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Behavioral/Cognitive

Stress-Induced Reinstatement of Nicotine Preference Requires Dynorphin/Kappa Opioid Activity in the Basolateral Amygdala

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The dynorphin (DYN)/kappa-opioid receptor (KOR) system plays a conserved role in stress-induced reinstatement of drug seeking for prototypical substances of abuse. Due to nicotine’s high propensity for stress-induced relapse, we hypothesized that stress would induce reinstatement of nicotine seeking-like behavior in a KOR-dependent manner. Using a conditioned place preference (CPP) reinstatement procedure in mice, we show that both foot-shock stress and the pharmacological stressor yohimbine (2 mg/kg, i.p.) induce reinstatement of nicotine CPP in a norbinaltorphimine (norBNI, a KOR antagonist)-sensitive manner, indicating that KOR activity is necessary for stress-induced nicotine CPP reinstatement. After reinstatement testing, we visualized robust c-fos expression in the basolateral amygdala (BLA), which was reduced in mice pretreated with norBNI. We then used several distinct but complementary approaches of locally disrupting BLA KOR activity to assess the role of KORs and KOR-coupled intracellular signaling cascades on reinstatement of nicotine CPP. norBNI injected locally into the BLA prevented yohimbine-induced nicotine CPP reinstatement without affecting CPP acquisition. Similarly, selective deletion of BLA KORs in KOR conditional knock-out mice prevented foot-shock-induced CPP reinstatement. Together, these findings strongly implicate BLA KORs in stress-induced nicotine seeking-like behavior. In addition, we found that chemogenetic activation of G<i>Gi</i>-linked intracellular signaling within CaMKIIα BLA neurons was sufficient to induce nicotine CPP reinstatement, identifying an anatomically specific intracellular mechanism by which stress leads to reinstatement. Considered together, our findings suggest that activation of the DYN/KOR system and G<i>Gi</i> signaling within the BLA is both necessary and sufficient to produce reinstatement of nicotine preference.

Key words: basolateral amygdala; conditioned place preference; kappa opioid receptors; nicotine; reinstatement; stress

Significance Statement

Considering the major impact of nicotine use on human health, understanding the mechanisms by which stress triggers reinstatement of drug-seeking behaviors is particularly pertinent to nicotine. The dynorphin (DYN)/kappa-opioid receptor (KOR) system has been implicated in stress-induced reinstatement of drug seeking for other commonly abused drugs. However, the specific role, brain region, and mechanisms that this system plays in reinstatement of nicotine seeking has not been characterized. Here, we report region-specific engagement of the DYN/KOR system and subsequent activation of inhibitory (G<i>Gi</i>-linked) intracellular signaling pathways within the basolateral amygdala during stress-induced reinstatement of nicotine preference. We show that the DYN/KOR system is necessary to produce this behavioral state. This work may provide novel insight for the development of therapeutic approaches to prevent stress-related nicotine relapse.

Introduction

Nicotine use is the leading cause of preventable death, being responsible for about one in every five deaths in the United States, and has strikingly high rates of relapse (Lerman et al., 2014). Relapse to smoking occurs in >90% of abstinent smokers and stress is the most common reason reported for relapse (al’Absi et al., 2007). In laboratory studies, both foot shock and the pharmacological stressor yohimbine have been shown to reinstate drug-
seeking behaviors reliably for prototypical substances of abuse such as cocaine, opiates, and nicotine (Sinha et al., 2011; Mantsh et al., 2016). Yohimbine is an α-2 adrenergic receptor antagonist that promotes responses of stress and dysphoria in humans and rodents and there is accumulating evidence that the dynorphin (DYN)/kappa-opioid receptor (KOR) system plays a role in these effects (Koob, 2013; Grella et al., 2014; Lalanne et al., 2014; Zhou and Leri, 2016). Conditioned place preference (CPP) procedures are especially useful to study nicotine reinstatement due to the importance of contextual cues in the nicotine addiction process (Subramaniam and Dani, 2015) and because cue-induced craving is a strong predictor of relapse to nicotine use in humans (Drougas et al., 1995; Janes et al., 2010). In addition, place preference procedures are increasingly being used in human drug abuse studies, providing more validity for CPP rodent models (Napier et al., 2013; Astur et al., 2016). Redila and Chavkin (2008), show that stress-induced reinstatement of cocaine CPP is blocked by the KOR antagonist norbinaltorphimine (norBNI). Forced swim stress has been shown to restate nicotine CPP in mice (Jackson et al., 2013; Titomanlio et al., 2014) and can be prevented by KOR antagonism (Jackson et al., 2013), suggesting that KOR activation through endogenous DYN released during stress is necessary for reinstatement. We recently showed that activating the KOR system with a KOR agonist is sufficient to induce reinstatement of both cocaine and nicotine CPP (Al-Hasani et al., 2013a; Al-Hasani et al., 2013b). However, how the KOR system plays a role in nicotine reinstatement is less understood. Here, we tested the hypothesis that foot-shock stress and the pharmacological stressor yohimbine would both produce endogenous DYN release and activate specific regional KORS and induce reinstatement of nicotine CPP.

The amygdala is known to be involved in reward-context/cue associations, specifically functioning to assign importance to reward associated cues and to continually update the value of those cues (Murray, 2007; Koob, 2009). The basolateral amygdala (BLA) is KOR rich (Mansour et al., 1988) and activation of DYN/KOR systems in the BLA has anxiogenic effects (Bruchas et al., 2009; Knoll et al., 2011; Crowley et al., 2016). KOR function in the BLA and associated circuits is activated by stress and plays a role in stress-induced dysphoria (Land et al., 2008). Nicotine CPP has been shown to be potentiated by stress via the BLA without affecting other anxiety-related behaviors (Smith et al., 2012). Activation of KORS with the KOR agonist U50,488 decreases synaptic transmission and LTP in the BLA and inhibits glutamatergic activity of BLA to bed nucleus of stria terminalis (BNST) projection neurons to mediate anxiogenic behavior (Huge et al., 2009; Crowley et al., 2016). Considering the effects of BLA KOR activation on anxiety-like behavior and on cellular activity within the BLA, we hypothesized that KOR signaling in the BLA may play a role in stress-induced nicotine CPP reinstatement. Specifically, we hypothesized that stress-induced activation of the endogenous DYN/KOR system within the BLA would be necessary for stress to restate nicotine CPP.

