). All sessions were for 15 min.

The day following the post-test, extinction sessions began. Extinction sessions were the same as the preference session (access to both compartments) and were placed in one of the following four groups (time injection given before extinction session): SB-334867 (1 h)–vehicle (30 min), DMSO-vehicle (1 h)–yohimbine (30 min), DMSO-vehicle (1 h)–vehicle (30 min), and SB-334867 (1 h)–yohimbine (30 min). Immediately before each session, all mice were given an i.p. saline injection. Time spent in each compartment and the distance traveled was monitored throughout every session.

The data are presented as percent (%) of pre-test preference in order to convey the magnitude of the change in preference more clearly and for statistical analysis purposes among many groups.

### Calcium Mobilization Assay

Human Embryonic Kidney (HEK-EM4) cells expressing the $OX_1R$ were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 20 mM HEPES (pH 7.3), 2 mM L-glutamine, 1 × antibiotic/antimycotic, and 100 μg/ml Zeocin. HEK-293 cell lines co-expressing rat $mGluR_2$ and G-protein-coupled inwardly rectifying potassium (GIRK) channels (for additional

information, see Jin *et al*, 2010; Niswender *et al*, 2008) were grown in growth media containing 45% DMEM, 45% Ham's F12, 10% FBS, 20 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 2 mM L-glutamine, 1 × antibiotic/antimycotic, 1 × non-essential amino acids, 700 μg/ml G418 (Mediatech, Inc., Herndon, VA), and 0.6 μg/ml puromycin dihydrochloride (Sigma-Aldrich, St Louis, MO) at 37 °C in the presence of 5% $CO_2$. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). The cells were plated in non-coated (HEK-EM4) or poly-D-lysine-pretreated (HEK-293) 96-well black-walled, clear bottom plates (Corning Life Sciences, Lowell, MA) at ~45 000 cells/well in growth media. Twenty-four hours later, 50 μl of calcium assay buffer (Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA), 20 mM HEPES, 2.5 mM probenecid (Sigma, St Louis, MO), pH 7.4) containing 2 μM Fluo-4 (Invitrogen) was added into each well and the cells were incubated for 1 h at room temperature in the dark. The cells were washed with assay buffer and then incubated in with either 80 μl (Figure 4a and c) or 40 μl (Figure 4b) of assay buffer for 10 min at room temperature in the dark. Compounds were dissolved in the assay buffer, and the responses were recorded on a FLEXstation benchtop scanning fluorometer (Molecular Devices, Sunnyvale, CA) at room temperature, with the settings of 485 nm for excitation and 525 nm for emission. For Figures 6a and c, 20 μl of a 5 × compound was added at the 20-s time point and the fluorescence was monitored for a total of 240 s. For Figure 6b, 40 μl of 2 × SB-334867 was added at the 20 s time point, followed by addition of 5 × compound at the 180 s time point and the fluorescence was monitored for a total of 240 s. Maximal responses were observed with ~1 μM orexin A in orexin 1 receptor expressing cells and with 100 μM ATP in $mGluR_2$-expressing cells. Thus, in each experiment the results are expressed as a percentage of the response obtained with 1 μM orexin A, or EC80 orexin A (300 nM) or 100 μM ATP and are described as percentage of activation. Prism software (GraphPad Software Inc., San Diego, CA) was used to plot fluorescence intensities and to calculate the EC80 values.

## *Ex Vivo* BNST Electrophysiology

*Brain slice preparation.* Brain slices from the dorsal anterolateral (dlBNST) were prepared as previously described (Egli *et al*, 2005; Weitlauf *et al*, 2004). Briefly, male C57Bl6/j mice (8–12 weeks old, Jackson Laboratories) were retrieved from the colony and allowed to rest in sound attenuating boxes for a minimum of 1 h, after which they were decapitated under anesthesia (Isoflurane). The brains were quickly removed and placed in ice-cold artificial cerebro-spinal fluid (ACSF) (in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 $NaH_2PO_4$, 10 glucose, and 26 $NaHCO_3$). Slices of 300 μM thickness were prepared using a vibratome (Leica). BNST slices containing anterior portions of BNST (bregma 0.26 mm to 0.02 mm) (Franklin and Paxinos, 1997) were identified using the internal capsule, anterior commissure, fornix, and stria terminalis as landmarks. Following slicing, hemisected slices were placed in an interface chamber (field recordings) or a holding chamber prior to transfer to a submerged perfusion chamber (whole-cell recordings) and were continuously perfused with oxygenated (95% $O_2$/5% $CO_2$) and heated (~28 °C) ACSF at a rate of 2 ml/min. Slices were allowed to equilibrate in normal ACSF for 1 h before experiments began. 1–3 hemisected slices were used per animal.

*Extracellular field recordings.* Low-resistance (2–3 MΩ) extracellular electrodes were pulled with borosilicate glass on a Flaming-Brown Micropipette Puller (Sutter) and were filled with ACSF. Following dissection, slices were immediately placed in the interface chamber and allowed a minimum 60 min recovery period. Stimulating electrodes consisted of twisted, insulated nichrome bipolar wire. Stimulating electrodes were placed on the dorsal anterolateral BNST (dlBNST) border of the internal capsule approximately 200–500 μm dorsal to the anterior commissure. To isolate excitatory transmission, all recordings were conducted in the presence of the $GABA_A$ receptor antagonist, picrotoxin (25 μM). Field potential responses were evoked at a frequency of 0.05 Hz using a stimulus range of 5–15 V at a duration of 100–150 μs. Baseline responses (60% of maximum evoked responses) were recorded for a minimum of 20 min before drug application. Experiments in which the N1 (Figure 1b) changed by >20% were discarded.

*Whole-cell patch-clamp recordings.* Whole-cell recordings were performed as previously reported (Grueter and Winder, 2005; Kash and Winder, 2006). dBNST neurons were visualized and recorded via standard IR-DIC methods. Recording electrodes (4–6 MΩ) were filled with the following for experiments examining excitatory transmission (in mM): 117 Cs gluconate, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl, 4 $Na_2ATP$, 0.3, $Na_2GTP$ (pH 7.2–7.4, Osm. 270–290). EPSCs of 100–250 pA were recorded at a frequency of 0.1 Hz while voltage-clamped at −70 mV in the presence of the $GABA_A$ receptor antagonist, picrotoxin (25 μM). After whole-cell configuration was achieved, cells were allowed to equilibrate a minimum of 5 min before baseline recordings were started. For experiments in which the effects of antagonists were determined, the antagonist was applied for at least 15 min before application of the agonist and remained on for the duration of the experiment. Access resistance, input resistance, and holding current were monitored continuously throughout the duration of the experiments. Recordings in which access resistance changed by more than 20% were excluded from analysis. Following an 8–10 min baseline period, 2 μM SB-334867, 1 μM JNJ-10397049, 100 nM orexin A or 100 nM reboxetine was bath applied for 35 (SB-334867 and JNJ-10397047) 10 (reboxetine) or 20 (orexin A) min. Baseline values were calculated as an average of 3 min directly before agonist application. Recorded data was analyzed via Clampfit 10.2 (Molecular Devices). Both the % coefficient of variation (CV) and paired-pulse ratio (PPR) were examined to assess alterations in presynaptic release probability. Paired pulses with a 70 ms interstimulus interval were given every 20 s. The PPR was the ratio of averaged amplitude of the second EPSC (EPSC2) to that of the first EPSC (EPSC1).

