# Kappa Opioid Receptor-Mediated Dysregulation of Gamma-Aminobutyric Acidergic Transmission in the Central Amygdala in Cocaine Addiction

***Supplemental Information***

## Supplemental Text

Our experiments were designed to accommodate the slow onset of specificity (24-48 h) and extended time course (> 3 weeks) of kappa-opioid receptor (KOR) blockade by norbinaltorphimine (nor-BNI) to produce long-lasting receptor inactivation to prevent or abolish cocaine-associated conditioning and production of anxiety-like behaviors. Although this property of nor-BNI might be considered a therapeutic advantage, further investigation is needed to understand the precise mechanisms associated with chronic KOR blockade, including alterations of downstream intracellular signaling (1). Interestingly, a recent report found that a combination of buprenorphine and naltrexone (expected to act as a physiological KOR antagonist) also blocked compulsive cocaine self-administration in rats, suggesting that acute KOR antagonism may be sufficient to dampen cocaine addiction-related behaviors (2).

## Supplemental Methods and Materials

### Animals

Male Wistar rats (*n* = 112, Charles River, Hollister, CA) weighing 250-300 g at the beginning of the experiments were used. Pairs of rats were housed in a room with artificial 12:12 h light/dark cycle (lights off at 9 AM), at constant temperature (20–22°C) and humidity (45–55%). All training and experimental sessions were conducted once a day during the nocturnal phase of the light/dark cycle, and each experiment used independent groups of rats. Animals were given *ad libitum* access to food and water throughout except during experimental test sessions. All procedures were conducted in the dark cycle and met the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

**Intravenous and Intracranial Surgery**

For cocaine self-administration studies, rats were catheterized with indwelling catheters (0.3 mm ID 0.64 mm OD; Dow Corning Co., Midland, MI) as previously described (3). After insertion into the vein, the proximal end of the catheter was anchored to the muscles underlying the vein with surgical silk. The distal end of the catheter was attached to a stainless-steel cannula bent at a 90° angle. The cannula was inserted in a support made by dental cement on the back of the animals covered with a plastic cap. For one week after surgery, rats were treated daily with 0.2 ml of the antibiotic Sodium Cefotaxime (262 mg/ml). For the duration of the experiments, catheters were daily flushed with 0.2-0.3 ml of heparinized saline solution. Animals' body weights were monitored every day. Training for cocaine self-administration began 1 week after the post-surgery recovery. For the behavioral tests, the animals underwent stereotaxic surgery in which bilateral cannula were implanted and aimed at the central amygdala (CeA) as described elsewhere (4, 5). Animals were anesthetized with halothane (1–2% v/v in oxygen) and placed in a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA). Cannula made of 23-gauge stainless steel tubing, 7 mm long, were secured to the surface of the skull by using three stainless steel screws and dental cement and sealed with a wire stylet. For bilateral cannula placements, the coordinates with reference to bregma for the CeA were as follows: (-2.6 AP, ± 4.2 ML, -6.6 DV). Drugs were injected through a stainless steel injector protruding beyond the cannula tip 1.5 mm. Coordinates were taken from Paxinos and Watson (6) and adjusted for the body weight of the animals. Rats were allowed to recover for at least 1 week following surgery.

**Drugs**

*Cocaine hydrochloride* (provided by the National Institute on Drug Abuse (Rockville, MD). For locomotor sensitization experiments, it was dissolved in an isotonic saline solution (0.9% NaCl) and injected at a dose of 20 mg/kg. Control animals received saline injections. Cocaine or saline was injected intraperitoneally (IP) at 1 ml/kg body weight when indicated, and infused in the right jugular vein during cocaine self-administration sessions. *Nor-BNI* was dissolved in distilled water and was infused bilaterally 1 day before the initiation of IP treatments with cocaine or saline. Infusion dose (2 ug/side), in a volume of 0.3 ul/side, was based on previous evidence demonstrating that this dose is effective in reducing excessive alcohol drinking in alcohol-dependent animals (7). *U50488* was provided by the National Institute on

Drug Abuse (Rockville, MD) and dissolved in isotonic saline. *D-AP5, CNQX, CGP 55845A, picrotoxin, and bicuculline* were purchased from Sigma Aldrich (St. Louis, MO).