Here, we report that DYN/KOR activity in the BLA is a key mediator of stress-induced nicotine reinstatement. We show that activation of Gαi signaling within the BLA is sufficient for nicotine CPP reinstatement and that BLA KORS are necessary for this effect. Our results provide new insights on how KORS transduce the effects of stress and add to the scientific basis for considering KOR antagonists as potential therapies for nicotine use disorders.

Materials and Methods

Animals
Male WT C57BL/6 mice, KOR<sup>−/−</sup>, DYN<sup>−/−</sup>, KOR conditional KO (cKO) (Van’t Veer et al., 2013a), and KOR cKO<sup>idTomato</sup> (a line generated by crossing KOR cKO mice with Ai9dTomato reporter mice) mice with a C57BL6 background weighing 25–30 g (8–10 weeks old) at the time of behavioral testing were used. Surgery was performed on 6–7-week-old mice to ensure appropriate age at time of CPP testing. Mice were housed 5–7 per cage and maintained in a holding room adjacent to the behavioral testing room for at least 7 days before testing to ensure access to food and water and were maintained on a 12 h light/dark schedule (lights on at 7:00 A.M. and off at 7:00 P.M.). All procedures were approved by the Animal Care and Use Committee of Washington University and were in accordance with National Institutes of Health guidelines.

Genotyping
Three- to 4-week-old mice were tail clipped to obtain DNA samples for genotyping. PCR screening was used with the following primers: KOR <sup>−/−</sup> (5′-GTC TAA TTG TTT GGC CAC TGC AGA TGC TGG-3′ and 3′-TAG ATG TTT GGT GGG TTC TTC TTT GGC CAC TGG-5′) yield a 450 bp product in WT mice and (5′-TGCG TCC TGC GCA GAA AGT ATC CAT CAT TGG C-3′ and 3′-GGG CAA CTT CAG CAA TAT CAC GGC TAG-5′) which yields a 380 PCR product in KO mice. The following primers were used to genotype DYN<sup>−/−</sup> mice: 5′-DYN (Mouse) 30 bp: ATC TCG TCA GAT TTG CCT CTT CCT GGT ACC CAC ATG-5′ and: 3′-DYN (Mouse) 30 bp: CAT AGC GCT TCT CCT ACC TCA TCT GAC (500 bp PCR product); 5′′-Neo (MG) 380 bp: TGC TCC TGG CAA GAG ATG CAT CAC GAT C-3′ and: 3′-Neo (MG) 380 bp: CGG CAA CCT CTT CAG CAA CAC GGG TAG (380 bp PCR product). KOR cKO mice: (5′-TAT GTT CTC CCT CAA GTG CCA CAC GCT GGA GTA CCA CAG TTG TAC CTA TGT GGA CAA GGA AGT ATC CAT CAT CAC GGC-3′) with PCR products of 399 bp for WT DNA, 459 bp for uncombined DNA, and 563 bp for the knock-out (Van’t Veer et al., 2013a). A9′ was confirmed with the following primers: A9 WT (Structural Bioinformatics) 20 bp: AAG GGA GCT GCA GTG GAT A; A9 WT REV (A9 Structural Bioinformatics) 20 bp: CCA AAA ATC TGT GGT GAG ATG TG; A9 WT Mutant FWD (A9 Structural Bioinformatics) 20 bp: CTG TCT CTC TCG CAA GGA ATG ATC CAT CAT CAC GGC C-3′ and: 3′-A9 WT Mutant REV (A9 Structural Bioinformatics) 20 bp: GCG ATT AAA CAC GCA GAG TAT CC-5′ with PCR products of 275 bp for WT and 150 bp for tdTomato positive.

Drug administration
(−) Nicotine hydrogencarbonate tartrate salt (Sigma-Aldrich, N5260) (0.5 mg/kg, corrected for the weight of the tartrate salt) was dissolved in saline and injected subcutaneously. Yohimbine hydrochloride (2 mg/kg) (Sigma-Aldrich, Y3125) was dissolved in sterile water and injected intraperitoneally. Norbinaltorphimine (norBNI dihydrochloride (log no. 13335–66A) was injected intraperitoneally (10 mg/kg) or intracranially (2.5 μg/μl), dissolved in saline or ACSF, respectively, as the vehicle. Clozapine N-oxide (CNO, 1 mg/kg, i.p.) was purchased from Enzo Life Sciences, BML-N5105-0025). CNO was made in 0.5% DMSO in saline.

Handling/habituation
Mice were transported to the CPP behavior testing room and handled once per day for at least 7 d before behavioral testing. Each mouse was scruffed, its belly rubbed, and head gently poked to habituate them to the testing environment. Testing began 48 h after genotyping. PCR screening was used with the following primers: KOR FWD (oIMR9020) 20 bp: AAG GGA GCT GCA GTG GAT A; 3′-A9 WT Neo (MGC) 380 30 bp: CGC CAA GCT CTT CAG CAA TAT CAC GGG TAG (380 bp PCR product). KOR cKO mice: (5′-TAT GTT CTC CCT CAA GTG CCA CAC GCT GGA GTA CCA CAG TTG TAC CTA TGT GGA CAA GGA AGT ATC CAT CAT CAC GGC-3′) with PCR products of 399 bp for WT DNA, 459 bp for uncombined DNA, and 563 bp for the knock-out (Van’t Veer et al., 2013a). A9′ was confirmed with the following primers: A9 WT (Structural Bioinformatics) 20 bp: AAG GGA GCT GCA GTG GAT A; A9 WT REV (A9 Structural Bioinformatics) 20 bp: CCA AAA ATC TGT GGT GAG ATG TG; A9 WT Mutant FWD (A9 Structural Bioinformatics) 20 bp: CTG TCT CTC TCG CAA GGA ATG ATC CAT CAT CAC GGC C-3′ and: 3′-A9 WT Mutant REV (A9 Structural Bioinformatics) 20 bp: GCG ATT AAA CAC GCA GAG TAT CC-5′ with PCR products of 275 bp for WT and 150 bp for tdTomato positive.