## Drugs

The following drugs were used for experiments: cocaine hydrochloride (National Institute on Drug Abuse, Bethesda, MD), SB-334867, 1-(2-methylbenzoxazol-6-yl)-3-[1,5] naphthyridin-4-yl urea hydrochloride; Tocris, Ellisville,

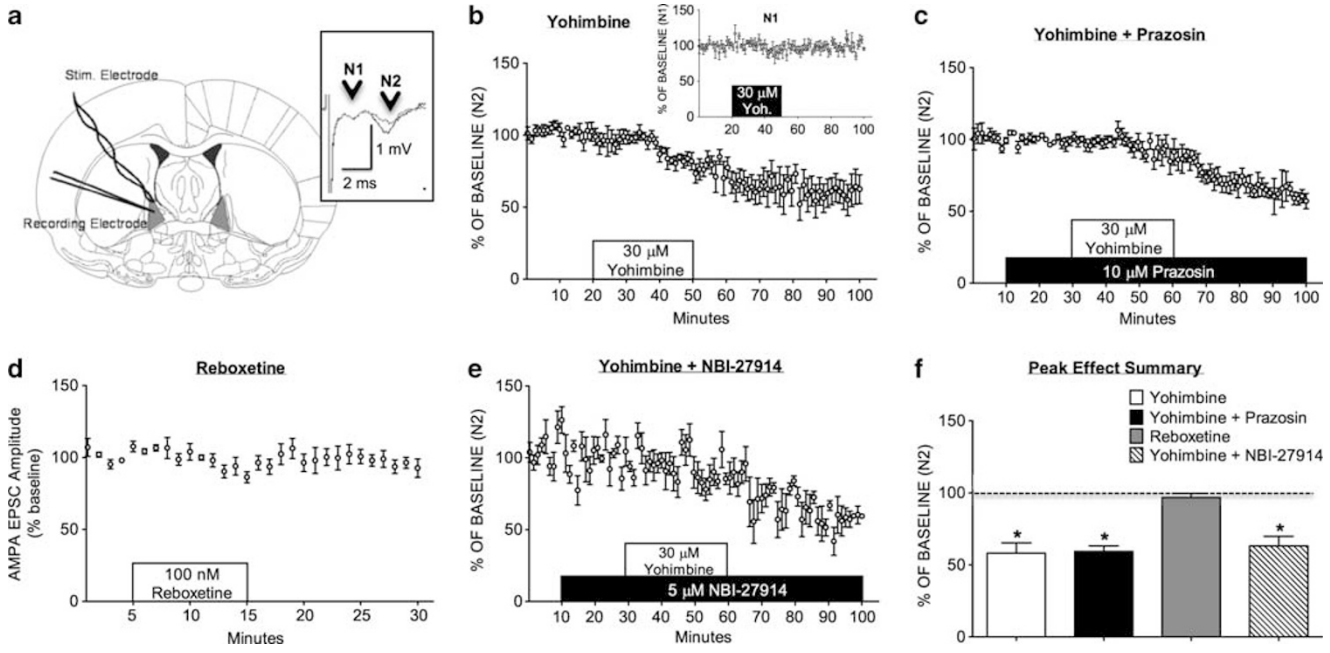


**Figure 1** Norepinephrine and corticotrophin releasing factor (CRF) receptor 1 ($CRFR_1$) antagonists do not block yohimbine-induced depression of excitatory transmission in the dorsal anterolateral bed nucleus of the stria terminalis (dlBNST). Extracellular field potential and whole-cell patch-clamp recordings of excitatory transmission in the dlBNST: (a) left, diagram of coronal section containing the dlBNST and demonstrating placement of stimulating and recording electrodes during electrophysiological recordings; right, a representative extracellular field potential recording trace depicting the TTX-sensitive N1 downward deflection and CNQX-sensitive N2 downward deflection. (b) 30 μM yohimbine inset: corresponding N1; six slices recorded from five mice. (c) 30 μM yohimbine in the presence of 10 μM prazosin; six slices recorded from four mice. (d) 100 nM Reboxetine; six slices recorded from four mice. (e) 30 μM yohimbine in the presence of 5 μM NBI-27914; six slices recorded from five mice. (f) Summary of 86–90 min for yohimbine, prazosin + yohimbine, Reboxetine, NBI-27914 + yohimbine. Data are represented as mean ± SEM, $*p<0.05$, $n=5$–6 slices per group.

Missouri), NBI-27914 (Sigma-Aldrich, St Louis, MO), yohimbine HCl (Sigma-Aldrich), methoxamine hydrochloride (Sigma-Aldrich), prazosin (Tocris), ATP (Sigma-Aldrich), carbachol (Sigma-Aldrich), orexin A (Ascent, Scientific, Princeton, NJ), JNJ-10397047 and 2-{4-[5-methyl-2-(2*H*-1,2,3-triazol-2-yl)benzoyl]-1,4-diazepan-1-yl}-quinazoline (MTBDQ; C Lindsley, Vanderbilt University), reboxetine (Tocris) and picrotoxin (Sigma-Aldrich). For the electrophysiology experiments, drugs were bath-applied at final concentrations, which are noted in the experimental design. DMSO is the solvent used for picrotoxin, NBI-27914, JNJ-10397047, prazosin, MTBDQ, orexin A, and SB-334867 in which the maximum final concentration of DMSO was 0.02% by volume. Methoxamine and yohimbine were dissolved in $ddH_2O$. Reboxetine was dissolved in 0.9% saline.

### Statistical Analysis

When determining whether a drug had a significant effect, or mice displayed a significant difference between their expressed pre- and post-test preference for the cocaine-paired chamber, a paired Student's *t*-test was used to compare the baseline value (or pre-test preference) to the drug effect value (or post-test preference). Unpaired Student's *t*-test was used for determining effects between two separate experimental conditions (eg, post-test preference for the cocaine paired chamber between $\alpha_{2A}$-AR-WT and KO mice). When comparing effects across more than two drug application experiments, a one-way analysis of variance (ANOVA) was used. For extinction of cocaine-CPP experiments, a two-way ANOVA repeated measures (RM) was used. Student Newman Keuls or Holmes-Sidak multiple comparison *post hoc* test were used; if equal variance criteria were not met, the Games-Howell *post hoc* test was used.

## RESULTS

### Norepinephrine and Corticotrophin Releasing Factor (CRF) Receptor 1 ($CRFR_1$) Antagonists Do Not Block Yohimbine-induced Depression of Excitatory Transmission in the dlBNST

A representative depiction of the location for all electrophysiological recordings of our stimulating and recording electrodes in slices containing the dlBNST is presented in Figure 1a (left) and a representative extracellular field potential recording trace depicting the TTX-sensitive N1 (used to monitor slice health) downward deflection and CNQX-sensitive N2 downward deflection (Figure 1a; right, boxed inset). Extracellular field potential recordings from the dlBNST revealed that a 30 min bath application of 30 μM yohimbine resulted in a depression of excitatory transmission ($58.3 \pm 6.9\%$ of baseline in 86–90 min, $t_5 = 4.507$, $p = 0.004$, $n = 6$ slices from five mice, Figure 1b), similar to our previous report (Davis *et al*, 2008) and the N1 was unaffected in these recordings (inset; Figure 1b). We previously demonstrated that (a) this effect is not fully mimicked by the more selective $\alpha_2$-AR antagonist

atipamezole, and was not disrupted in slices obtained from $\alpha_{2A}$-AR KO mice (Davis *et al*, 2008). As multiple actions of norepinephrine are disrupted in the BNST of these mice (Egli *et al*, 2005; McElligott *et al*, 2010), these data suggest that the actions of yohimbine on excitatory transmission are not likely mediated by $\alpha_2$-AR binding, or other norepinephrine dependent processes. It has recently been reported that the $\alpha_1$-AR antagonist prazosin can block yohimbine-induced drug-seeking behavior (Le *et al*, 2011). Thus, to further explore this issue, we assessed the ability of prazosin to block the depressant effects of yohimbine on excitatory transmission in the BNST. A 30 min application of 30 μM yohimbine in the presence of 10 μM prazosin depressed excitatory transmission (59.3 ± 3.9% of baseline in 86–90 min, $t_5 = 11.52$, $^*p < 0.001$, $n = 6$ slices from four mice, Figure 1c) in a manner that was not significantly different from that elicited by yohimbine alone (yohimbine in the presence of 10 μM prazosin compared with 30 μM yohimbine: $F_{3,18} = 0.1$, $p =$ n.s., Figure 1f). Finally, we assessed whether the norepinephrine transporter blocker reboxetine (100 nM), by elevating norepinephrine levels, could mimic the actions of yohimbine. Thus, if the available pool of synaptic norepinephrine in a deafferented slice is dynamic enough to be regulated by autoreceptors and produce the depressant effect elicited by yohimbine on excitatory transmission, we reasoned that reboxetine should mimic this effect. Utilizing whole-cell patch clamp recordings, a 10 min application of 100 nM reboxetine had no effect (96.8% of baseline in 25–30 min, $t_5 = 0.8$; $p =$ n.s., $n = 6$ slices from four mice, Figure 1d) on excitatory transmission in our experiments, as would be predicted in a deafferented slice where noradrenergic inputs are severed. These results suggest that modulation of extracellular norepinephrine levels is unlikely to contribute to the depressant effects of yohimbine on excitatory transmission in the dlBNST.

