



ARTICLE

α_{2A} -adrenergic heteroreceptors are required for stress-induced reinstatement of cocaine conditioned place preference

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The α_{2a} -adrenergic receptor (α_{2a} -AR) agonist guanfacine has been investigated as a potential treatment for substance use disorders. While decreasing stress-induced reinstatement of cocaine seeking in animal models and stress-induced craving in human studies, guanfacine has not been reported to decrease relapse rates. Although guanfacine engages α_{2a} -AR autoreceptors, it also activates excitatory G_i -coupled heteroreceptors in the bed nucleus of the stria terminalis (BNST), a key brain region in driving stress-induced relapse. Thus, BNST α_{2a} -AR heteroreceptor signaling might decrease the beneficial efficacy of guanfacine. We aimed to determine the role of α_{2a} -AR heteroreceptors and BNST G_i -GPCR signaling in stress-induced reinstatement of cocaine conditioned place preference (CPP) and the effects of low dose guanfacine on BNST activity and stress-induced reinstatement. We used a genetic deletion strategy and the cocaine CPP procedure to first define the contributions of α_{2a} -AR heteroreceptors to stress-induced reinstatement. Next, we mimicked BNST G_i -coupled α_{2a} -AR heteroreceptor signaling using a G_i -coupled designer receptor exclusively activated by designer drug (G_i -DREADD) approach. Finally, we evaluated the effects of low-dose guanfacine on BNST cFOS immunoreactivity and stress-induced reinstatement. We show that α_{2a} -AR heteroreceptor deletion disrupts stress-induced reinstatement and that BNST G_i -DREADD activation is sufficient to induce reinstatement. Importantly, we found that low-dose guanfacine does not increase BNST activity, but prevents stress-induced reinstatement. Our findings demonstrate a role for α_{2a} -AR heteroreceptors and BNST G_i -GPCR signaling in stress-induced reinstatement of cocaine CPP and provide insight into the impact of dose on the efficacy of guanfacine as a treatment for stress-induced relapse of cocaine use.

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INTRODUCTION

Stress is a precipitating factor for craving and relapse in cocaine use disorder (CUD) [1–3]; however, there are no FDA-approved medications for the treatment of relapse in CUD. α_{2a} -adrenergic receptor (α_{2a} -AR) agonists inhibit stress-induced reinstatement of operant drug-seeking and conditioned place preference (CPP), animal models useful in the study of stress-induced relapse [4–7]. In clinical laboratory studies, these compounds have been investigated for stress-induced cocaine craving [8, 9], but the application of full α_{2a} -AR agonists for the treatment of CUD has been limited due to adverse effects such as sedation and hypotension [10, 11]. Pretreatment with the α_{2a} -AR partial agonist guanfacine reduces stress-induced craving in female and stress-induced sympathetic tone in male CUD patients [12]. Guanfacine also decreases stress-induced craving of nicotine and alcohol in male and female patients, suggesting a potentially broad applicability for the treatment of stress-induced drug use [12, 13]. Although guanfacine decreases stress, craving, and withdrawal symptoms in clinical trials, it has not been reported to reduce relapse rates [14, 15].

The mechanisms underlying the anti-drug craving effects of guanfacine are unknown, but it has been suggested that guanfacine blunts stress responses through its actions at presynaptic G_i protein-coupled (G_i -coupled) autoreceptors, which decrease norepinephrine (NE) release [16, 17]. Pre-clinical studies show that at low doses, guanfacine blocks stress-induced activation of the extended amygdala, a group of brain regions that contains the central amygdala and bed nucleus of the stria terminalis (BNST) [18]. NE-signaling within the BNST is critical for stress-induced reinstatement of CPP, and previous research suggests that α_{2a} -AR autoreceptors might suppress stress-induced reinstatement by decreasing NE-mediated activation of BNST beta-adrenergic receptors [19–22].

Guanfacine also activates postsynaptic α_{2a} -AR heteroreceptors [23]. α_{2a} -AR heteroreceptors are expressed in non-adrenergic cells and regulate many of the pharmacological and physiological effects of α_{2a} -AR agonists, including analgesia, sedation, and improvements in cognition [24]. We previously reported that within the dorsal BNST (dBNST), a high dose of guanfacine increased cFOS, a proxy marker of cellular activity, via activation

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of α_{2A} -AR heteroreceptors and the subsequent blockade of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels [23, 25, 26]. Furthermore, functionally mimicking intra-dBNST α_{2A} -AR heteroreceptor signaling using virally expressed G_i -coupled designer receptors exclusively activated by designer drug (G_i -DREADDs) also increased dBNST cFOS proportionally to high-dose guanfacine, and produced anxiety-like responses [26]. Given the prominent role of BNST activity in stress-induced reinstatement of cocaine seeking behavior, and the interest in guanfacine as a potential treatment for CUD [9], understanding how α_2 -AR heteroreceptor signaling may regulate these behaviors is imperative. In this study, we demonstrate that α_2 -AR heteroreceptors are necessary for stress-induced reinstatement of CPP, and that mimicking their signaling in the dBNST using G_i -coupled DREADDs is sufficient to induce reinstatement. Finally, we show that a low dose of guanfacine does not increase dBNST activity but blocks stress-induced reinstatement.

METHODS AND MATERIALS

Animals

Male and female wild-type (WT), α_{2A} -AR knockout (KO), and KO mice re-expressing α_{2A} -ARs under the DBH promoter (HeteroKO), bred in house and maintained on a C57BL/6J background were used [26, 27]. For chemogenetic studies, male C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) were delivered at 6 or 7 weeks of age and acclimated for at least 1 week before surgical manipulations. For all conditioning experiments, mice were singly-housed at least 2 weeks prior to experiments. Male and female C57BL/6J mice were used for immunohistochemical experiments and were group-housed with 2–5 mice per cage. All procedures were approved by the Vanderbilt University animal care and use committee.