CPP
We used a modified three-chamber CPP apparatus consisting of two square boxes (27 cm × 27 cm) that served as the conditioning chambers separated by a small center area that served as the passage way (5 cm wide × 8 cm long) between boxes, as described previously (Land et al., 2008; Bruchas et al., 2011; Al-Hasani et al., 2013a; Al-Hasani et al., 2013b). Boxes had 2.5 cm black-and-white vertical stripes or horizontal lines.
Table 1. Average time spent in drug-paired side during each test for all groups

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pre</th>
<th>Post</th>
<th>Extinction</th>
<th>Reinstatement</th>
<th>Prime</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-only (foot shock)</td>
<td>471.1 (11.79)</td>
<td>482.6 (27.90)</td>
<td>518.7 (27.89)</td>
<td>523.7 (20.34)</td>
<td>489.4 (36.57)</td>
</tr>
<tr>
<td>Foot shock + saline</td>
<td>449.2 (10.30)</td>
<td>396.8 (19.51)</td>
<td>466.1 (17.79)</td>
<td>606.0 (26.96)</td>
<td>637.1 (73.91)</td>
</tr>
<tr>
<td>Foot shock + norBNI</td>
<td>438.2 (19.10)</td>
<td>586.2 (21.76)</td>
<td>480.9 (20.73)</td>
<td>471.7 (21.02)</td>
<td>572.9 (93.22)</td>
</tr>
<tr>
<td>Saline-only (yohimbine)</td>
<td>548.2 (16.21)</td>
<td>441.4 (56.13)</td>
<td>505.9 (52.09)</td>
<td>532.3 (51.26)</td>
<td>N/A</td>
</tr>
<tr>
<td>Yohimbine + saline</td>
<td>407.6 (18.33)</td>
<td>573.4 (24.71)</td>
<td>489.9 (25.14)</td>
<td>688.9 (47.47)</td>
<td>N/A</td>
</tr>
<tr>
<td>Yohimbine + norBNI</td>
<td>417.2 (23.49)</td>
<td>594.8 (19.04)</td>
<td>524.1 (24.27)</td>
<td>530.1 (32.79)</td>
<td>N/A</td>
</tr>
<tr>
<td>WT</td>
<td>461.0 (27.31)</td>
<td>558.2 (47.76)</td>
<td>382.4 (33.40)</td>
<td>520.1 (38.20)</td>
<td>784.8 (61.14)</td>
</tr>
<tr>
<td>KOR^{-/-} + yohimbine</td>
<td>482.0 (15.34)</td>
<td>594.7 (12.51)</td>
<td>470.4 (40.52)</td>
<td>497.3 (32.82)</td>
<td>825.5 (62.42)</td>
</tr>
<tr>
<td>DYN^{-/-} + yohimbine</td>
<td>396.4 (21.12)</td>
<td>541.5 (41.63)</td>
<td>431.8 (31.89)</td>
<td>446.3 (33.48)</td>
<td>590.8 (83.81)</td>
</tr>
<tr>
<td>mCitrine</td>
<td>445.8 (49.5)</td>
<td>613.0 (50.92)</td>
<td>585.1 (65.44)</td>
<td>547.9 (67.88)</td>
<td>N/A</td>
</tr>
<tr>
<td>Gi</td>
<td>461.5 (8.76)</td>
<td>611.8 (36.37)</td>
<td>564.2 (28.55)</td>
<td>684.3 (39.74)</td>
<td>N/A</td>
</tr>
<tr>
<td>BLA ACSF + yohimbine</td>
<td>423.5 (24.42)</td>
<td>581.2 (26.21)</td>
<td>487.4 (29.00)</td>
<td>618.4 (27.59)</td>
<td>106.0 (104.7)</td>
</tr>
<tr>
<td>BLA norBNI + yohimbine</td>
<td>419.4 (74.67)</td>
<td>568.2 (13.60)</td>
<td>433.8 (24.86)</td>
<td>487.2 (52.88)</td>
<td>531.5 (63.85)</td>
</tr>
<tr>
<td>KORcKO/BLA/GFP/foot shock</td>
<td>450.2 (20.22)</td>
<td>541.8 (20.76)</td>
<td>512.3 (27.74)</td>
<td>653.9 (49.18)</td>
<td>738.5 (82.66)</td>
</tr>
<tr>
<td>KORcKO/BLA/Cre/foot shock</td>
<td>458.8 (17.84)</td>
<td>576.9 (17.52)</td>
<td>509.4 (26.98)</td>
<td>511.1 (32.54)</td>
<td>670.4 (63.18)</td>
</tr>
</tbody>
</table>

Shown are the mean time (SEM) in seconds spent in the drug-paired side of the CPP apparatus during each stage of behavioral testing (pre, post, extinction, reinstatement, prime) for each group in each experiment in the study.

N/A, Not applicable.
2007), we were able to give mice 7 d to recover before the start of behavioral testing.

**Viral injections.** For KOR conditional KO studies, KOR floxed (KOR cKO; see Fig. 5A, left) mice were bilaterally injected into the BLA with either AAV5-Cre-GFP (8.1 × 10^{11} vg/ml) or the control virus AAV5-GFP, both purchased from UNC Viral Vector Core (see Fig. 5D–F). Then, 400 nl of virus was injected on each side at a rate of 100 nl/min. The syringle was left in place for an additional 8 min before retraction to avoid capillary effects, for a total injection time of 12 min per side. To confirm conditional deletion of KORs from KOR cKO mice and to visualize successful cre recombination, KOR cKO mice were also crossed with the cre-dependent Ai9 tDTomato reporter mouse line (Madisen et al., 2010), resulting in a new KOR cKOtdTomato mouse line. When KOR cKOtdTomato mice are injected with AAV-cre-GFP, KOR is conditionally deleted and tdTomato/GFP are simultaneously expressed in infected cells (see Fig. 5A–C) to confirm robust, virally induced cre recombinase activity.