Given the anxiogenic effect of yohimbine and the presence of CRF neurons and terminals in the BNST (Champagne *et al*, 1998), we next assessed if $CRFR_1$ may be the target for the depressant effects of yohimbine on excitatory transmission. A 30 min application of yohimbine in the presence of the $CRFR_1$ antagonist, NBI-27914, elicited a significant depression on excitatory transmission (63.2 ± 6.7% of baseline, $t_5 = 6.5$, $^*p = 0.0013$, $n = 6$ slices from five mice, Figure 1e and f), demonstrating that $CRFR_1$ does not mediate the yohimbine-induced depression of excitatory transmission in the BNST.

## Orexin Receptor (OxR) Antagonists Block Yohimbine-induced Depression of Excitatory Transmission in the dlBNST

Orexin A has been implicated in anxiety and reward-related behaviors (Buffalari and See, 2010; Dayas *et al*, 2008; Espana *et al*, 2010; Harris *et al*, 2005; Johnson *et al*, 2010; Lawrence *et al*, 2006; Richards *et al*, 2008), and the BNST receives dense orexinergic innervation (Baldo *et al*, 2003). To test the role of orexin signaling in the effects of yohimbine, we assessed the ability of OxR antagonists to block its actions. When applied alone, the $OX_1R$ antagonist, SB-334867 (5 μM), had no significant effect on excitatory transmission, even with extensive pretreatment (91.6 ± 3.8% of baseline in 86–90 min, $t_6 = 1.03$, $p =$ n.s., $n = 7$ slices from four mice; Figure 2a). However, pretreatment with 5 μM SB-334867 prevented yohimbine-induced depression of excitatory transmission (101.6 ± 2.6% of baseline in 86–90 min, $t_5 = 0.4$, $p =$ n.s, $n = 6$ from four mice, Figure 2b). Similarly, a 30 min application of a novel dual $OX_1R$ and $OX_2R$ antagonist, MTBDQ (1 μM), also had no effect on excitatory transmission (98.8 ± 2.6% % of baseline in 86–90 min, $t_5 = 1.2$, $p =$ n.s., $n = 6$ slices from three mice, Figure 2d). Similar to what was observed with the $OX_1R$, pretreatment with 1 μM MTBDQ prevented yohimbine-induced depression of excitatory transmission (101.1 ± 28.3% of baseline in 86–90 min, $t_6 = 0.1$, $p =$ n.s., $n = 7$ slices from five mice, Figure 2e). These results are summarized as peak drug effects at 86–90 min ($F_{4,26} = 9.9$; $^*p < 0.05$ for the yohimbine group compared with all other groups; Figure 2c).

## $OX_1R$-Dependent orexin A-Induced Depression of AMPA EPSCs in the dlBNST

Our findings suggest that an OxR agonist may mimic the effects of yohimbine on excitatory transmission in the dlBNST. Two recent studies reported that in the hippocampus and ventrolateral periaqueductal gray (vlPAG), orexin A elicited a depression of excitatory transmission in young animals (Ho *et al*, 2011; Selbach *et al*, 2010). We thus investigated the effects of exogenous orexin A on excitatory transmission in the dlBNST. Consistent with our hypothesis, in the majority of cells recorded (13 of 16 cells recorded from 10 mice), a 20 min application of 100 nM orexin A resulted in a slowly developing and significant reduction of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) EPSC amplitude (41.9 ± 7.0% decrease from baseline; $t_{12} = 6.3$, $^*p < 0.01$, $n = 13$, Figure 3a) and EPSC area (35.2 ± 6.2% decrease from baseline: $t_{12} = 6.6$, $^*p < 0.05$, Figure 3a *inset*), with no significant changes to EPSC kinetics (rise: $t_{12} = 0.4$, $p =$ n.s.; decay: $t_{12} = 0.2$, $p =$ n.s., Figure 3a *inset*). In the remaining cells (3 of 16 cells recorded), 20 min application of 100 nM orexin A had no significant effect on EPSC amplitude, area, or kinetics (data not shown). PPR and CV analysis was undertaken to investigate changes in presynaptic release probability. Interestingly, PPR ($t_{12} = 3.2$, $^*p < 0.05$, Figure 3b) and CV ($t_{12} = 3.4$, $^*p < 0.05$, Figure 3c) were increased compared with baseline, consistent with an orexin A-mediated decrease in presynaptic glutamate release probability.

As previous studies suggest the BNST has a moderate to high density of $OX_1R$ and $OX_2R$ mRNA and protein expression (Hervieu *et al*, 2001; Marcus *et al*, 2001), we next investigated whether the orexin A depression of excitatory transmission was $OX_1R$—or $OX_2R$-dependent. We found that pretreatment with the $OX_1R$ antagonist, SB-334867 (2 μM) attenuated the orexin A mediated depression with no significant changes in AMPA EPSC amplitude (6.6 ± 9.4% decrease from baseline, $t_4 = 0.2$; $p =$ n.s., $n = 5$ slices from five mice, Figure 3d). Consistent with these findings, no changes were observed in PPR ($t_4 = 0.1$; $p =$ n.s., Figure 3e) and CV ($t_4 = 0.1$; $p =$ n.s., Figure 3f). In contrast, pretreatment with the $OX_2R$ antagonist, JNJ-10397047 (1 μM) did not prevent a significant reduction in EPSC amplitude by orexin A (47.8 ± 4.9% decrease from baseline, $t_4 = 6.7$, $^*p < 0.05$, $n = 5$ slices from four mice, Figure 3g). Moreover, an increase in both PPR ($t_4 = 7.4$, $^*p < 0.05$, Figure 3h) and CV ($t_4 = 2.9$,