Behavior

Cocaine CPP training, extinction, and reinstatement testing was conducted as previously described [19]. Testing took place in open-field arenas with two-chamber preference inserts. During conditioning, mice received injections of cocaine (15 mg/kg i.p.) or saline and confined to alternating sides of the CPP apparatus. On the post-conditioning testing day and subsequent days, mice were allowed to move freely between sides. During extinction training, mice were placed in the CPP apparatus daily until they reached the extinction criterion. Mice underwent reinstatement testing 24 h after reaching the extinction criterion. For stress-induced reinstatement of CPP, mice underwent forced swim stress in a beaker of warm water (22–26°C) for 6 min and were placed in the CPP apparatus. For pharmacological studies, WT mice were injected with guanfacine (0.15 mg/kg, i.p.) 30 min prior to forced swim stress. To control for potential stress-induced biases in side occupancy, mice underwent a modified mock CPP procedure in which they only received injections of saline during the conditioning phase (see Supplement for more details on all procedures).

Stereotaxic surgery

Intracranial virus injections bilaterally targeting the dBNST with AAV5-CaMKII α -hM4Di-mcherry or AAV5-CaMKII α -mcherry were performed as previously described [26, 28]. All mice were allowed to recover for at least 3 weeks prior to behavioral testing.

Chemogenetic induction of reinstatement testing

hM4Di- and mcherry-injected mice underwent cocaine CPP conditioning and extinction as described above. Twenty-four hours after reaching the extinction criterion, mice received injections of clozapine-N-oxide (CNO) (3 mg/kg i.p.) [26] 30 min prior to being placed in the CPP apparatus.

Guanfacine-induced cFOS upregulation assay

The cFOS upregulation assay was conducted as previously described [26, 29]. WT mice received i.p. injections of either vehicle (saline) or guanfacine (0.15 mg/kg or 1 mg/kg), and 90 min later, were transcardially perfused. Brain sections containing the dBNST were harvested and processed using immunohistochemistry (see Supplement for details).

Statistics

Data are represented as means, means \pm SEM, or medians. All statistics were run using Prism 8 (GraphPad, La Jolla, CA). Differences between groups were assessed using *t*-tests, one-way, or 2-way repeated measures ANOVAs. Differences in standard deviation between groups were assessed using the Brown-Forsythe test, when significant differences in standard deviation were found using this test, they are reported in the text. Significance was set at $\alpha = 0.05$. When significant main effects were obtained using ANOVA testing, appropriate post-hoc comparisons between groups were performed (see Supplement for detailed list of statistical results).

RESULTS

α_{2A} -AR full or heteroreceptor deletion does not impact the acquisition or extinction of cocaine CPP

Consistent with previous studies [30, 31], we determined that conditioning increased the amount of time WT, KO, and HeteroKO mice spent on the cocaine-paired side during the post-conditioning session, with a main effect of conditioning (pre-conditioning test session (PRE) vs post-conditioning test session (POST), ($F_{2, 30} = 128.7, p < .0001$; two-way RM ANOVA), and a trend towards a main effect of genotype (WT, KO, or HeteroKO), ($F_{2, 30} = 2.95, p = .057$) that is likely due to the HeteroKO mice spending less time in the cocaine-paired side when compared to WTs and KOs (in sec: 819 ± 113 for WT, 821 ± 138 for KO, and 723 ± 59 for HeteroKO). The genotype \times conditioning interaction was not significant ($F_{2, 30} = .99, p = .38$) (Fig. 1c). We found no between-genotype differences in the increase in time on the paired side following conditioning, represented by the preference score (one-way ANOVA), or differences in locomotion during the post-conditioning session (two-way RM ANOVA) (Fig. 1d, e).

Next, we determined the impact of full or heteroreceptor deletion on the extinction of cocaine CPP (see Materials and Methods, Fig. 1f). We found no differences in the latency to reach the extinction criterion between groups (one-way ANOVA; in days: 3 ± 1 for WT, KO, and HeteroKO) (Fig. 1f). Taken together, these findings suggest that α_{2A} -AR auto- and heteroreceptors do not play a role in the acquisition or extinction of cocaine CPP.

α_{2A} -AR full or heteroreceptor deletion disrupts stress-induced reinstatement of CPP

We next determined the impact of α_{2A} -AR KO or HeteroKO on stress-induced reinstatement of cocaine CPP. Twenty-four hours after reaching the extinction criterion, WT, KO, and HeteroKO mice underwent stress-induced reinstatement testing (Fig. 2a). There was a main effect of stress (extinction session (EXT) vs stress-induced reinstatement session (STRESS)) but no significant genotype \times stress interaction (stress $F_{1, 28} = 15.01, p = .0006$ interaction $F_{2, 28} = 2.31, p = .11$, 2-way RM ANOVA). Sidak's multiple comparisons post-hoc test revealed that stress only significantly increased time in the paired side in WT mice when compared to the last day of extinction (EXT vs STRESS: WT $p < .01$, KO $p = .78$, HeteroKO $p = .15$) (Fig. 2b). Stress induced a significant difference in the variance of the preference score of WT, KO, and HeteroKO mice (Brown Forsythe test; $F_{2, 27} = 5.67, p < .001$) that was driven by a stress-induced increase in standard deviation (SD) in KO and HeteroKO mice (WT SD = 7.21, KO SD = 24.51, HeteroKO SD = 26.31) (Fig. 2c). There was a main effect of stress on locomotion, but no effect