For DREADD experiments, AAV5-CaMKIIα-HA-hM4D(i)-IRES-mCitrine (2.7 × 10^{12} VM/ml) or AAV5-CaMKIIα-mCitrine (control virus), purchased from UNC Virus Core was injected bilaterally into the BLA (BLA coordinates ML ± 3.1, AP − 1.8, DV − 5) at a volume of 300 nl over 3 min per side. The syringe was left in place for 10 min total to allow for viral diffusion. Mice were given 3 weeks for viral expression before behavioral testing (see Fig. 6A–C).

**Immunohistochemistry.** Mice were transcardially perfused with ice-cold 4% paraformaldehyde 90 min after yohimbine-induced reinstatement and tissue was stained to visualize c-fos expression. Mice that had surgery before testing were perfused to confirm viral expression in the BLA (see Figs. 5E,F, 6A–C). Mice with no reporter gene expression on both sides of the brain or misplaced expression were excluded from behavioral analysis.

After perfusion, brains were stored in 30% sucrose solution until sectioning (30 μm) and staining. Briefly, as described previously (Al-Hasani et al., 2013b; McCall et al., 2015), for c-fos IHC, sections (6–8 sections per well, 2 ml volume for all washes and incubations) were washed 3 times in PBS and then blocked with blocking buffer (PBS with 5% normal goat serum and 0.3% Triton X-100) for 1 h at room temperature. Sections were then incubated overnight at 4°C in blocking buffer containing the primary antibody for c-fos (c-fos rabbit polyclonal IgG; Santa Cruz Biotechnology, sc-52 RRID:AB_2106783,1:500). After 24 h of primary antibody incubation, sections were washed 3 times in PBS before secondary antibody incubation (Alexa Fluor 594 goat-anti-rabbit IgG; Thermo Fisher Scientific, R37117 RRID:AB_2556545,1:1000) and staining (Neurotrace 435/455 Blue Fluorescent Nissl stain; ZomThermo Fisher Scientific, N21479). Sections were incubated in labeled cells was reported. Counting was performed blinded times in PBS and 3 more times in PB. Tissues from mice that were perfused just to confirm viral expression were not stained for c-fos, so staining began with the Neurotrace step. Free-floating sections were mounted in 0.1 PB and coverslips were applied over mounted sections with Vectashield Hard Set mounting medium (Vector Laboratories, H-1400 RRID:AB_2336787). Slides were imaged on both epifluorescent and confocal microscopes. For quantification, images were acquired with the same gain and exposure time across brain regions and treatment groups.

**Cell quantification.** C-fos+ cells were quantified as described previously (Al-Hasani et al., 2013b; Kim et al., 2013; McCall et al., 2015). Briefly, images from 3 consecutive sections from n = 6 mice per group were taken on a epifluorescent microscope. Images were broken down into individual channels (Neurotrace: blue channel; c-fos: red channel) and analyzed separately. ImageJ (RRID:SCR_003070) particle analysis was used to count the number of c-fos-positive cells relative to the number of Neurotrace-positive cells within a given region. Images were overlaid and the percentage of c-fos-positive cells within each channel was recorded. The percentage of A9 tDTomato and GFP coexpression in the BLAs of KORcKOtdTomato mice injected with AAV5-cre-GFP was done the same way; however, the individual channels were different (Neurotrace: blue channel-not shown; GFP: green channel; and tDTomato: red channel). The percentage of GFP/tDTomato-colabeled cells is presented as the percentage of Neurotrace-positive cells within a given region.

**Data analysis and statistics.** Two-way (on time/stage) repeated-measures (RM)-ANOVA were used to compare differences in preference scores and locomotor responses (separately) between groups during each stage of testing. In cases in which groups were unequal for each stage of testing (because groups were split to move on to nicotine-prime reinstatement testing or perfused after reinstatement testing), prime data were analyzed separately with one-way RM-ANOVA or paired t test as appropriate. One-way ANOVAs were used to analyze c-fos quantification data. Significant ANOVAs were followed by Bonferroni post hoc comparisons and statistically significant differences were determined at p < 0.05. All data and statistical analysis was performed using Prism version 6 or 7 (GraphPad, RRID:SCR_002798).

**Results.**

**KOR activation is necessary for foot-shock stress-induced nicotine reinstatement.** To determine whether stress would reinstate a previously extinguished CPP for nicotine, we exposed mice to 15 min of unavoidable intermittent foot shock before reinstatement testing (see Fig. 1A for experimental timeline and foot-shock protocol). We found that foot shock reliably reinstated nicotine CPP and that this effect is blocked by pretreatment with norBNI (10 mg/kg, i.p; n = 12–16 per group, two-way RM-ANOVA, F_{(2,40)} = 16.84, p < 0.001; Fig. 1C). Post hoc comparisons showed a significant increase in reinstatement scores in the foot shock + saline group compared with saline-only controls and stressed mice pretreated with norBNI (all comparisons p < 0.001; see Table 1 for comparison of time spent in the drug-paired side during each stage of testing). Furthermore, norBNI did not have an effect on nicotine-prime reinstatement in nicotine-conditioned mice (n = 6–9 per group, one-way ANOVA, F_{(1,18)} = 9.25, p = 0.002), consistent with previous reports (Schindler et al., 2010; Jackson et al., 2013). Post hoc comparisons showed that a nicotine prime injection reinstated nicotine conditioned mice regardless of norBNI pretreatment (saline-only compared with foot shock + saline, p = 0.002; saline-only compared with foot shock + norBNI, p = 0.037; Fig. 1C). As expected, nicotine decreased locomotor behavior during conditioning sessions in both groups of nicotine-conditioned mice compared with saline-treated controls (n = 12–16 per group, two-way RM-ANOVA, F_{(2,40)} = 11.29, p < 0.001; Fig. 1D). No differences were seen in locomotor behavior during drug-free testing (see Fig. 1A,B for timeline and representative tracks of distance traveled during each testing time point). However, during the nicotine prime test, nicotine treatment again decreased locomotor responses compared with saline controls only in mice from the foot shock + saline group (two-way RM-ANOVA, F_{(2,40)} = 23.16, p < 0.001; multiple-comparisons p = 0.0004). norBNI treatment before stress reinstatement (24 h earlier) attenuated nicotine’s hypolocomotive effects during the prime test because there was no significant difference between this group and saline controls (p = 0.08; Fig. 1E). These data suggest that KOR activity is necessary for stress-induced reinstatement, but not prime reinstatement of nicotine CPP, and that the effect is specific to stress and not prime-induced reinstatement.