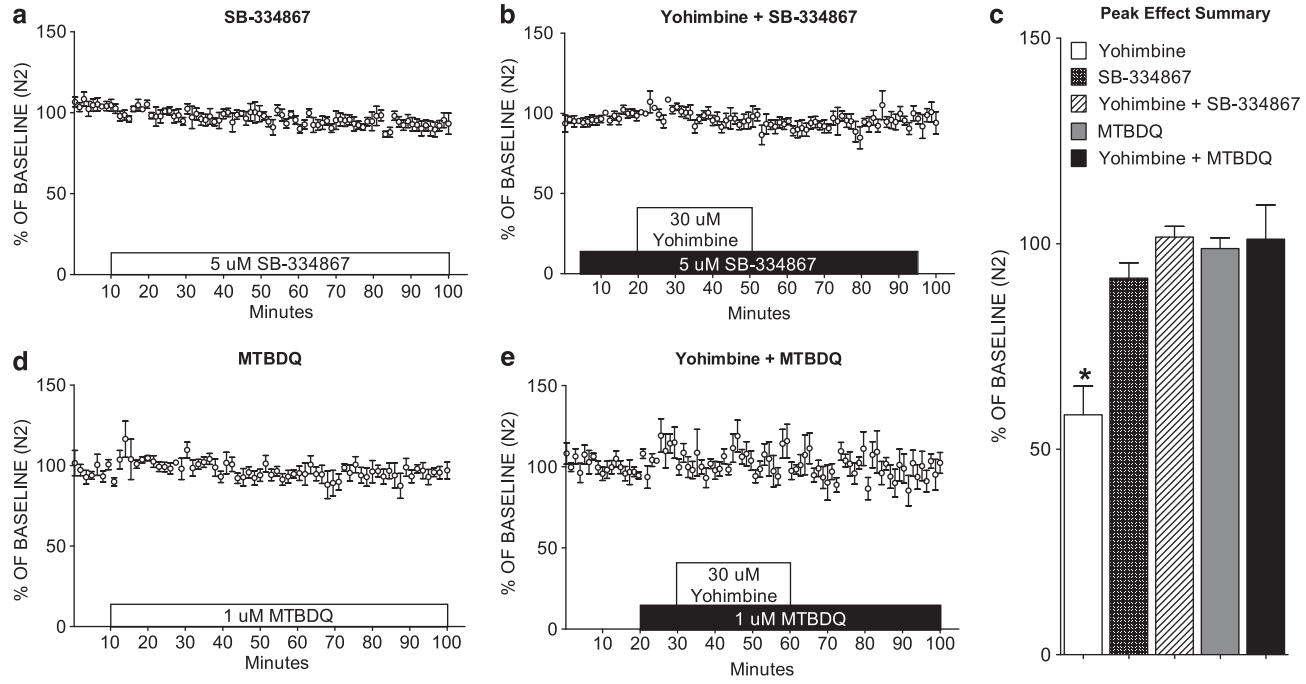


**Figure 2** Orexin receptor (OxR) antagonists block yohimbine-induced depression of excitatory transmission in the dorsal anterolateral bed nucleus of the stria terminalis (dlBNST). Extracellular field potential recordings of excitatory transmission in the dlBNST. (a) 5 μM SB-334867; seven slices recorded from four mice. (b) 30 μM yohimbine in the presence of 5 μM SB-334867; six slices recorded from four mice. (c) Summary of 86–90 min for yohimbine, 2-{4-[5-methyl-2-(2*H*-1,2,3-triazol-2-yl)benzoyl]-1,4-diazepan-1-yl}quinazoline (MTBDQ), MTBDQ + yohimbine, SB-334867, SB-334867 + yohimbine. (d) 1 μM MTBDQ; six slices recorded from three mice. (e) 30 μM yohimbine in the presence of 1 μM MTBDQ; seven slices recorded from five mice. Data are represented as mean ± SEM, $^*p > 0.05$, $n = 6$–7 slices per group.

$^*p < 0.05$, Figure 3i) were observed, indicating that application of orexin A in the presence of an $OX_2R$ antagonist did not effect presynaptic release probability. These results strongly suggest that orexin A acts at the $OX_1R$ to depress excitatory transmission in dlBNST.

## Yohimbine Does Not Directly Activate $OX_1R$ Signaling

Our results demonstrate both an $OX_1R$ and novel dual $OX_1R/OX_2R$ antagonist attenuate yohimbine-induced depression of excitatory transmission (Figure 2), and these depressant effects of yohimbine on excitatory transmission are mimicked by orexin A in an $OX_1R$-dependent manner (Figure 3), indicating that yohimbine depresses excitatory transmission in dlBNST by recruiting OxR signaling. This could be accomplished either via direct action of yohimbine on Ox1R, or via regulation of orexin release to activate the receptor. To address the former issue, we investigated the ability of yohimbine to act as a direct ligand and activate the $OX_1R$ utilizing a stable $OX_1R$ cell line and calcium mobilization assay. Increasing concentrations of orexin A, yohimbine, and ATP were applied to HEK-EM4 cells stably expressing the $OX_1R$ and calcium mobilization was measured. The $EC_{50}$ for orexin A was $\sim 72 \pm 6$ nM, the $EC_{50}$ for yohimbine $> 1$ mM, and the $EC_{50}$ for ATP was $709 \pm 40$ nM in HEK-EM4 cells expressing the $OX_1R$ (Figure 4a). In the HEK-EM4 $OX_1R$ expressing cells the $OX_1R$ antagonist SB-334867 fully inhibited the effects of an $EC_{80}$ concentration of orexin A ($IC_{50} \sim 109 \pm 3$ nM), whereas it had no effect on the signal produced by 1 mM yohimbine ($IC_{50} > 1$ mM; Figure 4b). To determine whether the mM yohimbine concentrations required to observe a calcium mobilization response were related to $OX_1R$ expression, carbachol, yohimbine, and ATP were applied to HEK 293 cells co-expressing $mGluR_2$ and GIRKs. The $EC_{50}$ for carbachol was $> 30$ μM, $EC_{50}$ for yohimbine $> 1$ mM, and the $EC_{50}$ for ATP was $420 \pm 83$ nM (Figure 4c). All experiments were performed in triplicate. These results suggest that yohimbine does not directly recruit $OX_1R$ signaling and more likely acts by indirectly promoting orexin release to influence orexin receptor signaling.

## Yohimbine-Induced Depression of Excitatory Transmission in the dlBNST is Absent in Prepro-orexin Knockout Mice

If yohimbine acts on dlBNST excitatory transmission via promoting the release of orexin, it would be predicted that this effect would be absent in dlBNST slices prepared from prepro-orexin knockout (Ox-KO) mice. Thus, we assessed the ability of yohimbine to depress excitatory transmission in the dlBNST in Ox-KO mice (Willie *et al*, 2003). Yohimbine (30 μM, 30 min) had no significant effect on excitatory transmission in the dlBNST ($94.9 \pm 7.2\%$ of baseline in 86–90 min, $t_4 = 0.5$, $p =$ n.s., $n = 5$ slices from three mice, Figure 5) in slices from Ox-KO mice; consistent with the idea that yohimbine acts via promoting orexin release in the dlBNST to depress glutamatergic transmission.