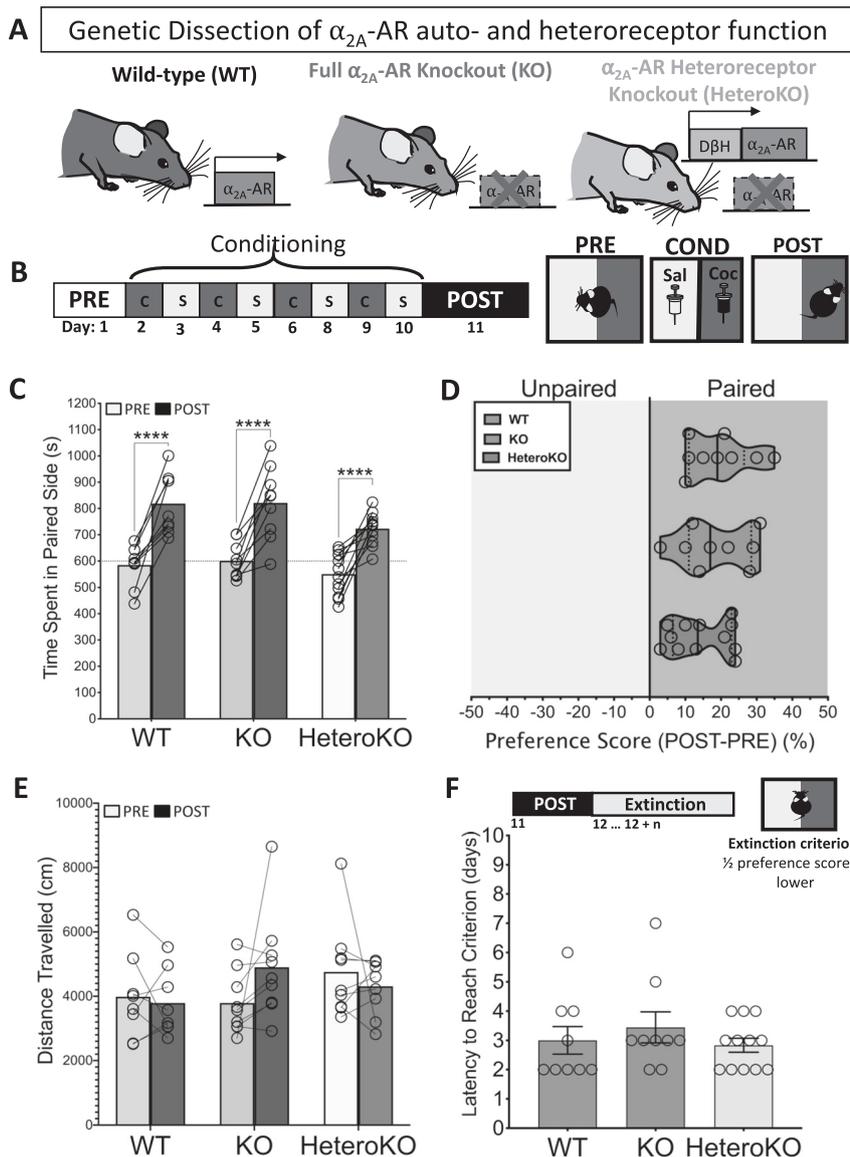


Fig. 1 Full or heteroreceptor α_{2A} -AR KO does not disrupt the acquisition or extinction of cocaine CPP. **a** Genetic model used to determine relative α_{2A} -AR auto- and heteroreceptor function. **b** Timeline and schematic of conditioned place preference (CPP) procedure. **c** Conditioning increased the amount of time mice from all genotypes spent on the paired side when compared to the pre-conditioning session. Data displayed as means with superimposed individual points. **d** There was no difference between genotypes in the preference scores (% change in time spent on the paired side from the pre- and post-conditioning sessions). Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **e** There were no intra-genotype differences in the distance travelled in the CPP apparatus during the pre- and post-conditioning sessions. **f** There were no inter-genotype differences in the latency to reach the extinction criterion. Data displayed as means plus individual points, or means and individual data points \pm SEM (**e**) (WT $n = 9$, KO $n = 8$, HeteroKO $n = 12$) (**** $p < .0001$) (PRE: pre-conditioning session, POST: post-conditioning session).

of genotype, or the interaction (stress $F_{1, 22} = 76.20, p < .0001$, genotype $F_{2, 22} = .03, p = .97$, interaction $F_{2,22} = .45, p = .64$; 2-way RM ANOVA; Sidak's multiple comparisons post-hoc test, EXT vs STRESS: WT $p < .0001$ KO $p < .0001$, HeteroKO $p = .0005$) (Fig. 2d). These findings suggest that stress reinstates cocaine CPP in WT mice, and that this reinstatement might be disrupted in KO mice. Furthermore, this disruption is not ameliorated by re-expression of α_{2A} -AR autoreceptors in heteroKO mice.

α_{2A} -AR full or heteroreceptor deletion does not produce stress-dependent biases in side occupancy in cocaine-naïve mice
The above findings suggest that, by suppressing locomotion, stress may spuriously increase time on one side of the chamber in a subset of KO and HeteroKO mice, independent of the retrieval of

cocaine CPP. We tested this hypothesis by conducting a mock saline CPP experiment followed by stress-induced reinstatement testing. Mice were subjected to a CPP, extinction, and reinstatement procedure as described above, but only received injections of saline during the conditioning stage (mock conditioning) (Fig s1A). Mock conditioning, genotype, or the mock conditioning \times genotype interaction, did not increase time in a randomly-paired side (two-way RM ANOVA), or alter the preference score of WT, KO, or HeteroKO mice (one-way ANOVA) (Fig s1B, C). Interestingly, mock conditioning decreased locomotion in WT and HeteroKO mice, but failed to do so in full KO mice (in cm: 3247 ± 937 for WT, 5115 ± 537 for KO, and 3537 ± 1618 for HeteroKO). This decrease in locomotion was statistically significant, with main effects for both the genotype and mock