**Operant Training and Cocaine Self-Administration**

Three groups of animals, including cocaine-naïve controls, short access (ShA), and long access (LgA) cocaine self-administering rats ($n$ = 9-13/group) were used for this study. ShA and LgA animals were trained to self-administer cocaine in the operant chambers under an FR-1 schedule of reinforcement, in which every response in the active lever resulted in the delivery of 0.1 ml of cocaine solution (0.5 mg/kg/infusion). The ShA group self-administered cocaine for 7-13 consecutive sessions for 1 h daily, while the LgA animals received cocaine for 6 h per day for at least 17 consecutive sessions. After recovery from surgery, intravenously catheterized animals ($n$ = 20) were trained to self-administer cocaine (0.5 mg/kg/infusion) under a fixed-ratio schedule for an hour per day in operant chambers described previously (8). After 10-22 days of cocaine self-administration with daily one-hour access, short access rats (ShA, $n$ = 9) were sacrificed for electrophysiological studies. In the remaining rats, the session duration was extended to six hours per day (long access, LgA).  After a minimum of 17 sessions of cocaine self-administration with extended access, LgA rats ($n$ = 13) were sacrificed and electric recording of gamma-aminobutyric acid (GABA) neurons in the CeA was performed. Five of the 13 LgA animals were administered 5-HT1A receptor ligands (8-OH-DPAT and WAY100635) as part of another study; however, similar electrophysiological data were obtained from the naïve and 5-HT1A ligand treated animals. Small batches of animals were sacrificed on separate days to accommodate electrophysiological recordings at consistent withdrawal times (1 d withdrawal) for each subject.

**Electrophysiology**

*Slice preparation.* We prepared CeA slices as previously described (9, 10) from male Wistar rats (556.9 ± 16 g) that were anesthetized with isoflurane (1-3%) and decapitated. We cut transverse slices 300-400 µm thick on a Vibratome Series 3000 (Technical Products International, St. Louis, MO), incubated them in an interface configuration for about 30 min, and then completely submerged and continuously superfused (flow rate of 2-4 ml/min) them with warm (31$^{o}$C), equilibrated with 95% O2/5% CO2 artificial cerebrospinal fluid (ACSF) of the following composition in mM: NaCl, 130; KCl, 3.5; $NaH_2PO_4$, 1.25; $MgSO_4$•7H2O, 1.5; $CaCl_2$,

2.0; $NaHCO_3$, 24; glucose, 10. Drugs were added to the ACSF from stock solutions to obtain final concentrations in the superfusate.

*Intracellular recordings.* We recorded from CeA neurons (primarily from the medial subdivision of the CeA) with sharp micropipettes filled with 3M KCl using discontinuous current-clamp mode (10, 11). We held most neurons near their resting membrane potential. Data were acquired with an Axoclamp-2A preamplifier (Axon Instruments, Foster City, CA) and stored for later analysis using pClamp software (Axon Instruments, Foster City, CA). We evoked pharmacologically isolated $GABA_A$ receptor-mediated inhibitory postsynaptic potentials (IPSPs) by stimulating locally within the CeA through a bipolar stimulating electrode while superfusing the slices with the glutamate receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and DL-2-amino-5-phosphonovalerate (APV; 30 μM), and the $GABA_B$ receptor antagonist (CGP 55845A; 1 μM). At the end of the recording we often superfused 30 μM bicuculline (or 50 μM picrotoxin) to confirm the $GABA_A$ergic nature of the IPSP, and these antagonists completely blocked IPSPs. To determine the synaptic response parameters for each cell, we performed an input-output (I-O) protocol (9, 10) consisting of a range of five current stimulations (50-250 mA; 0.125 Hz), starting at the threshold current required to elicit an IPSP up to the strength required to elicit the maximum amplitude. These stimulus strengths were maintained throughout the entire duration of the experiment. We established the stability of IPSPs by stimulating for at least 15 min prior to beginning experiments. In another protocol, we used four consecutive stimuli (30-second intervals) at the half maximal amplitude determined from the I-O relationship (12). We examined paired-pulse facilitation (PPF) in each neuron using paired stimuli at 50 msec inter-stimulus intervals (10, 12). The stimulus strength was adjusted such that the amplitude of the first IPSP was 50% of maximal, determined from the I-O relationship. We calculated the PPF ratio as the second IPSP amplitude over that of the first IPSP. All measures were taken before cocaine or kappa opioid agonist/antagonist superfusion (control), during their superfusion (10-25 min), and following washout (20-30 min). To determine the synaptic response parameters for each cell, we performed an input-output (I-O) protocol (9, 10) and the stimulus strengths were maintained throughout the entire duration of the experiment.