**KOR activation is necessary for yohimbine-induced nicotine reinstatement.** To determine whether the widely used pharmacological stressor yohimbine (Lé et al., 2005; Banna et al., 2010; Mantsch et al., 2010; Mantsch et al., 2016) would also induce nicotine CPP rein-
KOR activation is necessary for foot-shock stress-induced nicotine reinstatement. A, Timeline of behavioral testing schedule and protocol. B, Representative traces of locomotor behavior (distance traveled) during each stage of testing (pre, post, extinction, foot shock reinstatement, prime). C, Foot-shock stress before testing (day 8) reinstates nicotine CPP in a norBNI (10 mg/kg, i.p.)-sensitive manner. D, Locomotor responses are decreased during nicotine treatment (days 2 and 4, P.M. session) compared with saline-treated controls. E, Neither foot-shock stress nor norBNI pretreatment changes locomotor behavior during reinstatement testing. n = 12–16 per group, *p < 0.05.

To corroborate our pharmacological studies and determine whether the DYN/KOR system is necessary for stress-induced reinstatement and if genetic deletion of KOR or DYN affects reinstatement, we tested additional groups with or of either the KOR gene (KOR^-/-) or pre-pro-DYN (DYN^-/-) knock-out mice against their littermate controls. In these groups, we found that KOR and DYN KO mice displayed CPP scores comparable to WT control groups (no significant differences in CPP scores during postreinstatement test). However, neither KOR^-/- or DYN^-/- mice showed reinstatement of nicotine CPP after yohimbine, whereas a priming injection of nicotine induced reinstatement in both groups (two-way RM-ANOVA, main effect treatment, n = 5–9 per group, F(2,26) = 5.08, p = 0.02; post hoc comparisons only showed significant differences during reinstatement: KOR^-/- compared with WT, p = 0.02; DYN^-/- compared with WT, p = 0.05; Fig. 2E, Table 1). KOR^-/- and DYN^-/- mice showed locomotor responses to nicotine during conditioning similar to WT controls (main effect treatment not significant F(2,18) = 1.81, p = 0.18; Fig. 2F). Similarly, no significant differences were detected in distance traveled during preference and reinstatement testing (main effect treatment not significant F(2,18) = 0.18, p = 0.84; Fig. 2G). Together, these results suggest that, like foot-shock stress, the DYN/KOR system is engaged by the pharmacological stressor yohimbine and that both KOR and pre-pro-DYN play a critical role in the reinstatement of nicotine CPP.

KOR activation is necessary for increased BLA c-fos expression after nicotine CPP reinstatement

To investigate the regional effects of stress and yohimbine on neuronal activity, we performed immunohistochemistry (IHC) to visualize c-fos, the commonly used immediate to early gene marker of recent cellular activity (Minatohara et al., 2015). To determine whether yohimbine-induced reinstatement produced changes in neural activity throughout the brain, we perfused mice 90 min after the reinstatement test (2 h after yohimbine injection) and used IHC to visualize c-fos expression (Fig. 3A) in regions...
shown previously to be KOR sensitive (Land et al., 2008; Bruchas et al., 2011; Li et al., 2012; Crowley et al., 2016). We found that the percentage of c-fos+ cells in the BLA was significantly greater in yohimbine-treated mice than in saline controls (Fig. 3B, n = 5–6 mice per group, one-way ANOVA, $F_{(2,13)} = 32.87, p < 0.0001$). However, corresponding to our behavioral results, mice pretreated with norBNI before yohimbine reinstatement had significantly fewer c-fos+ cells in the BLA compared with yohimbine/saline-treated mice ($p < 0.01$). No changes were seen in the total number of Nissl-stained cells in any of the groups ($F_{(2,13)} = 0.102, p = 0.90$; Fig. 3C for representative images). In other brain regions that we visualized, we failed to detect norBNI-sensitive changes that paralleled our behavioral results (Table 2). The percentage of c-fos+ cells in the central amygdala (CeA) was increased compared with saline controls after yohimbine-treatment regardless of norBNI pre-treatment (n = 3–6 mice per group, one-way ANOVA, $F_{(2,12)} = 9.39, p = 0.003$). No changes were seen between any groups in the percentage of c-fos+ cells in the BNST (n = 3–5 mice per group; one-way ANOVA, $F_{(2,10)} = 3.78, p = 0.07$). The percentage of DRN c-fos+ cells was also increased after yohimbine reinstatement (n = 2–6 mice per group; one-way ANOVA, $F_{(2,11)} = 54.27, p = 0.001$). Post hoc comparisons showed that, although both yohimbine-treated groups of mice had significantly more c-fos+ cells than saline controls, the increase was significantly greater in mice pretreated with norBNI compared with yohimbine/saline-treated mice (all $p < 0.05$). Together, these data support the conclusion that c-fos activity in the BLA is altered in a norBNI-sensitive manner after yohimbine reinstatement, suggesting that DYN/KOR system is regulating this system in response to yohimbine exposure. The changes in BLA c-fos expression that we observed corresponded to the changes that we saw in behavior, indicating that KOR signaling within the BLA may be important in mediating this behavior.