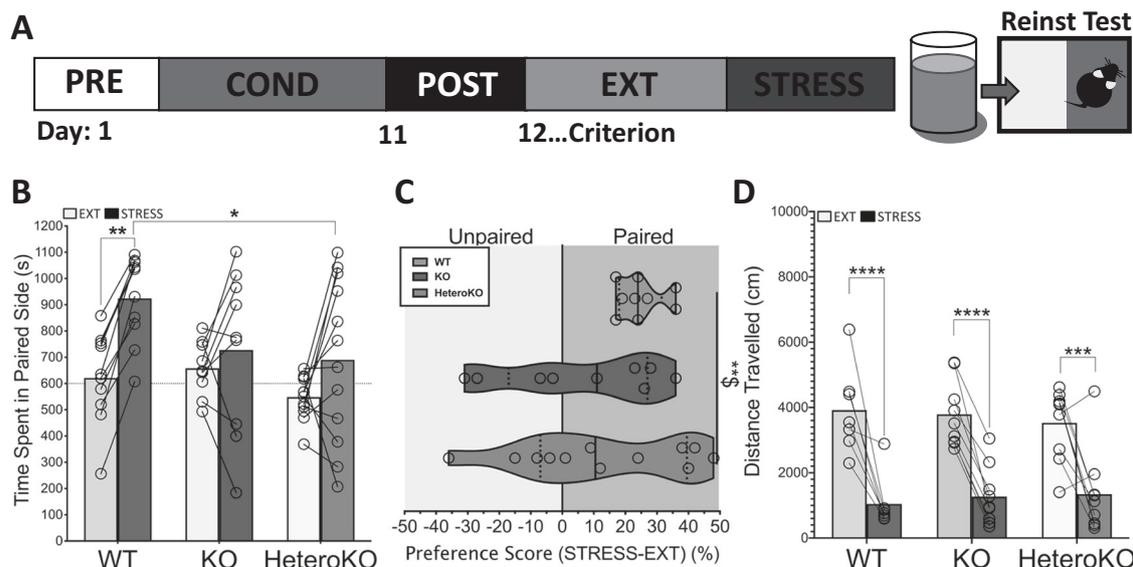


Fig. 2 Full or heteroreceptor α_{2A} -AR KO disrupts stress-induced reinstatement of cocaine CPP. **a** Timeline and schematic of stress-induced reinstatement of CPP procedure. **b** during the stress test session (STRESS), 6 min of forced swim stress increased the time WT mice spent on the paired side compared to the last day of extinction (EXT) and this increase was different from heteroKO mice. Data displayed as means plus individual points. **c** There was a difference between genotypes in the variance of preference scores (Brown-Forsythe test for differences in SD, $***p < .01$). Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **d** There was an intra-genotype difference in distance travelled in the CPP apparatus during EXT and STRESS. Data displayed as means plus individual points (WT $n = 9$, KO $n = 8$, HeteroKO $n = 12$) ($**p < .001$, $****p < .0001$) (EXT: last extinction session, STRESS: stress-induced reinstatement session).

conditioning factors as well as the interaction (mock conditioning $F_{1, 27} = 4.12$, $p = .017$, genotype $F_{2, 27} = 11.54$, $p = .029$, interaction $F_{2, 27} = 4.88$, $p < .01$; two-way RM ANOVA, Sidak's multiple comparisons test, PRE vs POST: WT $p < .01$, KO $p = .92$, HeteroKO $p < .05$) (Fig s1D). The higher level of locomotion in KO mice following repeated exposure to the chamber is consistent with previous findings suggesting that autoreceptors play a role in suppressing spontaneous hyper-locomotion [27].

Twenty-four hours after the last mock extinction training session, mice were subjected to 6 min of forced swim stress and placed in the CPP apparatus (Fig. 3a). Cocaine-naïve mice did not show a significant change in preference for a randomly-paired side of the chamber after stress (two-way RM ANOVA), and we found no inter-genotype differences in preference score of the cocaine-naïve WT, KO, and HeteroKO mice (one-way ANOVA) (Fig. 3b, c). Interestingly, stress increased the SD of the preference score across genotypes when compared to the mock post-conditioning test session (26.65 vs 12.56 for WT, 21.76 vs 14.25 in KO, and 19 vs 11.51 for HeteroKO). This increase in SD demonstrates an enhanced variability in how much time mice spent on each side of the chamber. KO mice showed increased locomotion during the last day of mock extinction, which was significantly different from WT, with main effects of genotype and stress but no interaction (genotype $F_{2, 20} = 3.77$, $p < .05$, stress $F_{1, 20} = 68$, $p < .0001$, interaction $F_{2, 20} = 1.71$, $p = .205$, two-way ANOVA; Sidak's multiple comparisons test, EXT vs STRESS: WT $p < .05$, KO $p < .0001$, HeteroKO $p < .01$). However, mice across all genotypes showed a stress-induced suppression in locomotion during the mock reinstatement test with a main effect of stress ($F_{1, 20} = 68$, $p < .0001$; two-way RM ANOVA). (Fig. 3d). Taken together, these findings indicate that stress suppresses locomotion and increases variability in side occupancy in cocaine-naïve mice and suggests that variability increases due to decreased movement between sides of the CPP chamber.

Activation of dBNST G_i -coupled GPCR signaling reinstates cocaine CPP

We previously reported that α_{2A} -AR heteroreceptors mediate increases in dBNST activity produced by α_{2A} -AR agonists such as

guanfacine, as measured by cFOS upregulation [26]. Due to the fact that α_{2A} -AR receptors are G_i -coupled GPCRs, we previously employed a G_i -coupled DREADD strategy to mimic α_{2A} -AR heteroreceptor signaling and found hM4Di, expressed under the CaMKII α promoter, activation is both sufficient to increase dBNST cFOS in a similar proportion of cells as guanfacine and occlude guanfacine-induced cFOS upregulation, suggesting the recruitment of an overlapping populations of neurons [26]. Due to the prominent role of the BNST in stress-induced reinstatement [32, 33], we investigated whether hM4Di enhancement of dBNST activity would be sufficient to reinstate cocaine CPP. C57BL/6J mice were micro-injected in the dBNST with either AAV5-CaMKII α -mcherry or AAV5-CaMKII α -hM4Di:mcherry, which produced robust expression in this region (Fig. 4b, c, sF2). Following 4 weeks of recovery, mice underwent CPP training, testing, and extinction as described above (sF3. A). Conditioning increased time in the paired side for both mcherry- and hM4Di-expressing mice with a main effect of conditioning, but no effects of virus (mcherry vs hM4Di) or the interaction (conditioning $F_{1, 12} = 66.28$, $p < .0001$, virus $F_{1, 12} = .00$, $p = .996$, interaction $F_{1, 12} = 1.26$, $p = .282$; two-way RM ANOVA; Sidak's multiple comparisons test, PRE vs POST: mcherry $p < .01$, hM4Di $p < .0001$) (Fig s3B). We also found no inter-condition differences in preference score (unpaired t -test, $p = .275$) (Fig s3C), or latency to reach the extinction criterion (unpaired t -test, $P = .593$) (Fig s3D). Twenty-four hours after reaching the extinction criterion, mcherry- and hM4Di-expressing mice were injected with CNO (3 mg/kg, i.p.) as previously described [26] and following a 30-min period, mice underwent reinstatement testing (Fig. 4a). CNO treatment significantly increased the time hM4Di-expressing mice spent on the paired side when compared to the last day of extinction training, but failed to do so in mcherry-expressing mice (Fig. 4d). There was a significant main effect for CNO (EXT vs CNO sessions) and a trend towards significance for the interaction, but no effect of virus (CNO $F_{1, 11} = 8$, $p < .05$, interaction $F_{1, 11} = 4.8$, $p = .0506$; virus $F_{1, 11} = 1.43$, $p = .25$, two-way RM ANOVA, Sidak's multiple comparisons test, EXT vs CNO: mcherry, $p = .9$, hM4Di $p < .01$). There was a significant difference between the preference score of