*Whole-cell patch-clamp recording of miniature inhibitory postsynaptic currents (IPSCs). W*e recorded from CeA neurons visualized in brain slices (300 mm) using infrared differential interference contrast optics and a CCD camera (EXi Aqua, QImaging). A 60x water immersion

objective (Olympus) was used for identifying and approaching CeA neurons. Whole-cell voltage-clamp recordings were made with a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 2-5 kHz, digitized (Digidata 1440A; Molecular Devices), and stored on a PC using pClamp 10 software (Axon Instruments). All voltage-clamp were performed in a gap-free acquisition mode with a sampling rate per signal of 10 kHz. Patch pipettes (4-8 MΩ) were pulled from borosilicate glass (Warner Instruments) and filled with an internal solution composed of (in mM): 145 KCl; 0.5 EGTA; 2 $MgCl_2$; 10 HEPES; 2 Na-ATP; 0.2 Na-GTP. GABAergic miniature IPSCs (mIPSCs) were recorded in the presence of 10 μM CNQX, 30 μM APV, 1 μM CGP 55845A, and 1 μM tetrodotoxin (TTX). Drugs were constituted in ACSF and applied by bath superfusion. All 62 cells were clamped at -60 mV for the duration of the recording. In all experiments, series resistance (<10 MΩ) was continuously monitored with a 10 mV hyperpolarizing pulse and experiments with > 20% change in series resistance were not included in the final analysis. Frequency, amplitude, and kinetics of miniature IPSCs were analyzed using a semi-automated threshold-based mini detection software (Mini Analysis, Synaptosoft Inc., Fort Lee, NJ). To accurately determine the mIPSC amplitude, only mIPSCs that were > 5 pA were accepted for analysis. The choice of this cutoff amplitude for acceptance of mIPSCs was made to obtain a high signal-to-noise ratio. Averages of mIPSC characteristics were based on a minimum time interval of 3-5 min and a minimum of 50 events. All detected events were used for event frequency analysis, but superimposed events were eliminated for amplitude and decay kinetic analysis. All data are expressed as mean ± SEM.

**Bilateral Intra-Central Amygdala Microinjections**

For intra-CeA microinjections, the stylet was removed from the guide cannula, and an 8.5-mm long, 30-gauge injector was connected to a calibrated polyethylene tube inserted through the guide and placed into the CeA. A volume of 0.3 ul/side was infused, and the injectors were left in the cannula for an additional 60 s to avoid backflow leakage. After completion of the injection, the stylets were replaced. The location of each cannula was confirmed at the end of the experiment via injection and visualization of methylene blue solution. Rats were deeply anesthetized with pentobarbital and then injected with dye, following the same procedure used for intra-CeA injections. Only those animals with accurate placements were considered for statistical analysis (see **Figure S3**).