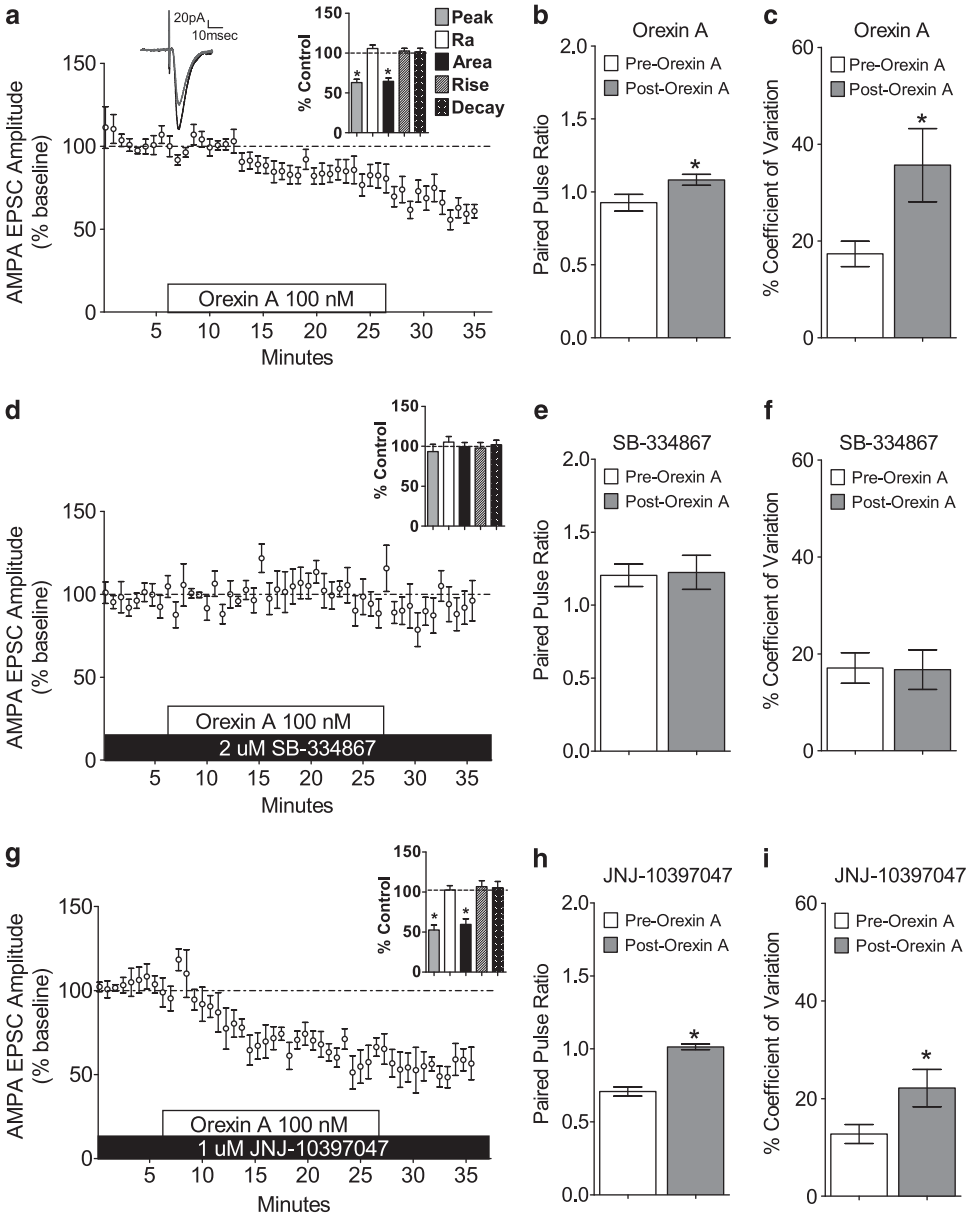


**Figure 3** $OX_1R$-dependent orexin A-induced depression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) EPSCs in the dlBNST. Whole-cell patchclamp recordings of AMPA EPSCs in the dlBNST. (a) Time course showing the effect of 100 nM orexin A on the peak amplitude of AMPA EPSCs in 13 out of 16 cells recorded from 10 mice; inset: representative trace from whole-cell recording (baseline: black line; post-orexin A: blue line); boxed inset: Summary of cell parameters monitored throughout recordings: amplitude, access resistance (Ra), area, rise time, and decay time of EPSCs. * indicated significant difference from baseline. (b) PPR of pre-orexin A and post-orexin A application. (c) % coefficient of variation (CV) in pre-orexin A and post-orexin A events. (d) Time course showing the effect of 100 nM orexin A in the presence of 2 μM of the $OX_1R$ (orexin receptor 1) antagonist, SB-334867, on the peak amplitude of AMPA EPSCs in 5 out of 5 cells recorded from five mice. Boxed Inset: Summary of cell parameters monitored throughout recordings: amplitude, access resistance (Ra), area, rise time, and decay time of EPSCs * indicated significant difference from baseline. (e) In the presence of SB-334867, PPR of pre-orexin A and post-orexin A application. (f) In the presence of SB-334867, % CV of pre-orexin A and post-orexin A events. (g) Time course showing the effect of 100 nM orexin A in the presence of 1 μM of the $OX_2R$ antagonist, JNJ-10397047, on the peak amplitude of AMPA EPSCs in 5 out of 5 cells recorded from four mice. Boxed Inset: Summary of cell parameters monitored throughout recordings: amplitude, access resistance (Ra), area, rise time, and decay time of EPSCs * indicated significant difference from baseline. (h) In the presence of JNJ-10397047, PPR of pre-orexin A and post-orexin A application. (i) In the presence of JNJ-10397047, % CV of pre-orexin A and post-orexin A events. The color reproduction of the figure is available at the *Neuropsychopharmacology* journal online.

### $\alpha_1$-AR-Induced Depression of Excitatory Transmission in the dlBNST is Not Blocked by an $OX_1R$ Antagonist

As shown above, orexin A, acting at the $OX_1R$, depresses excitatory transmission in the dlBNST. Similarly, previous work in our lab has demonstrated that several other Gq-linked receptors (eg, $\alpha_1$-AR, mGluR1, mGluR5) cause a depression of excitatory transmission in the dlBNST (Gosnell *et al*, 2011; Grueter *et al*, 2006; McElligott *et al*, 2010; McElligott and Winder, 2008, 2009). Although the $\alpha_1$-AR antagonist prazosin did not block yohimbine-induced depression of excitatory transmission (see above), to further

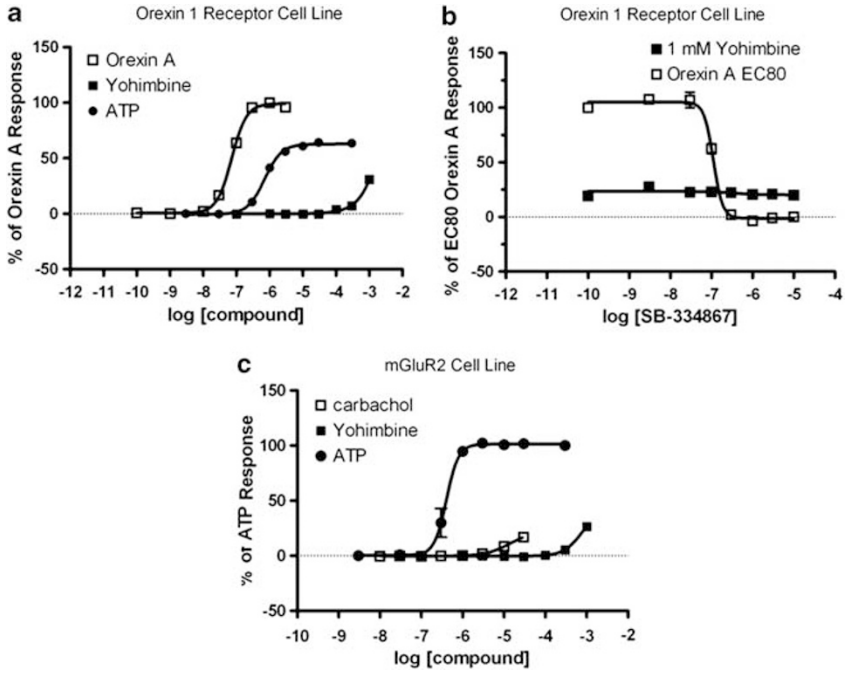


**Figure 4** Yohimbine does not directly activate $OX_1R$ signaling. Concentration–response curves (CRCs) of (a) orexin A, yohimbine, and ATP in HEK-EM4 cells expressing orexin 1 receptor ($OX_1R$-Cs). (b) Effects of 1 mM yohimbine or EC80 orexin A in SB-334867 pre-treated $OX_1R$-Cs. (c) Carbachol, yohimbine, and ATP in HEK 293 cell co-expressing rat $mGluR_2$ and G-protein-coupled inwardly rectifying potassium (GIRK) channels ($mGluR_2$ Cell Line). All data are expressed as the mean of a minimum of three independent experiments performed in triplicate. Data are represented as mean ± SEM.