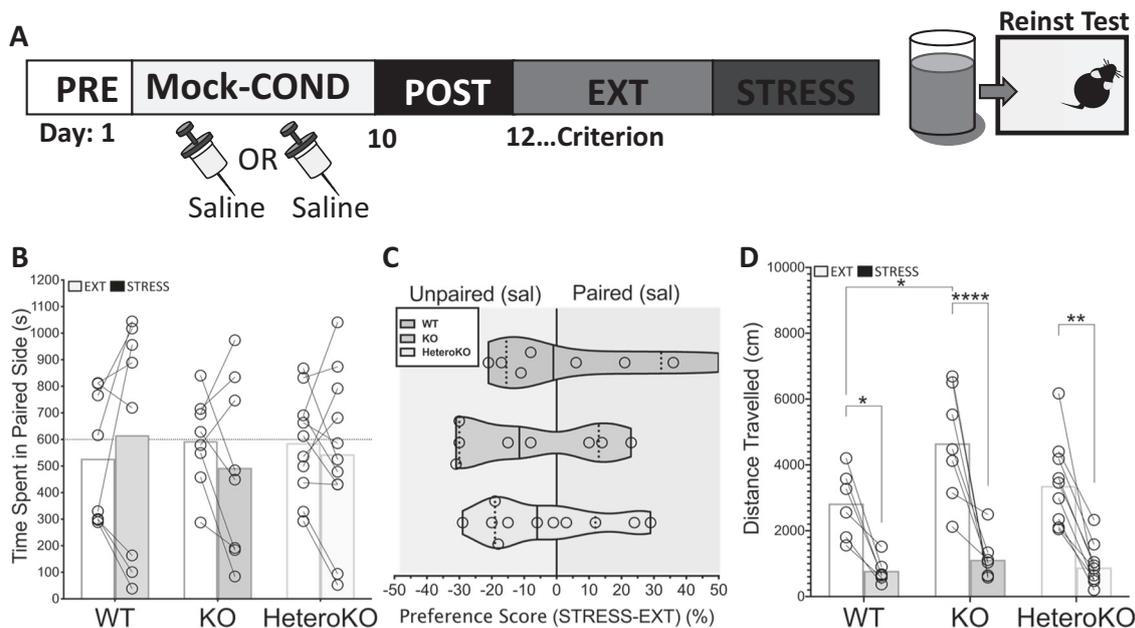


Fig. 3 Full or heteroreceptor deletion of α_{2A} -ARs does not induce stress-dependent biases in side occupancy in cocaine-naïve mice. **a** Timeline and schematic of mock conditioning, extinction, and stress-induced reinstatement test. **b** Stress did not change time in a randomly paired side across genotypes in cocaine-naïve mice. **c** Stress did not produce differences in the preference score between genotypes. **d** Stress suppressed locomotion across genotypes and there was a difference in locomotion between WT and KO mice during the last day of mock extinction (EXT) (WT $n = 8$, KO $n = 8$, HeteroKO $n = 11$). Data displayed as means with individual data points (B and C) or medians (solid lines) and quartiles (dashed lines with individual data points (C and G) ($*p < .05$, $**p < .01$, $****p < .0001$) (EXT: last extinction session, STRESS: stress-induced reinstatement session).

hM4D_i- and mcherry-expressing mice (unpaired t -test, $t_{2,11} = 2.239$, $p < .05$) (Fig. 4e). CNO-treatment did not alter locomotion in hM4D_i- or mcherry-expressing mice (two-way RM ANOVA) (Fig. 4f). Further, we found that in an additional group of mice in which the location of mcherry or hM4D_i was off target (Fig s4A), CNO did not induce reinstatement, or alter locomotion (Fig s4B-D). Taken together, these findings suggest that engagement of G_i-GPCR signaling in the dBNST is sufficient to reinstate cocaine CPP without impacting locomotion.

A low dose of guanfacine that does not increase cFOS within the BNST blocks stress-induced reinstatement of cocaine CPP

While non-specific α_2 -AR agonism blocks stress-induced reinstatement of drug seeking, high doses of these compounds have negative effects, which limit their clinical applications [10]. We previously reported that a 1 mg/kg dose of the α_{2A} -AR partial agonist guanfacine, which produces a strong sedative effect, increased dBNST activity as measured by cFOS upregulation [26]. Given the prominent role of BNST activity in reinstatement of drug seeking and anxiety-like behaviors [34–36], we hypothesized that a lower dose of guanfacine that shows antidepressant- and anxiolytic-like effects (0.15 mg/kg), would not increase cFOS in the dBNST but would block stress induced reinstatement [37, 38]. To determine dose-dependent changes in dBNST activity, we assessed cFOS expression within the dBNST following guanfacine administration [26]. Mice were injected with either saline (vehicle, VEH), low (0.15 mg/kg), or high dose (1 mg/kg) guanfacine (Fig. 5a). As we previously reported, high dose guanfacine increased the number of cFOS positive cells within the dBNST, however, this effect that was not present in VEH- or low dose-treated mice ($F_{2,14} = .154$, $p < .0001$; one-way ANOVA, Sidak's multiple comparisons test: VEH vs 0.15 mg/kg $p = .88$, VEH vs 1 mg/kg $p < .0001$) (Fig. 5b, c).