## Locomotor Activity Testing

Repeated exposure to psychostimulants produces locomotor sensitization, with underlying plastic mechanisms initiating as early as the first exposure (13). Increased locomotor activity following repeated cocaine exposure (Figure 7) is thought to model the sensitization of incentive salience attributed to unique, cocaine-conditioned environments (14, 15). With the development of more elaborate animal models of cocaine addiction including the extinction-reinstatement paradigm (16, 17), substantial neuroanatomical and neuropharmacological overlap has been shown to exist between the mechanisms underlying locomotor sensitization and reinstatement to cocaine-seeking behavior (18-20). Based on our electrophysiological data, we hypothesized that bilateral injection of nor-BNI in the CeA would modify the ability of subsequent chronic cocaine injections to induce locomotor sensitization. A separate set of animals ($n$ = 21) underwent stereotaxic surgeries for bilateral CeA cannulation. Following basal locomotor activity measurement, animals were divided into four identical groups: cocaine/veh; cocaine/nor-BNI; saline/veh, and saline/nor-BNI ($n$ = 5-6/group). Sixteen identical hanging wire cages (26 cm wide × 35 cm long × 20 cm high) were used to monitor locomotor activity following daily injections of cocaine (20 mg/kg) or saline (1 mg/mL) in this context ($n$ = 5-6/group). Each cage was equipped with two pairs of infrared emitter–detector photocells that were positioned along the long axis 1 cm from the floor and 8 cm from the front and back of the cage. Photocell interruptions served as a measure of locomotor activity and were recorded by a PC for every 5 minutes. Rats were habituated to the cages for two consecutive days prior to the start of the experiment for 2 h and equally sorted in four groups. White noise (70 dB) was present during habituation and testing for the entire duration of the experiment. On the days of testing, rats were acclimated to the cages for 1 h prior to injection of cocaine or saline at the onset of the dark cycle and the photobeam breaks were recorded every 5 min for 1 h. Each separate cage was cleaned carefully before the next animals entered.

## Shock-Probe Defensive Burying Paradigm

Cocaine-treated rats ($n$ = 20) received IP cocaine injections once daily, 20 mg/kg, for 14 consecutive days in their home cage, while control animals ($n$ = 20) received IP injections with an isotonic saline solution in their home cage. Half of the rats in each group received intra-CeA nor-BNI, while the other half received intra-CeA saline before these repeated treatment regimens.

The cocaine dose used in these experiments, and the time periods of withdrawal testing (48 h) following completion of the treatments, was based on previous reports (21) demonstrating increased anxiety-like behavior following this regimen and on preliminary studies in our laboratories. Defensive burying behavior is believed to represent an active coping mechanism modeling anxiety-like behavior after confronting an aversive stimulus (22, 23). The defensive burying apparatus was a modified home cage with 5-cm wood chip bedding material evenly distributed throughout the cage. The shortest end of the cage contained a 0.75-cm hole through which a probe delivering a 1.5 mA electric shock upon contact was inserted. Animals received only one shock; afterwards the probe was de-energized and the animal's behavior was recorded for 15 min. Prior contact with the probe resulted in the rat piling bedding material with treading-like movements of the forepaws and shoveling movements of the head, often directed toward the shock probe. The latency to start burying, duration of burying (time spent pushing the bedding material toward the probe), and height of the bedding material at the junction between the probe and the wall at the end of the 15-min test were used as measures of anxiety-like behavior. Tests were performed 48 h after the last saline or cocaine injection. Subjects were placed in the test chambers for 45–60 min habituation sessions on 2 consecutive days before the testing day. The probe was not present during these habituation sessions. The bedding material was changed and the chamber cleaned between each habituation and testing trial. In the current study we employed a relatively high cocaine dose (20 mg/kg) for challenges since it is known to generate negative withdrawal symptoms (21, Figure 7) in addition to locomotor sensitization. Similar to escalated cocaine self-administrating animals, administration of (and/or withdrawal from) this higher dose may have more fully engaged CeA dynorphin/KOR systems.

**Additional Statistical Analysis**

The mIPSC results were evaluated with cumulative probability analysis, and statistical significance was determined using the Kolmogorov-Smirnov, non-parametric two-sample test (24) with $p < 0.05$ considered significant for each neuron. The pooled data for each experimental condition were then analyzed by paired $t$-test analyses for individual means comparisons to evaluate single drug (cocaine, U50488, Nor-BNI) effects within the same group or one-way repeated measures ANOVA to evaluate different treatment effects (Nor-BNI, Nor-BNI +

Cocaine) within the same group or with two-way repeated measures ANOVA. We accepted statistical significance at the $p < 0.05$. All averaged values are presented as mean ± SEM.