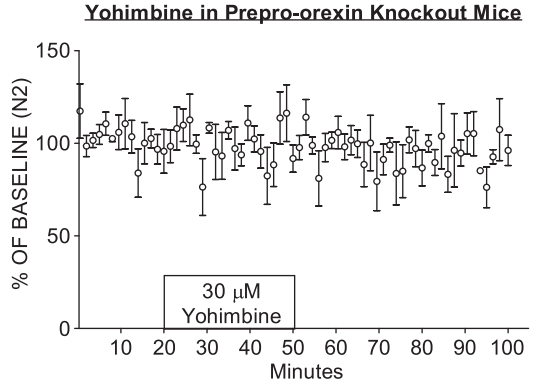


**Figure 5** Yohimbine-induced depression of excitatory transmission in the dlbnst is absent in prepro-orexin (ox) knockout mice. Extracellular field potential recordings of excitatory transmission in the dlBNST of prepro-orexin knockout (Ox-KO) mice. 30 μM yohimbine. Data are represented as mean ± SEM, $p$ = n.s., $n = 5$ slices from three mice.

address possible activation of common downstream effectors, we investigated if an $OX_1R$ antagonist could block methoxamine (a Gq-linked $\alpha_1$-AR)-induced depression in the dlBNST. A 30 min application of methoxamine (100 μM) elicited a significant depression in excitatory transmission (66.1 ± 3.5% of baseline in 86–90 min, $t_5 = 6.2$, $^*p < 0.05$, $n = 6$ slices from four mice, Figure 6a). A 30 min application of 100 μM methoxamine in the presence of 5 μM SB-334867 still elicited a significant depression of excitatory transmission (71.7 ± 4.3% of baseline in 86–90 min, $t_6 = 7.5$, $^*p < 0.05$, $n = 7$ slices from four mice, Figure 6b) that was not significantly different from the depression elicited by 100 μM methoxamine alone ($F_{2,17} = 10.9$, $^*p < 0.05$ for methoxamine compared to SB-334867, $^*p < 0.05$ for SB-334867 + methoxamine compared with SB-334867, $p$ = n.s. for methoxamine compared with methoxamine + SB-334867; Figure 6c). Taken together, these results demonstrate that the $\alpha_1$-AR mediated depression of excitatory transmission is independent from the yohimbine-inducible and $OX_1R$-dependent depression of excitatory transmission observed in the dlBNST.

### $OX_1R$ Antagonist, SB-334867, Blocks Yohimbine-induced Impairment of Extinction of Cocaine-CPP in $\alpha 2_A$-AR Wild-Type and KnockOut Mice

Recent studies have demonstrated that an $OX_1R$ antagonist, SB-334867, can attenuate yohimbine-induced reinstatement of drug-seeking behavior (Richards *et al*, 2008; Zhou *et al*, 2011). However, the role of $OX_1Rs$ in the extinction of stress-induced increases in cocaine-seeking behavior is unknown. Although our understanding of the neural mechanisms and substrates critical to reinstatement of drug-seeking behavior have increased substantially in recent years, the role of a potentially therapeutically viable method to help achieve and maintain abstinence, extinction training, has lagged considerably (Millan *et al*, 2011; Myers and Carlezon, 2010). To determine if systemic administration of an $OX_1R$ antagonist (SB-334867) can attenuate yohimbine-induced impairment of extinction of cocaine-CPP, $\alpha_{2A}$-AR wild-type ($\alpha_{2A}$-AR-WT) and knockout ($\alpha_{2A}$-AR-KO) mice were tested using a previously employed cocaine-CPP and extinction paradigm (Davis *et al*, 2008). $\alpha_{2A}$-AR-WT and KO littermate mice display cocaine-CPP when tested during a 15-min post-test session (post-test preference expressed compared with pre-test

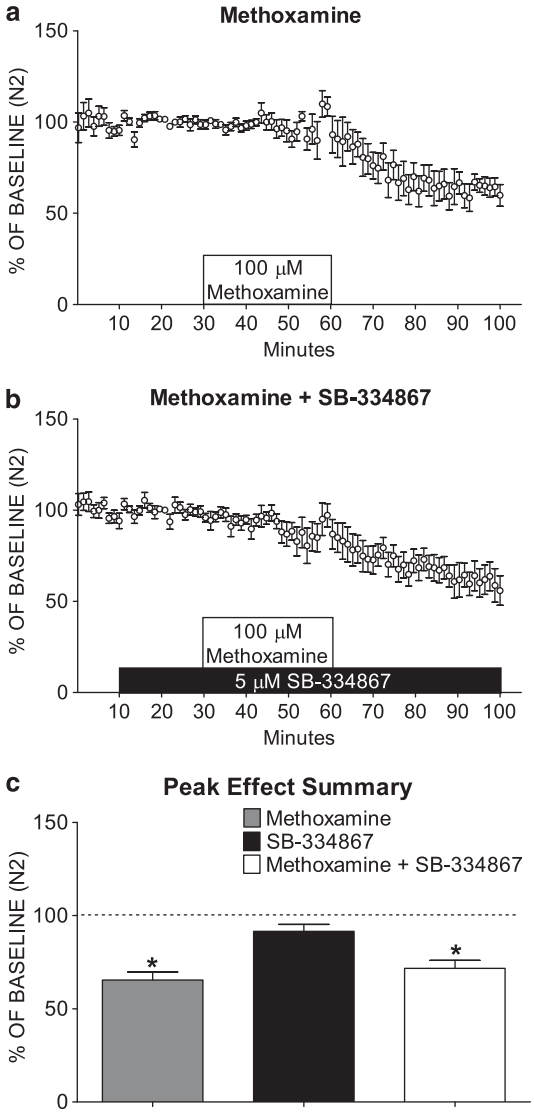


**Figure 6** $\alpha_1$-AR-induced Depression of Excitatory Transmission in the dlBNST is Not Blocked by an $OX_1R$ Antagonist. Extracellular field potential recordings of excitatory transmission in the dlBNST (a) 100 μM methoxamine; $n=6$ slices from four mice; (b) 100 μM methoxamine in the presence of 5 μM SB-334867; $n=7$ slices from four mice; (c) summary of 86–90 min for methoxamine and SB-334867 + methoxamine. Data are represented as mean ± SEM, $^*p<0.05$, $n=5$–7 slices per group.