We next examined the potential anti-reinstatement effects of low dose guanfacine. WT mice that had undergone CPP training and extinction were injected with either VEH or low dose guanfacine, 30 min prior to stress (Fig. 5d). While we did not find

main effects of drug (VEH vs GUAN) or stress, we found a main effect of the treatment \times session interaction (Drug $F_{1,12} = .01$, $p = .9$, stress $F_{1,12} = 1.49$, $p = .24$, interaction $F_{1,12} = 5.843$, $p = .032$; two-way RM ANOVA), driven by a stress-induced increase in time on the paired side in VEH-treated mice that was not present in guanfacine-treated mice (Fig. 5e). Guanfacine-treated mice also showed a significant decrease in preference score when compared to VEH-treated mice ($t = 2.417$, $df = 12$, $p < .05$; unpaired t -test) (Fig. 5f). While stress significantly suppressed locomotion in both groups, there were no differences in locomotion in VEH- and guanfacine-treated mice following stress, and no main effect of drug, suggesting that low dose guanfacine treatment did not have further sedative effects (interaction $F_{1,12} = 10.34$, $p < .01$, drug $F_{1,12} = .18$, $p = .67$, stress $F_{1,12} = 222.5$, $p < .0001$; two-way RM ANOVA, Sidak's multiple comparisons test, STRESS: VEH vs guanfacine $p = .44$, EXT vs STRESS: VEH $p < .0001$, guanfacine $p < .0001$) (Fig. 5g). Taken together, these findings suggest low dose guanfacine does not increase the activity of the BNST but blocks stress-induced reinstatement without impacting locomotion.

DISCUSSION

α_{2A} -AR agonists such as guanfacine have risen in popularity for the treatment of several psychiatric conditions [39, 40]. α_{2A} -AR agonists target autoreceptors expressed in adrenergic neurons and heteroreceptors expressed in non-adrenergic cells. Determining the relative contributions of these receptor populations to the pharmacological effects of α_{2A} -AR agonists and behavior has been challenging, due to the inability to distinguish these populations using conventional pharmacological or genetic approaches [27]. In the current study, we used selective genetic deletion models, behavior, histology, and chemogenetics to define the role of α_{2A} -AR heteroreceptors on stress-induced reinstatement of cocaine CPP.

In our unbiased two-sided CPP apparatus, stress reinstated cocaine CPP in all wild-type mice, but only a fraction of α_{2A} -AR full

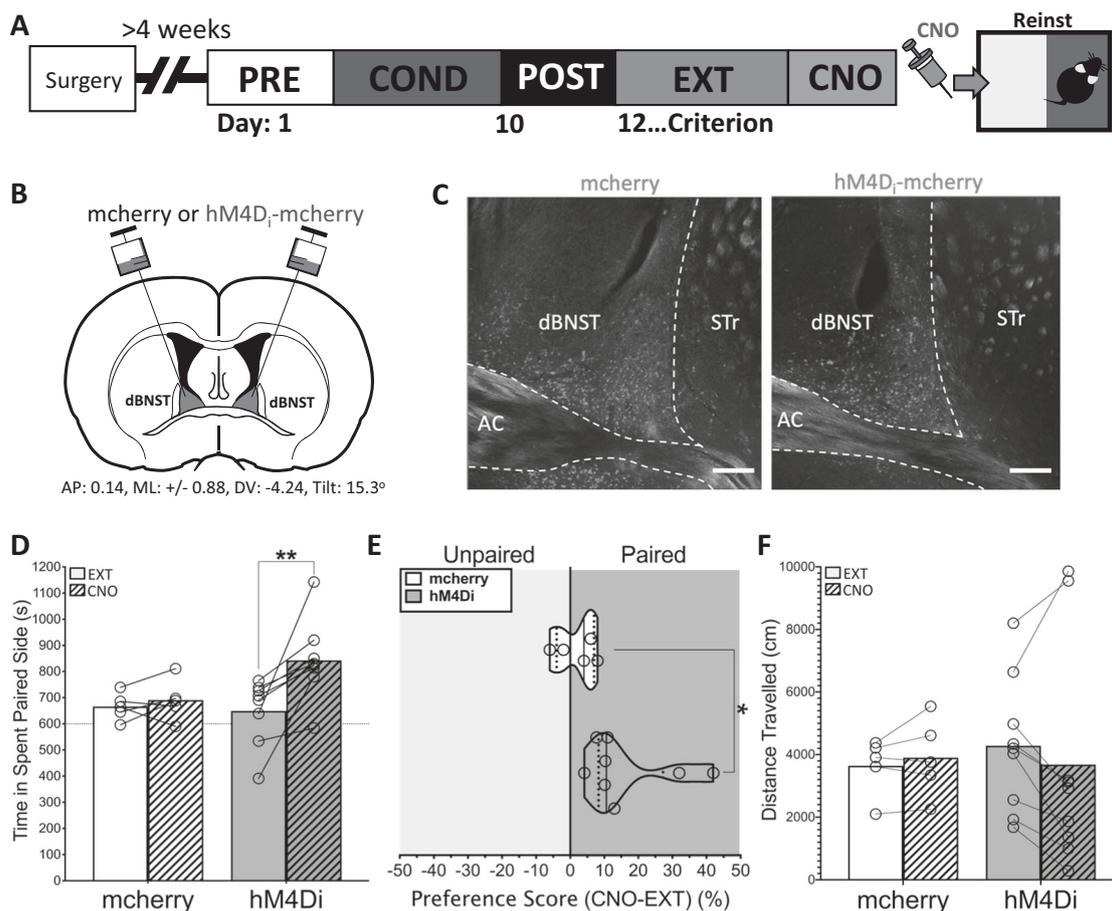


Fig. 4 Activation of G_1 signaling within the BNST reinstates cocaine CPP. **a** Timeline and schematic showing surgery, recovery and CNO-induced reinstatement of CPP procedure. **b** Schematic and coordinates of injection site for mcherry and mcherry-tagged hM4Di. **c** Low magnification (10 \times) images showing spread of mcherry and mcherry-tagged hM4Di in BNST. Scale bar = 200 μ m. **d** CNO treatment (CNO) increased the time hM4Di-expressing mice spent on the paired side when compared to the last day of extinction (EXT) but did not increase the time in mcherry-expressing controls. Data displayed as means plus individual data points. **e** CNO treatment increased the CPP score of hM4Di-expressing mice compared to mcherry-expressing controls. Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **f** CNO treatment did not alter locomotion in mcherry- or hM4Di-expressing mice when compared to the EXT session. Data displayed as means with individual data points (mcherry $n = 5$, hM4Di = 8) ($p < .05$) (EXT: last extinction session, CNO: CNO-induced reinstatement session).