**Table S1**. Basal membrane properties of central amygdala neurons from cocaine naïve, short access (ShA), and long access (LgA) rats.

| | **Naïve-control ($n$ = 26)** | **ShA ($n$ = 24)** | **LgA ($n$ = 25)** |
|---|---|---|---|
| Resting Membrane Potential (mV) | -78.6 ± 0.8 | -78.2 ± 1.1 | -78.8 ± 1.1 |
| Input Resistance (MΩ) | 119.6 ± 7.6 | 115.7 ± 7.1 | 114.8 ± 7.4 |
| Membrane Capacitance (pF) | 105.9 ± 11.3 | 99.3 ± 8.7 | 103.6 ± 6.1 |

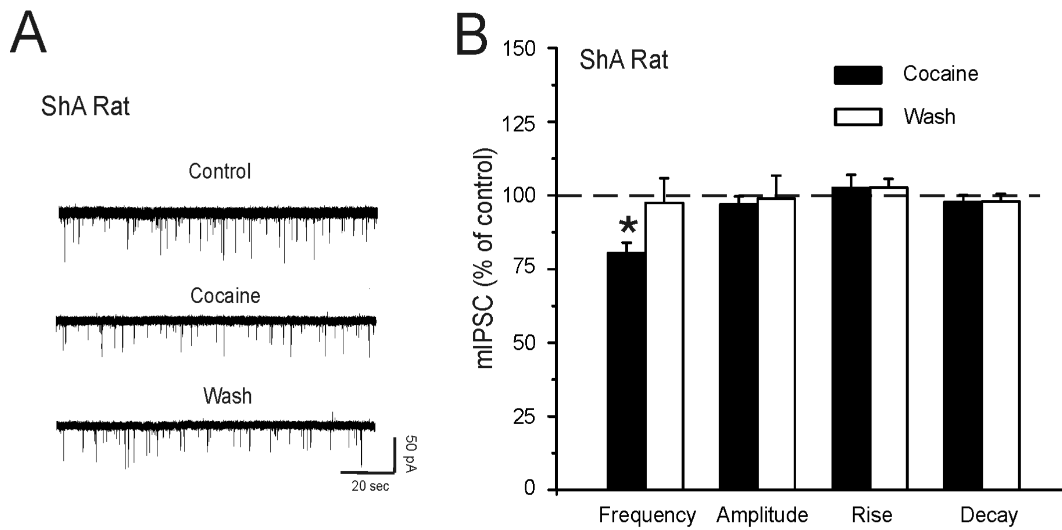


**Figure S1.** Acute application of cocaine decreases spontaneous GABAergic transmission in CeA neurons from ShA rats. (**A**) Representative mIPSC recordings in CeA neurons from a ShA rat during control, application of 1 uM cocaine, and washout. Cocaine decreased mIPSC frequency but not amplitude or kinetics. (**B**) Mean ± SEM frequency, amplitude, rise, and decay time of mIPSCs for 10 CeA neurons of ShA rats. Cocaine significantly (*$p < 0.05$) decreases the mean mIPSC frequency but does not change mean mIPSC amplitude, rise, or decay time. CeA, central amygdala; GABA, gamma aminobutyric acid; mIPSC, miniature inhibitory postsynaptic current; ShA, short access.