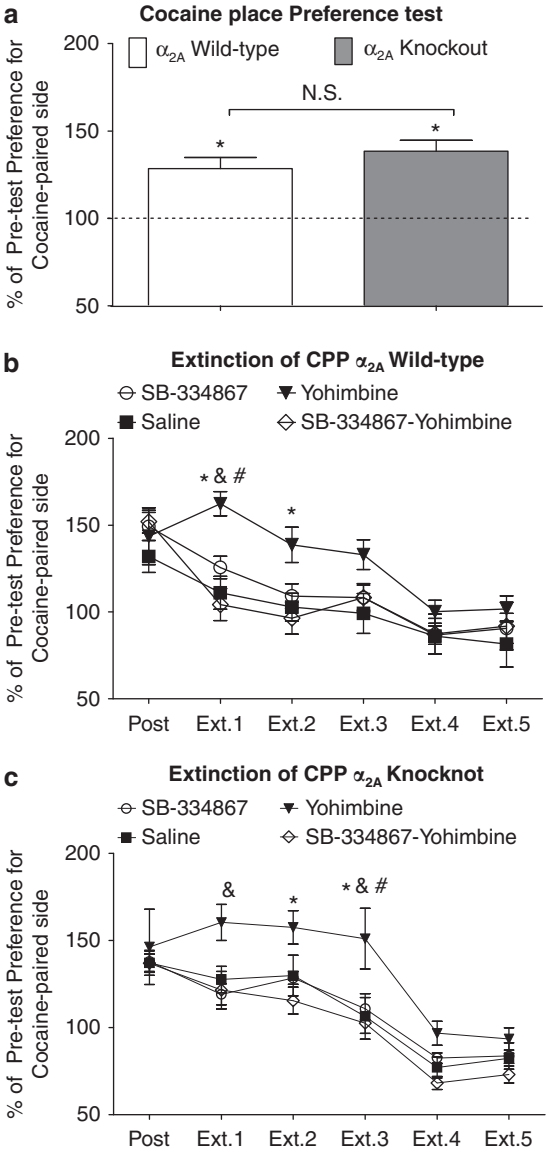


**Figure 7** $OX_1R$ antagonist, SB-334867, blocks yohimbine-induced impairment of extinction of cocaine-CPP in $\alpha 2_A$-AR wild-type and knockout mice. Magnitude of cocaine place preference assessed in a drug-free state during the pre and post preference test. All mice were conditioned with a 20 mg/kg dose of cocaine to the cocaine paired side (see Patients and Methods for additional details). (a) $\alpha_{2A}$-AR wild-type and knockout mice display cocaine-CPP. (b) Mice treated with yohimbine (5 mg/kg; WT-Yoh) display impaired extinction of cocaine-CPP on extinction (Ext) days 1 and 2 but not on extinction days 3, 4, and 5. SB-334867 (15 mg/kg; WT-SB) and SB-334867 and yohimbine (5 and 15 mg/kg respectively; WT-SB-Yoh) mice display levels similar to saline (WT-Sal) mice on all extinction days. (c) Mice treated with yohimbine (5 mg/kg; KO-Yoh) display impaired extinction of cocaine-CPP on extinction (Ext) days 1, 2, and 3 but not on extinction days 4 and 5. SB-334867 (15 mg/kg; KO-SB) and SB-334867 and yohimbine (5 mg/kg and 15 mg/kg respectively; KO-SB-Yoh) mice display levels similar to saline (KO-Sal) mice on all extinction days. Data are represented as mean ± SEM. $^*p<0.05$ when YOH group is significantly different from SB-YOH, $^{\#}p<0.05$ when YOH group is significantly different from SAL, $^{\&}p<0.05$ when YOH group is significantly different from SB group.

values for *$\alpha_{2A}$-AR-WT:* $t=4.7$, $^*p<0.01$, $n=51$, Figure 7a; post-test preference expressed compared with pre-test values for *$\alpha_{2A}$-AR-KO:* $t=8.9$, $^*p<0.01$, $n=32$, Figure 7a). The magnitude of preference expressed did not differ between $\alpha_{2A}$-AR-WT and $\alpha_{2A}$-AR-KO mice. ($t_{81}=1.1$, $p=$n.s., Figure 7a). In the WT mice cohort, mice were assigned to one of four groups and treated with yohimbine 30 min before the extinction session (5 mg/kg; WT-YOH), SB-334867 1 h before the extinction session (15 mg/kg; WT-SB), saline 1 h and 30 min before the extinction sessions (WT-Sal) or mice pre-treated with SB-334867 1 h before and yohimbine 30 min before the extinction sessions (WT-SB-YOH). Doses used for yohimbine and SB-334867 were determined based on previous studies (Davis *et al*, 2008; Richards *et al*, 2008). In WT mice, two-way ANOVA RM revealed a significant effect (Figure 7b) of time ($F_{5,260}=19.89$), treatment ($F_{3,260}=2.92$), and time x treatment interaction ($F_{15,260}=1.76$). Post-hoc analysis revealed a significant effect of WT-YOH on extinction day one compared to WT-SB-YOH ($t=3.867$;

$^{*}p<0.05$), WT-SAL ($t=3.174$; $^{\#}p<0.05$), and WT-SB ($t=2.689$; $^{\&}p<0.05$) and a significant effect of WT-YOH on extinction day 2 compared to WT-SB-YOH ($t=2.859$; $^{*}p<0.05$). No differences were found on distance traveled in any $\alpha_{2A}$-AR WT group tested during extinction days 1–5 (data not shown). These results demonstrate that systemic administration of an antagonist of the $OX_1R$ prevents yohimbine-mediated impairment of extinction of cocaine-CPP, yet the $OX_1R$ alone had no effect on the rate of extinction.

Given the putative role of the $\alpha_2$-AR in mediating the anxiogenic and drug-seeking related effects of yohimbine, we next investigated if an antagonist of the $OX_1R$ could still alter the yohimbine-mediated increase in time spent in the chamber previously associated with cocaine in mice lacking the $\alpha_{2A}$-AR. In the $\alpha_{2A}$-AR-KO cohort, mice were assigned to 1 of 4 groups and treated with yohimbine 30 min before the extinction session (5 mg/kg; KO-YOH), SB-334867 1 h before the extinction session (15 mg/kg; KO-SB), saline 1 h and 30 min prior (KO-Sal) or mice pre-treated with SB-334867 1 h prior and yohimbine 30 min before the extinction sessions (KO-SB-YOH). In KO mice, two-way ANOVA RM revealed a significant effect (Figure 7c) of time ($F_{5,129}=42.50$), treatment ($F_{3,129}=5.61$), but not time x treatment interaction. *Post-hoc* analysis revealed a significant effect of KO-YOH on extinction day one compared to KO-SB-YOH ($t=4.291$; $^{*}p<0.05$), and KO-SB ($t=3.1$; $^{\&}p<0.05$); KO-YOH on extinction day two compared to KO-SB-YOH ($t=3.0351$; $^{*}p<0.05$); KO-YOH on extinction day three compared to KO-SB-YOH ($t=3.782$; $^{*}p<0.05$), KO-SAL ($t=3.346$; $^{\#}p<0.05$), and KO-SB ($t=3.015$; $^{\&}p<0.05$). No differences were found on distance traveled in any $\alpha_{2A}$-AR wild-type group tested during extinction days 1–5 (data not shown). Taken together, these results demonstrate that an $OX_1R$ antagonist can prevent yohimbine-induced impairment of extinction, both in wild-type and $\alpha_{2A}$-AR-KO mice.

## DISCUSSION

We found that yohimbine depresses excitatory transmission in the dlBNST via $OX_1R$-dependent signaling, and this depressant effect was blocked both by an $OX_1R$ and a novel dual $OX_1R/OX_2R$ antagonist, but not antagonists of $\alpha_1$-AR and $CRFR_1$, nor mimicked by a norepinephrine transporter blocker. Moreover, the depressant effects of yohimbine on excitatory transmission in the BNST were mimicked by orexin A acting at the $OX_1R$ but not $OX_2R$, demonstrating a novel action of orexin in the BNST. Additionally, we found that yohimbine did not activate $OX_1Rs$ in a stable cell line, and yohimbine depression of excitatory transmission was absent in slices prepared from OX-KO mice. Yohimbine likely depresses excitatory transmission in BNST through orexin-dependent activation of $OX_1R$ to decrease glutamate release probability, as whole-cell recordings demonstrated an $OX_1R$-dependent increase in both PPR and CV. In parallel, we find that the ability of yohimbine to impair extinction of cocaine-CPP in $\alpha_{2A}$-AR-WT and $\alpha_{2A}$-AR-KO mice is blocked by systemic administration of the $OX_1R$ antagonist SB-334867.

### Yohimbine Recruits $OX_1R$ Signaling to Depress Excitatory Transmission in the BNST

Utilizing extracellular recordings, we replicated previous whole-cell recordings demonstrating yohimbine depresses excitatory transmission in dlBNST (Davis *et al*, 2008). Yohimbine-induced depression is intact in $\alpha_{2A}$-AR KO mice, suggesting an off-target action of the drug. While yohimbine could be acting through antagonism of $\alpha_{2B}$-and/or $\alpha_{2c}$-AR subtypes (Altman *et al*, 1999), the $\alpha_2$-AR agonist UK 14 304 depresses excitatory transmission but is attenuated rather than mimicked by the $\alpha$2-AR antagonist, atipamezole (Davis *et al*, 2008; Egli *et al*, 2005). Furthermore, it is unlikely that the effects observed here involve autoreceptor functions of $\alpha_2$-AR activity, because the norepinephrine transport blocker reboxetine did not mimic yohimbine, and the $\alpha_1$-AR antagonist prazosin did not block it. Yohimbine is known to have relatively moderate- to high-affinity for a number of GPCRs other than $\alpha_2$-ARs, including serotonin (5-HT)(1A), 5-HT(1B) receptors among others (Millan *et al*, 2000). Indeed, the actions of yohimbine on reinstatement of cocaine- and alcohol-seeking has been shown to involve 5HT and $\beta$-adrenergic receptors ($\beta$-ARs) (Dzung Le *et al*, 2009; Fletcher *et al*, 2008; Sakurai *et al*, 1998). However, the actions of $\beta$-AR and 5HT receptor agonists on excitatory transmission in BNST differ from the known actions of yohimbine at these receptors (Guo and Rainnie, 2010; Hammack *et al*, 2009; Nobis *et al*, 2011). For example, previous work from our group demonstrated a $\beta$-AR agonist enhances excitatory transmission in BNST (Nobis *et al*, 2011). Moreover, a 5HT-1A and 5HT-2C agonist has no effect and a 5HT-1B agonist depresses excitatory transmission in the BNST (Guo *et al*, 2009; Guo and Rainnie, 2010), yet yohimbine is known to act as an antagonist at 5HT-1B and has no significant affinity for $\beta$-ARs (Millan *et al*, 2000), suggesting a different target may better explain the effects of yohimbine in BNST.