and HeteroKO mice. Following stress, we observed a high degree of variability in the amount of time α_{2A} -AR KO and HeteroKO mice spent on the two sides of the apparatus. This increase in variability was also found in cocaine-naïve mice from all genotypes, but not in cocaine-treated WT. Notably, stress reduces locomotion in the CPP assay independent of genotype or drug exposure history. Therefore, our data suggest that in α_{2A} -AR full and heteroreceptor knockouts, stress-induced reinstatement of CPP is replaced by increased occupancy in an arbitrary side of the chamber, due to an overall reduction in activity rather than side preference. While this is a parsimonious interpretation of the data, currently we cannot unequivocally rule out the possibility that some of the α_{2A} -AR full and HeteroKO mice show stress-induced reinstatement. Regardless of this limitation, it is still clear that α_{2A} -AR heteroreceptors positively regulate reinstatement behavior.

Our previous findings have demonstrated functional recovery of autoreceptors in the dBNST in this model [26], suggesting the surprising result that α_{2A} -AR heteroreceptor deletion disrupts the ability of stress to drive reinstatement of preference for the cocaine-paired side. It is possible that within the dBNST, α_{2A} -AR auto- and heteroreceptors play opposing roles, where autoreceptors might prevent reinstatement by decreasing NE release, heteroreceptor, and beta-adrenergic signaling [20, 21]. Conditions

in which large amounts of NE are released into the BNST, such as chronic stress, might override autoreceptor regulation to lead to reinstatement [41, 42].

We used a chemogenetic approach to mimic dBNST α_{2A} -AR heteroreceptor signaling to determine the effects of G_1 -GPCRs on reinstatement of cocaine CPP. We found that acute activation of BNST hM4Di was sufficient to induce reinstatement of CPP. This G_1 -DREADD-induced reinstatement is consistent with our findings showing that activation of G_1 -DREADDs within the BNST increases activity and anxiety-like responses [26]. Previous work has shown that activation of intra-BNST G_1 -DREADDs can inhibit drug consumption or the acquisition of CPP [43–46]. Notably, these studies focused on alcohol consumption and CPP acquisition without a focus on reinstatement. Thus, our study provides novel insight into the potential role of G_1 -GPCR signaling on stress-induced reinstatement of cocaine CPP. Additionally, we previously reported that direct α_{2A} -AR activation or G_1 -DREADD mimicking of α_{2A} -AR signaling in excitatory inputs onto BNST CRF cells decreases stress-induced cFOS [29]. Taken together, these findings highlight the heterogeneous nature of α_{2A} -AR regulation of BNST activity. Future studies will aim to determine the role of different BNST cell populations and projections in the pro-reinstatement effects of G_1 -coupled GPCR signaling.