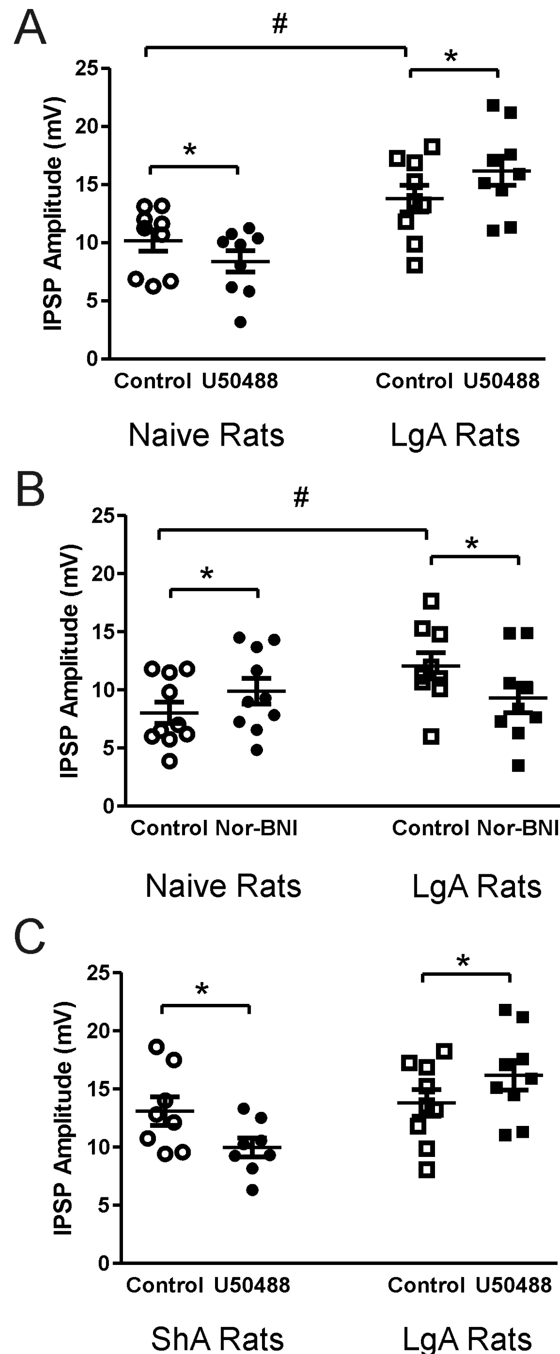


**Figure S2.** (**A**) Scatter plots of evoked IPSP amplitudes from cocaine naïve and LgA rats before and during U50488 application. **p* < 0.05, drug effect within group; #*p* < 0.05, baseline difference between groups. (**B**) Scatter plots of evoked IPSP amplitudes from cocaine naïve and LgA rats before and during nor-BNI application. **p* < 0.05, drug effect within group; #*p* < 0.05, baseline difference between groups. (**C**) Scatter plots of evoked IPSP amplitudes from ShA and LgA rats before and during nor-BNI application. **p* < 0.05, drug effect within group. Note that the IPSP was evoked at 50% maximal amplitude determined from the I/O relationship. IPSP, inhibitory postsynaptic potential; LgA, long access; ShA, short access.