**DYN/KOR activity within the BLA is required for stress-induced nicotine CPP reinstatement**

To test the hypothesis that functional KOR signaling in the BLA is required for yohimbine-induced reinstatement, we locally injected the long-lasting KOR antagonist norBNI (Bruchas et al., 2016). We found that the percentage of c-fos+ cells in the BLA was significantly greater in yohimbine-treated mice than in saline controls ($p < 0.01$). However, no changes were seen in the total number of Nissl-stained cells between any groups in the BLA. In other brain regions that we visualized, we failed to detect norBNI-sensitive changes that paralleled our behavioral results (Table 2). The percentage of c-fos+ cells in the central amygdala (CeA) was increased compared with saline controls after yohimbine-treatment regardless of norBNI pre-treatment (n = 3–6 mice per group, one-way ANOVA, $F_{(2,12)} = 9.39, p = 0.003$). No changes were seen between any groups in the percentage of c-fos+ cells in the BNST (n = 3–5 mice per group; one-way ANOVA, $F_{(2,10)} = 3.78, p = 0.07$). The percentage of DRN c-fos+ cells was also increased after yohimbine reinstatement (n = 2–6 mice per group; one-way ANOVA, $F_{(2,11)} = 54.27, p = 0.001$). Post hoc comparisons showed that, although both yohimbine-treated groups of mice had significantly more c-fos+ cells than saline controls, the increase was significantly greater in mice pretreated with norBNI compared with yohimbine/saline-treated mice (all $p < 0.05$). Together, these data support the conclusion that c-fos activity in the BLA is altered in a norBNI-sensitive manner after yohimbine reinstatement, suggesting that DYN/KOR system is regulating this system in response to yohimbine exposure. The changes in BLA c-fos expression that we observed corresponded to the changes that we saw in behavior, indicating that KOR signaling within the BLA may be important in mediating this behavior.
Figure 3. KOR activation is necessary for increased BLA c-fos expression after yohimbine-induced reinstatement. A, Timeline of behavioral events/treatment before perfusions after yohimbine-induced reinstatement to visualize c-fos expression within the BLA. B, Percentage of neurons with both c-fos + and Nissl stain is increased compared with saline controls or mice pretreated with norBNI (n = 6 mice per group; *p < 0.05), of c-fos + cells shown in C. C, Representative confocal images (20 ×) of BLA c-fos expression from each group quantified in B. Nissl (blue) and c-fos (red) staining. White arrow points to c-fos + and Nissl + -labeled cell. Scale bars, 50 μm.

Table 2. Mean percentage of c-fos +/Nissl + -colabeled cells in the BLA, CeA, BNST, and DRN after yohimbine-induced nicotine CPP reinstatement

<table>
<thead>
<tr>
<th>Region</th>
<th>Treatment Group</th>
<th>Saline-only</th>
<th>Yohimbine/saline</th>
<th>Yohimbine/norBNI</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLA</td>
<td>Saline-only</td>
<td>2.01 (0.66)</td>
<td>52.45 (4.75)</td>
<td>25.53 (7.62)</td>
</tr>
<tr>
<td></td>
<td>Yohimbine/saline</td>
<td>0 (0)</td>
<td>40.71 (3.36)</td>
<td>31.96 (7.38)</td>
</tr>
<tr>
<td>CeA</td>
<td>Saline-only</td>
<td>0 (0)</td>
<td>46.33 (7.39)</td>
<td>37.05 (14.99)</td>
</tr>
<tr>
<td></td>
<td>Yohimbine/norBNI</td>
<td>6.77 (1.37)</td>
<td>34.96 (2.89)</td>
<td>74.08 (4.50)</td>
</tr>
</tbody>
</table>

Shown are the mean and (SEM) percent of c-fos + cells in the BLA, central amygdala (CeA), bed nucleus of the stria terminalis (BNST), and dorsal raphe nucleus (DRN) after yohimbine reinstatement for each group of mice.

*Significantly different from saline-only group.

**Significantly different from yohimbine/saline group.

2007; Melief et al., 2011; Al-Hasani et al., 2015) bilaterally into the BLA before the start of behavioral testing. KOR antagonism in the BLA (see Fig. 4A, B for a timeline for injections and behavior) before behavioral testing prevents yohimbine-induced nicotine CPP reinstatement without affecting nicotine CPP acquisition or prime reinstatement (n = 8–12 per group, F(2,48) = 7.61, p = 0.01; post hoc showed only yohimbine reinstatement scores differed significantly between the two groups p = 0.0007; Fig. 4C,D). Nicotine significantly decreased locomotion during P.M. conditioning in both groups of mice compared with A.M. locomotor responses (main effect conditioning session F(2,73) = 102.2, p < 0.0001; post hoc comparisons p = 0.01 both groups). However, mice locally injected with norBNI in the BLA showed significantly less nicotine-induced hypolocomotion during P.M. conditioning sessions compared with vehicle-treated controls (main effect of treatment F(1,25) = 8.18, p = 0.008; Fig. 4E). No differences were seen in locomotor behavior during CPP or reinstatement testing (F(1,18) = 1.89, p = 0.18; Fig. 4F).