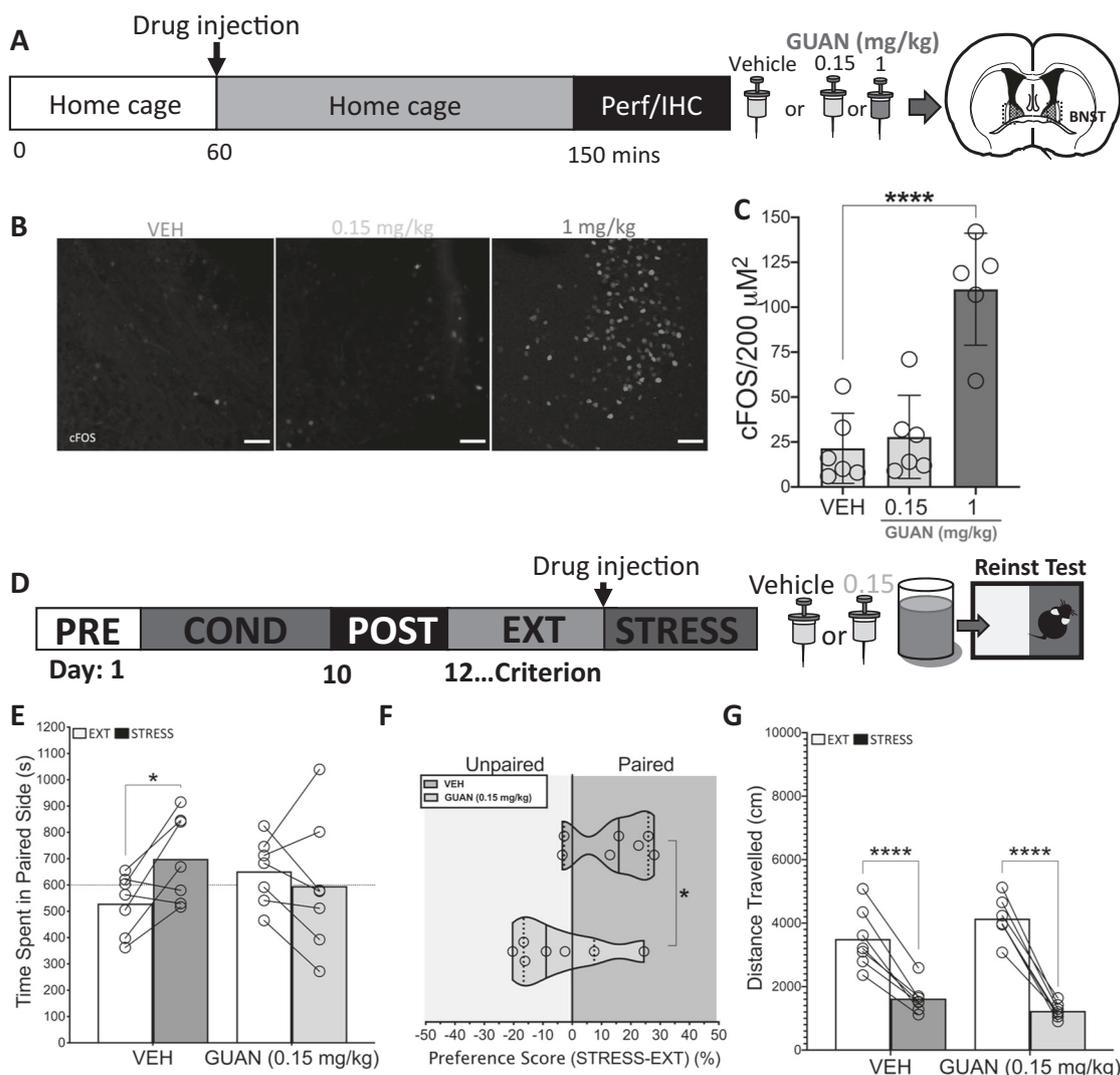


Fig. 5 A dose of guanfacine that does not increase BNST cFOS blocks stress-induced reinstatement of cocaine CPP. **a** Timeline and schematic of drug injection and immunohistochemistry experiment. Following an hour of acclimation mice were injected with saline (Vehicle, VEH) or guanfacine (GUAN) and sacrificed 90 min post-injection. **b** High magnification (x20) images showing cFOS staining in dorsal BNST. Scale bar = 50 μm. **c** 1 mg/kg injection of GUAN increased cFOS within the BNST. (VEH *n* = 6, 0.15 mg/kg *n* = 6, 1 mg/kg *n* = 5). **d** Timeline and schematic of stress-induced reinstatement blockade experiment. Mice were injected with VEH or GUAN (0.15 mg/kg) 30 min prior to stress. **e** Stress increased time in the paired side in VEH-treated mice but failed to do so in GUAN-treated mice. Data displayed as means plus individual data points. **f** There was a difference in the CPP score of VEH-treated and GUAN-treated mice. Data displayed as individual points overlaid on top of medians (solid lines) and quartiles (dashed lines). **g** Stress suppressed locomotion in VEH- and GUAN-treated mice but there were not inter-treatment differences in locomotion. Data displayed as means plus individual data points (VEH *n* = 7, GUAN *n* = 7) (**p* < .05, *****p* < .0001) (EXT; last extinction session, STRESS; stress-induced reinstatement test session).

A pro-reinstatement role of heterosynaptic α_{2A} -ARs contrasts with previous reports suggesting that systemic administration of the nonselective α_2 -AR antagonist yohimbine and the α_{2A} -AR antagonist BRL44408 reinstates CPP [19]. While yohimbine antagonizes α_{2A} -ARs and increases norepinephrine levels, it is also a 5-HT_{1A} receptor agonist [47, 48]. Yohimbine-induced reinstatement of cocaine- and food-seeking, or cocaine CPP is not blocked by application of the α_{2A} -AR agonist clonidine [19, 49]. Indeed, yohimbine's effects on drug-associated behaviors require orexin and serotonin 5-HT_{1A} receptor signaling [30, 50]. Furthermore, we have previously reported that within the BNST, yohimbine produces α_{2A} -AR independent, orexin receptor-1 dependent decreases in excitatory transmission [30]. Therefore, our current findings add to the growing body of evidence suggesting that the pro-reinstatement effects of yohimbine may not be dependent on modulation of adrenergic signaling. BRL44408 also reinstated CPP,

presumably through inhibition of α_{2A} -AR autoreceptors [7]. The location of these receptors mediating the pro-reinstatement effects of this compound remain unknown, but would be predicted to involve presynaptic terminal α_{2A} -ARs [29].

As we previously found that a high dose of guanfacine increases BNST activity, we next sought to determine if a low dose of guanfacine, which has been previously reported to reduce anxiety-like behaviors, would lack excitatory effects in the BNST [37, 38]. We replicated our previous finding showing that a high dose of guanfacine increases cFOS in the BNST, but found that the low dose did not. This suggests that a low dose of guanfacine does not engage BNST α_{2A} -AR heteroreceptors. We also found that low dose guanfacine blocked stress-induced reinstatement of CPP without impacting locomotion. While previous work has shown that higher doses of the α_{2A} -AR agonist clonidine do not block stress-induced reinstatement [19], the strong hypo-locomotive

effects of the 1 mg/kg dose of guanfacine prevented us from testing this dose in the current study. Future studies will aim to determine the effects of intra-dBNST manipulation of α_2A -AR signaling. These findings may inform the development of dose-targeted guanfacine for the treatment of substance use disorders and are congruent with recent reports suggesting that the efficacy of guanfacine in some clinical applications is dose-dependent [51, 52].

Notably, while α_2A -AR agonism shows sex-dependent effects in patients with CUD [12], we did not find any apparent sex differences in the effects of α_2A -AR full or heteroreceptor KO, therefore data from male and female mice were combined in our statistical analyses (see Supplemental Figs. 5–8 for values separated by sex). However, one limitation of the current study is that it is not sufficiently powered to analyze more subtle sex differences. While a recent report showed that the low dose of guanfacine used in the current study equally prevented forced swim-induced reinstatement of nicotine CPP in male and female mice [18], future investigations will be aimed at determining potential sex differences in α_2A -AR regulation of BNST activity as well as low-dose guanfacine on cocaine CPP reinstatement.

Our findings demonstrate a previously unknown role of α_2A -AR heteroreceptors in stress-induced reinstatement of cocaine-associated behaviors. Additionally, we also expand on previous reports suggesting that guanfacine should be further explored as a potential treatment for CUD and other drug use disorders.

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AUTHOR CONTRIBUTIONS

R.E.P. conceived the study, designed and performed experiments, and co-wrote the paper. A.B. analyzed data, validated viral deposits, and contributed to writing. B.P.N. piloted behavioral studies. N.A.H. performed surgery, assisted in experimental design, and analysis. O.M.F. performed surgery. S.P., R.G., and L.H. provided materials and resources. D.G.W. conceived the study, designed experiments, and co-wrote the paper.

ADDITIONAL INFORMATION

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