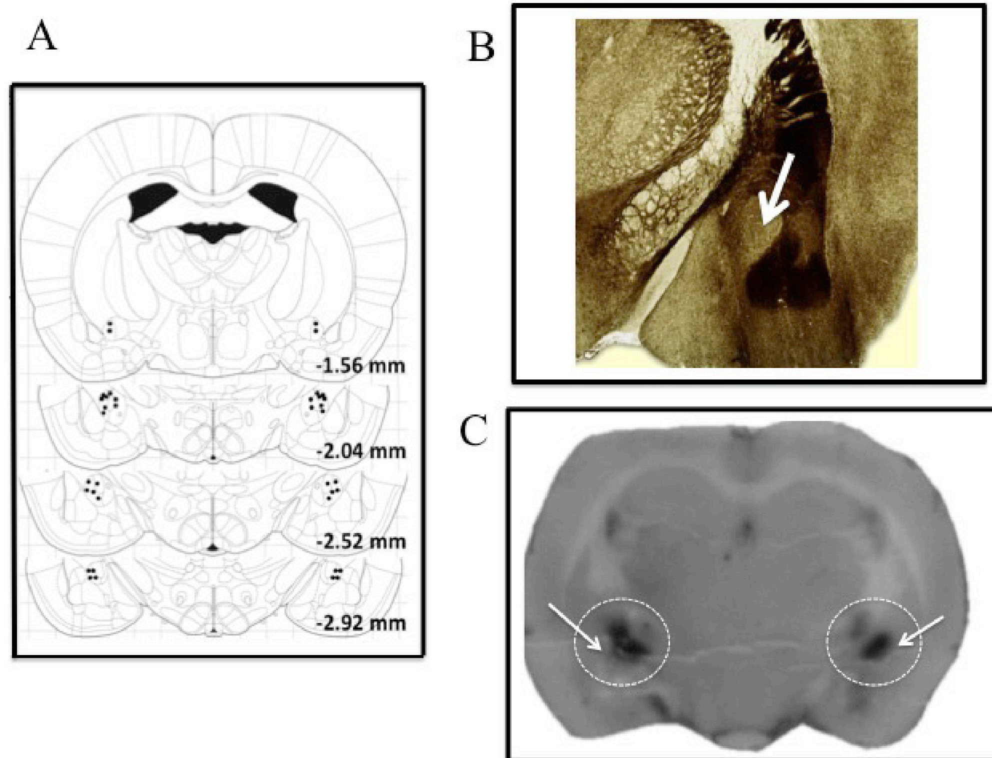


**Figure S3.** Schematic sections from the rat brain showing injections into the central nucleus of the amygdala (CeA). (**A**) Histological reconstructions showing correct (filled circles) injections into the CeA. Data presented in the figures are indicative of the criteria used for identification of correct cannulas sites. (**B**) Panel is from the atlas of Paxinos and Watson (6), reprinted with permission. (**C**) Representative photomicrograph showing the extent of typical cannula diffusion.

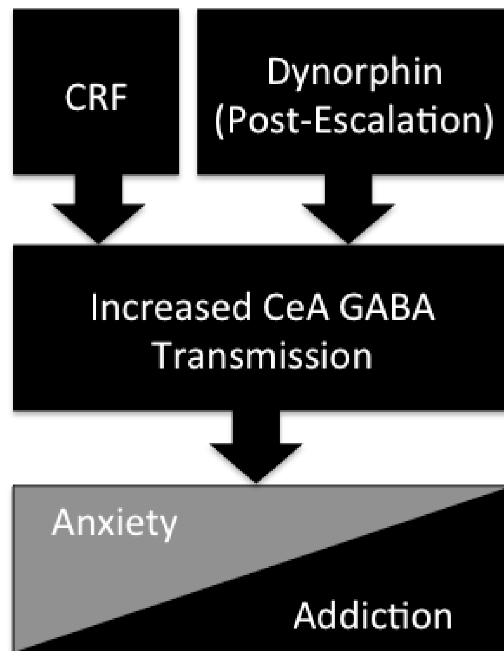


**Figure S4.** Proposed interaction of CRF and dynorphin signaling in the CeA. Increases in CRF and dynorphin activation of CeA GABAergic transmission in cocaine-escalated animals are hypothesized to underlie the generation of negative emotional states (e.g., anxiety) and ultimately facilitate the transition to cocaine addiction. Blockade of KORs with nor-BNI diminishes CeA GABAergic signaling after escalation, which may not only preclude the effects of dynorphin but may also dampen the effects of a potentiated CRF system on GABA activity and production of negative motivational states driving cocaine addiction. CeA, central amygdala; CRF, corticotropin-releasing factor; GABA, gamma aminobutyric acid; KOR, kappa-opioid receptor.

## Supplemental References


1. Bruchas MR, Chavkin C (2010): Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology (Berl)*. 210:137-147.

2. Wee S, Vendruscolo LF, Misra KK, Schlosburg JE, Koob GF (2012): A combination of buprenorphine and naltrexone blocks compulsive cocaine intake in rodents without producing dependence. *Sci Transl Med*. 4:146ra110.

3. Wee S, Specio SE, Koob GF (2007): Effects of dose and session duration on cocaine self-administration in rats. *J Pharmacol Exp Ther*. 320:1134-1143.

4. Kallupi M, Cannella N, Economidou D, Ubaldi M, Ruggeri B, Weiss F, *et al.* (2010): Neuropeptide S facilitates cue-induced relapse to cocaine seeking through activation of the hypothalamic hypocretin system. *Proc Natl Acad Sci U S A*. 107:19567-19572.