To test the hypothesis that KOR expression within the BLA is necessary for stress-induced reinstatement, we used a recently developed mouse line to delete KORs selectively within certain cell types or regions (Van’t Veer et al., 2013a) and crossed it with the Ai9 tdTomato reporter mouse line to generate a new KOR cKO mouse line (Fig. 5A, B). Colabeling of GFP cells and tdTomato cells was observed in 80.56% of BLA cells, indicating that these cells had successful recombination and KOR deletion (Fig. 5C). Conditional deletion of BLA KORs in KOR cKO mice significantly prevented foot-shock stress-induced nicotine CPP reinstatement (n = 6–9 per group, two-way RM-ANOVA, F(2,26) = 4.32, p = 0.024). Post hoc tests confirmed that the only significant difference between groups was during the reinstatement test, in which KOR cKO mice injected with AAV-cre-GFP had significantly lower reinstatement scores than AAV-GFP-injected controls (p = 0.011; Fig. 5G). No differences in locomotor behavior were seen between groups during conditioning (F(1,16) = 0.002, p = 0.97; Fig. 5H) or preference/reinstatement testing (F(1,16) = 0.95, p = 0.34; Fig. 5I). Together, these data demonstrate that BLA KORs are necessary for yohimbine and foot-shock stress-induced reinstatement, but not for acquisition of the initial association or subsequent nicotine-primed reinstatement, indicating that BLA KORs are critical mediators of stress-induced nicotine reinstatement. In addition, the localized viral expression shown in Figure 5, E and F, helps to demonstrate...
that the effect of local norBNI antagonism is not due to an off-target effect at other receptors in other regions.

**Chemogenetic activation of Gαi signaling in the BLA is sufficient to induce reinstatement of nicotine CPP**

Our prior report suggested that systemic injection of the KOR agonist U50,488 was sufficient to reinitiate nicotine CPP, but the regional effect was not defined (Al-Hasani et al., 2013a). In addition, recent reports indicate a critical role of KORs in the BLA in generating anxiogenic behavior and showed that their actions on excitatory cells in the BLA are critical for producing this negative affective state (Bruchas et al., 2009; Crowley et al., 2016). Furthermore, KORs are well known to couple to Gαi signaling pathways to mediate their effects on neurotransmitter release (Al-Hasani and Bruchas, 2011). Therefore, to determine whether Gαi signaling within BLA excitatory cells plays a role in stress-induced reinstatement of nicotine CPP, we virally injected AAV5-CaMKIIα-hM4D(Gi)–mCitrine DREADD (Rei et al., 2015; Roth, 2016) into the BLA (Fig. 6A for timeline). Activation of Gαi signaling within BLA CaMKIIα neurons (Fig. 6B,C for injection diagram, confocal images of viral expression and illustrations showing spread of viral expression) with CNO 30 min before reinstatement testing, induced reinstatement to nicotine CPP in DREADD–injected mice compared with mice injected with control virus (n = 9 hM4D(G)i) mice and n = 10 mCitrine controls, two-way RM-ANOVA, F(1,17) = 16.09, p = 0.0009; Fig. 6D). A two-way RM-ANOVA revealed that, although hM4D(G)i mice were significantly less active during the CNO reinstatement test than during the nicotine CPP posttest, locomotor responses did not differ between hM4D(G)i and control mice during the CPP posttest or reinstatement test (F(1,17) = 12.39, p = 0.003; Fig. 6E) during the test. As a nonbehavioral confirmation of DREADD activation by CNO, we looked at c-fos expression after CNO reinstatement and saw no costaining of c-fos and mCitrine in the BLA. However, there was c-fos expressed in the CeA of the same section, confirming that the lack of c-fos in the BLA was due to CNO activation of the DREADDs (Fig. 6B). Together, these data suggest that inhibition of CaMKIIα neurons through Gαi activation after nicotine conditioning and extinction is sufficient to induce reinstatement of nicotine CPP and suggest an anatomically specific intracellular mechanism by which stress can lead to reinstatement of nicotine preference.

**Discussion**

The principal findings reported here are as follows: (1) both yohimbine and foot-shock stress reliably reinitiate nicotine seeking-like behavior in a CPP model in mice in a DYN/KOR-dependent manner; (2) the role of KOR in reinstatement is specific to stress because nicotine–prime reinstatement was unaffected by either systemic KOR antagonism or global knock-out of DYN/KOR; (3) activation of Gαi signaling in the BLA is sufficient to induce nicotine reinstatement; and finally (4) BLA KORs play a key role in mediating stress, but not prime-induced nicotine CPP reinstatement. These findings indicate that BLA KORs play a critical role in stress-induced nicotine reinstatement by establishing that elevated signaling via Gαi–coupled pathways is sufficient to produce this behavior. Together, our data suggest that, during stress, KOR inhibitory signaling within the BLA may serve to change behavioral responses to environmental stimuli such as those previously associated with the rewarding effects of nicotine.

**Jackson et al. (2013)** found that systemic treatment with norBNI blocks forced swim-stress-induced nicotine CPP reinstatement. Here, we extend this initial report and now show that the DYN/KOR system within the BLA is necessary stress-induced nicotine reinstatement. We extend the results of Jackson et al. (2013) by showing that two additional stressors (yohimbine and foot shock) reliably reinitiate CPP for nicotine in mice. This KOR effect is specific to stress because norBNI did not block nicotine–primed reinstatement in WT mice. In addition, we report that KOR and DYN KO mice show similar locomotor responses to nicotine and condition similarly to WT controls, but genetic disruption of the KOR or DYN system results in blockade of stress-induced nicotine CPP reinstatement. Although slightly more variable than WT controls, KOR and DYN KO mice still show nicotine–primed reinstatement. Therefore, the effects of DYN/KOR system disrup-
tion are likely not mediated by nonspecific effects on learning or memory processes, consistent with previous reports for the KOR system in cocaine reinstatement (Land et al., 2008; Redila and Chavkin, 2008; Schindler et al., 2010; Bruchas et al., 2011). Rather, this system is activated during stress and may potentially be increasing the valence of cues previously associated with nicotine.