Based on previous studies that had separately shown that the BNST is an important site for yohimbine actions in stress and anxiety-related behavior (Buffalari and See, 2010; Johnson *et al*, 2010), we hypothesized that yohimbine interacts with orexin signaling to alter glutamatergic transmission. Indeed, yohimbine-induced depression was sensitive to $OX_1R$ and a dual $Ox1R/OX_2R$ antagonism, and mimicked by exogenous orexin A application. Finally, an absence of yohimbine-induced depression on excitatory transmission in the Ox-KO confirms the necessity of the orexin system in mediating the effects of yohimbine on excitatory transmission in the BNST.

### Orexin A Depresses Excitatory Transmission in the BNST in an $OX_1R$-Dependent Manner

Our results indicate a novel role for orexin A in depressing excitatory transmission in the dlBNST of adult mice, while studies in other brain regions have predominantly demonstrated excitatory roles (Bisetti *et al*, 2006; Burlet *et al*, 2002; Follwell and Ferguson, 2002; Hagan *et al*, 1999; Sakurai *et al*, 1998; Shin *et al*, 2009; Shirasaka *et al*, 2001; Soffin *et al*, 2002; Sutcliffe and de Lecea, 2000; Yang *et al*, 2003). This dichotomy of actions of GPCRs on neuronal function is not unusual. The Gq-linked mGluR5 and $\alpha_1$-AR both induce

depression of excitatory transmission yet enhance NMDA receptor function (Grueter and Winder, 2005; Kirkwood *et al*, 1999; McElligott and Winder, 2009; McElligott *et al*, 2010). Moreover, two recent studies reported that in the hippocampus and ventrolateral periaqueductal gray (vlPAG), orexin A elicited a depression of excitatory transmission in young animals (Ho *et al*, 2011; Selbach *et al*, 2010).

### Yohimbine Acts Indirectly at the $OX_1R$

Our results with the $OX_1R$ cell line indicate that yohimbine is not a direct ligand for $OX_1R$. Indeed, a concentration of 1 mM yohimbine and above was necessary to observe any calcium mobilization. This effect was not blocked by the $OX_1R$ antagonist, SB-334867, and was also evident in a cell line expressing mGluR2 rather than $OX_1R$, indicating that it was independent of $OX_1R$ signaling. It should be noted, however, that we cannot exclude the possibility that $OX_1R$ *in vivo* responds differently to yohimbine than in cell lines. For example, Gq-linked elevations in postsynaptic $Ca^{2+}$ levels have been shown to lead to endocannabinoid-mediated retrograde signaling (Wettschureck *et al*, 2006). Within the BNST, activation of the cannabinoid 1 receptor (CB1) depresses excitatory transmission (Grueter *et al*, 2006; Puente *et al*, 2010). As our PPR and CV analysis indicate a decrease in presynaptic glutamate release probability, future studies will investigate the potential role for CB1-mediated retrograde signaling on the $OX_1R$-dependent yohimbine-induced depression of excitatory transmission. Our results demonstrate that deletion of orexin via the use of OX-KO mice blocked the yohimbine-induced depression of excitatory transmission and it seems most likely that in the BNST yohimbine acts indirectly to promote orexin A release to influence synaptic function and impact behavior.

### $OX_1R$ Mediates Yohimbine-impaired Cocaine Extinction

As we found that the depressant effects of yohimbine in the BNST require orexin signaling, we wondered whether behavioral actions of yohimbine would also require the actions of this neuropeptide. To date, studies on the role of the orexin system on cocaine relapse behavior have focused primarily on reinstatement. In the present study, we sought to investigate the potential involvement of orexin in mediating the effects of stress on cocaine extinction.

We found that systemic $OX_1R$ antagonism blunted yohimbine-impaired extinction of cocaine-CPP in $\alpha_{2A}$ -AR WT and KO mice. Consistent with our findings that the $OX_1R$ is involved in extinction of cocaine reward are reports that the $OX_1R$ mediates yohimbine-induced reinstatement of alcohol- and cocaine-seeking behavior (Buffalari and See, 2010; Zhou *et al*, 2011). Recent studies have also highlighted the interaction of yohimbine with non-$OX_1R$ neuropeptides, such as $CRFR_1$ (Marinelli *et al*, 2007). However, given that (1) Brown *et al* (2009) demonstrated that a CRFR antagonist failed to block yohimbine-induced reinstatement of cocaine-seeking behavior and (2) we found no effect of the $CRF_1$ antagonist, NBI-27914, on yohimbine-induced depression of excitatory transmission, it seems unlikely but remains to be examined the behavioral effects of $CRFR_1$ antagonism on yohimbine-induced impairment of extinction of cocaine-CPP. Moreover, a role for norepinephrine involvement in our reported behavior also seems unlikely, as an $OX_1R$ antagonist was similarly effective at blocking yohimbine-impaired cocaine-CPP extinction in both $\alpha_{2A}$ -AR WT and KO mice. Indeed, previous work demonstrated that the $\alpha_2$-AR antagonist, atipamezole, also failed to impair extinction of cocaine-CPP (Davis *et al* 2008). On alcohol self-administration and reinstatement, the effects of yohimbine are not mimicked by a highly selective $\alpha_2$-AR antagonist, RS-79948, nor were the effects of yohimbine on self-administration blocked by the $\alpha_2$-AR agonist clonidine (Dzung Le *et al*, 2009). Similarly, neither clonidine nor prazosin block yohimbine-induced reinstatement of cocaine-seeking behavior (Brown *et al*, 2009).

### Conclusions

Taken together, these data demonstrate an important new mechanism for orexin action, and show that significant behavioral actions of yohimbine may be independent of adrenergic signaling and instead require $OX_1R$ in the BNST. Moreover, our data does not preclude the additional possibility that orexin acts upstream of yohimbine to facilitate yohimbine-induced impairment of excitatory transmission. However, our data do strongly suggest little role, if any, for the norepinephrine system in the depressant effects of yohimbine on glutamatergic transmission. Given the pivotal role stress has during withdrawal in enhancing susceptibility to cocaine-seeking behavior, our data highlight an important role for orexin in mediating stress-induced impairment of the extinction of cocaine-seeking behavior. Moreover, our data implicating a role for $OX_1R$ in stress- and reward-related cocaine extinction behavior is paralleled by changes in neuronal function in the BNST and reveals a novel action of orexin in depressing excitatory transmission, likely through a presynaptic decrease in the release probability of glutamate. Our data indicate that continued investigation of the actions of orexin on synaptic plasticity in the BNST, a region critical to the integration of stress and cocaine reward information, is clearly warranted. Moreover, as our study establishes yohimbine lacks direct agonist activity at the $OX_1R$, future studies should address the precise target(s) and underlying mechanism by which yohimbine affects orexin release and/or signaling in the dlBNST.

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## DISCLOSURE

The authors declare no conflict of interest.

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