5. Kallupi M, de Guglielmo G, Cannella N, Li HW, Calo G, Guerrini R, *et al.* (2013): Hypothalamic neuropeptide S receptor blockade decreases discriminative cue-induced reinstatement of cocaine seeking in the rat. *Psychopharmacology (Berl)*. 226:347-355.

6. Paxinos G, Watson C (1998): *The rat brain in stereotaxic coordinates*. San Diego: Academic Press.

7. Walker BM, Koob GF (2008): Pharmacological evidence for a motivational role of kappa-opioid systems in ethanol dependence. *Neuropsychopharmacology*. 33:643-652.

8. Wee S, Orio L, Ghirmai S, Cashman JR, Koob GF (2009): Inhibition of kappa opioid receptors attenuated increased cocaine intake in rats with extended access to cocaine. *Psychopharmacology (Berl)*. 205:565-575.

9. Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR (2003): Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. *Proc Natl Acad Sci U S A*. 100:2053-2058.

10. Roberto M, Madamba SG, Stouffer DG, Parsons LH, Siggins GR (2004): Increased GABA release in the central amygdala of ethanol-dependent rats. *J Neurosci*. 24:10159-10166.

11. Haubensak W, Kunwar PS, Cai H, Ciocchi S, Wall NR, Ponnusamy R, *et al.* (2010): Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature*. 468:270-276.

12. Cruz MT, Herman MA, Cote DM, Ryabinin AE, Roberto M (2013): Ghrelin increases GABAergic transmission and interacts with ethanol actions in the rat central nucleus of the amygdala. *Neuropsychopharmacology*. 38:364-75.

13. Vanderschuren LJ, Schoffelmeer AN, Mulder AH, De Vries TJ (1999): Dopaminergic mechanisms mediating the long-term expression of locomotor sensitization following pre-exposure to morphine or amphetamine. *Psychopharmacology (Berl)*. 143:244-253.

14. Robinson TE, Berridge KC (1993): The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev*. 18:247-291.

15. Vanderschuren LJ, Kalivas PW (2000): Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berl)*. 151:99-120.

16. de Wit H, Stewart J (1981): Reinstatement of cocaine-reinforced responding in the rat. *Psychopharmacology (Berl)*. 75:134-143.

17. Shalev U, Grimm JW, Shaham Y (2002): Neurobiology of relapse to heroin and cocaine seeking: a review. *Pharmacol Rev*. 54:1-42.

18. De Vries TJ, Schoffelmeer AN, Binnekade R, Raaso H, Vanderschuren LJ (2002): Relapse to cocaine- and heroin-seeking behavior mediated by dopamine D2 receptors is time-dependent and associated with behavioral sensitization. *Neuropsychopharmacology*. 26:18-26.

19. Edwards S, Whisler KN, Fuller DC, Orsulak PJ, Self DW (2007): Addiction-related alterations in D1 and D2 dopamine receptor behavioral responses following chronic cocaine self-administration. *Neuropsychopharmacology*. 32:354-366.

20. Steketee JD, Kalivas PW (2011): Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. *Pharmacol Rev*. 63:348-365.

21. Basso AM, Spina M, Rivier J, Vale W, Koob GF (1999): Corticotropin-releasing factor antagonist attenuates the "anxiogenic-like" effect in the defensive burying paradigm but not in the elevated plus-maze following chronic cocaine in rats. *Psychopharmacology (Berl)*. 145:21-30.

22. Korte SM, Korte-Bouws GA, Bohus B, Koob GF (1994): Effect of corticotropin-releasing factor antagonist on behavioral and neuroendocrine responses during exposure to defensive burying paradigm in rats. *Physiol Behav*. 56:115-120.

23. Edwards S, Koob GF (2012): Experimental psychiatric illness and drug abuse models: from human to animal, an overview. *Methods Mol Biol*. 829:31-48.

24. Van der Kloot W (1991): The regulation of quantal size. *Prog Neurobiol*. 36:93-130.