As a pharmacological stressor, yohimbine has generated considerable discussion and debate. Recently, Chen et al. (2015) found that...
the ability of yohimbine to reinstate operant behavior was dependent on the presence of a discrete cue after responding regardless of whether the cue was paired with a reward. Our results increase the understanding of the similarities between the mechanisms of yohimbine and stress. For example, if yohimbine acts by increasing reward-cue valence, then this may also be the mechanism by which stress reinstates drug seeking. Previous reports have in fact suggested that stress modifies the salience of cues and can thus likewise regulate behavioral responding (Schindler et al., 2010). Our results raise the possibility that this may also underlie our observations here, although this hypothesis requires further investigation. If yohimbine acts by increasing the salience of cues rather than inducing a stressful experience, our results show that the DYN/KOR system plays a role in this increased valence of cue-associations within the BLA, which in the case of nicotine CPP, leads to reinstatement. This may provide evidence that the role of DYN/KOR in both yohimbine and foot-shock stress-induced reinstatement of nicotine CPP likely involves an interaction with the cholinergic, noradrenergic, and corticotropin-releasing factor (CRF) systems (Kang-Park et al., 2015; Bashiri et al., 2016), which are critical in nicotine-seeking behaviors (Yamada and Bruijnzeel, 2011; Picciotto and Mineur, 2014). However, it is still possible that yohimbine and foot-shock stress may use different neural circuits to cause nicotine reinstatement. Future studies that fully characterize relationships among discrete cues and nicotine seeking and self-administration behaviors, as well as a complete neural circuit analysis for DYN/KOR, are needed in future studies to resolve these outstanding questions.

We report that KOR activity in the BLA is a key mediator of stress-induced nicotine reinstatement. Furthermore, local antagonism or selective deletion of KORs in the BLA was sufficient to block stress-induced nicotine CPP reinstatement. Again, this effect is stress specific because these manipulations did not affect nicotine prime reinstatement. Systemic yohimbine treatment has been shown to increase neuronal activity and extracellular noradrenaline levels within the BLA (Buffalari and Grace, 2009). Consistent with this previous work, we showed increased expression of c-fos, a marker of recent cellular activity, in the BLA after yohimbine-induced reinstatement that was attenuated in mice pretreated with norBNI, indicating the necessity of DYN/KOR activity during reinstatement testing to induce reinstatement. Together, our results show a potential mechanism by which modulation of BLA KOR-coupled intracellular signaling cascades during stress can result in nicotine CPP reinstatement. A potential explanation for our results is that we may be inducing an anxiety-like state similar to that produced by KOR activation (Bruchas et al., 2009) and stress (Kim et al., 2005). The neural adaptations that take place within the BLA (and associated circuits) during stress can change the behavioral response to a nicotine-associated context, thus inducing nicotine reinstatement.

However, this hypothesis requires thorough manipulations of BLA signaling pathways and behavioral experiments.

Inhibition of excitatory signaling within the BLA through Gopi activation plays a role in the regulation of synaptic plasticity and LTP induction in the BLA (Li and Rainnie, 2014). In our study, Gopi DREADD activation was also sufficient to induce nicotine CPP reinstatement. This is consistent with a recent report showing that activation of Gopi DREADDs in the BLA reduces stress-induced impairments in learning and memory tasks (Rei et al., 2015) and is consistent with a prior report that showed systemic KOR activation is sufficient to reinstate nicotine CPP (Al-Hasani et al., 2013a). However, some studies contradict this result and show that optogenetic or pharmacological inhibition of BLA neurons promotes reinstatement to drug seeking and reward-related behaviors (Lalumiere, 2014; Sciascia et al., 2015). These studies used optogenetics to inhibit terminals from specific projections originating in the BLA, including the prelimbic cortex and nucleus accumbens core (Britt et al., 2012; Stefanik and Kalivas, 2013), and, given the recent evidence for heterogeneity of BLA projection neurons (Namburi et al., 2015; Crowley et al., 2016), helps to explain these differences. It has also been shown that activation of BLA terminals in the CeA, a projection often implicated in reinstatement of drug seeking and CPP (Mantsch et al., 2016), decrease anxiety-like behaviors, whereas inhibition of this projection has an anxiogenic effect (Tye et al., 2011). In ad-

Figure 6. Chemogenetic activation of Gopi signaling in the BLA is sufficient to induce reinstatement of nicotine CPP. A, Diagram of hM4D(Gi) DREADD viral injections into BLA and subsequent behavioral experiments. B, Diagram of viral injection into the BLA (left) and IHC 20× confocal images of hM4D(Gi) DREADD (middle) and control (mCitrine packaged under CamKIIα promoter with no receptor, right) viral expression in the BLA. mCitrine (cyan), c-fos (red), and Nissl (blue). Scale bars, 50 μm. C, Diagram with marks that indicate viral spread in mice included in behavioral dataset. D, Activation of hM4D(Gi) DREADDs in the BLA by CNO is sufficient to induce nicotine CPP reinstatement. E, No differences in locomotor activity before or after DREADD activation between Gopi and controls. n = 9–10 per group, *p < 0.01.
dition, a recent report showed that optogenetic activation of BLA neurons projecting to the BNST decreases anxiety-like behavior, but that KOR activation blocks this anxiolytic effect and inhibits BLA glutamate transmission within this projection (Crowley et al., 2016). Future work may help to establish whether KORs within this BLA–BNST circuit play a role in nicotine CPP reinstatement. Although we are not yet able to address the issue of cell type with respect to our pharmacologic and viral manipulations within the BLA, our results provide a foundation for future studies to build from when looking at the role of BLA KOR circuitry. Further, our DREADD CaMKII data, as well as the recent report from Crowley et al. (2016), suggest that excitatory neurons in the BLA are a likely mediator.

Here, we demonstrate region specific engagement of the DYN/KOR system within the BLA during stress-induced reinstatement of nicotine preference. These findings suggest that BLA KORs play a critical role in stress-induced nicotine seeking by establishing that elevated signaling via G2-coupled pathways is sufficient to produce behaviors in mice thought to reflect the persistence of nicotine addiction in humans. This work may provide novel insights for the development of therapeutic approaches to prevent stress-related nicotine relapse. Future studies will attempt to further dissect this BLA circuitry to identify cell-type specificity of these KOR circuits involved with stress-induced reinstatement of nicotine CPP and where and how endogenous DYN tone (cell types) is engaged by stress-induced nicotine interactions. These studies also further support recent efforts for using KOR antagonists as therapeutics for relapse to drug seeking.

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