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Heroin addiction engages negative emotional learning brain circuits in rats

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Conflict of Interest

The authors have declared that no conflict of interest exists.

One sentence summary:

Stimuli paired with heroin withdrawal after compulsive-like heroin intake activate negative emotional learning brain circuits in rats.
Abstract

Opioid use disorder is associated with the emergence of persistent negative emotional states during drug abstinence that drive compulsive drug taking and seeking. Functional magnetic resonance imaging (fMRI) in rats identified neurocircuits that were activated by stimuli that were previously paired with heroin withdrawal. The activation of amygdala and hypothalamic circuits was related to the degree of heroin dependence, supporting the significance of conditioned negative affect in sustaining compulsive-like heroin seeking and taking, and providing neurobiological insights into the drivers of the current opioid crisis.
Introduction

The United States leads the world in the opioid dependence and overdose crisis (1). New conceptual frameworks and therapeutic targets are needed to more effectively treat OUD and curb opioid overdose deaths. Individuals with OUD are hypothesized to compulsively use opioid drugs to avoid the severe negative emotional states (e.g., dysphoria, pain, anxiety, and depression) that are experienced during abstinence (2). In both clinical and preclinical models, environmental stimuli can become conditioned to these negative emotional states, which can then maintain compulsive opioid use on their own (3–5). Individuals with OUD report withdrawal symptoms and drug craving when encountering drug-related stimuli (6).

Stimuli that are conditioned to opioid withdrawal may perpetuate and reinstate drug seeking by generating negative emotional states that are relieved by drug taking via negative reinforcement (2, 4, 7). Identifying the brain circuits that support motivational aspects of conditioned withdrawal may provide insights into the long-term neuroplasticity-based consequences that frustrate therapeutic interventions in OUD (2, 8). Thus, we hypothesized that conditioned withdrawal would engage brain circuitry that is involved in negative emotional learning. In the present study, we found that cue-induced conditioned withdrawal engaged brain emotional systems in a rat model of heroin dependence.
Results and Discussion

To test our hypothesis, we utilized a behavioral model in which cues were paired with heroin withdrawal-induced negative emotional-like states (4). Rats were first trained to self-administer heroin (60 µg/kg/intravenous infusion) in short-access (ShA; 1 h/day) or long-access (LgA; 12 h/day) sessions that were designed to model non-dependent, controlled use versus dependent, compulsive heroin use (9). LgA rats rapidly escalated their heroin intake, whereas ShA exhibited stable drug intake (Figure 1A).

In the conditioning phase, rats were treated with saline or naloxone (120 µg/kg, s.c.) 30 minutes into each heroin self-administration session. Naloxone competes with heroin at µ-opioid receptors and in this dose range precipitates motivational signs of withdrawal (e.g., place aversion, increased intracranial self-stimulation thresholds), but not somatic signs of withdrawal (e.g. ‘wet dog’ shakes) in opioid-dependent rats (4, 9–11). The treatments were paired with distinct olfactory cues (lemon- or vanilla-scented bedding) in the self-administration chamber. The cue pairings lasted 30 min to coincide with the short-acting pharmacological effect of naloxone. After the cue pairing, ShA rats were returned to their home cages and LgA rats completed their 12 h session without olfactory cues (i.e., unscented bedding).

Naloxone treatment increased heroin intake relative to saline treatment in both self-administration groups (Figure 1B). Heroin intake remained stable across cue pairings (Supplemental Figure S1). As naloxone has a greater affinity for the µ-opioid receptor than heroin, naloxone likely immediately produced opioid withdrawal in LgA rats. This withdrawal effect might have been relieved by the elimination of naloxone concomitantly with increases in heroin self-administration. An alternative explanation for the increase in heroin intake during
naloxone treatment may be to maintain hedonic tone that is disrupted by naloxone, and that rats
that self-administer more heroin have increased tolerance to heroin.

Earlier work demonstrated that presentation of a compound auditory and visual cue
previously paired with naloxone treatment increased intracranial self-stimulation thresholds and
motivated heroin intake during heroin self-administration in LgA rats, suggesting that the cue
became conditioned to naloxone-precipitated withdrawal (4). Here we confirmed that an
olfactory cue previously paired with naloxone similarly increased heroin intake in LgA, but not
ShA rats, when presented in the absence of naloxone during heroin self-administration
(Supplemental Figure S2). Additionally, presentation of an olfactory cue previously paired with
naloxone in LgA rats produced greater reinstatement of heroin seeking after extinction compared
with an odor previously paired with saline (Supplemental Figure S3).

To explore the brain circuits that underlie conditioned withdrawal, we presented the
olfactory cues alone to lightly anesthetized rats that were subjected to spontaneous heroin
withdrawal during fMRI BOLD signal acquisition 24 h after their last cue conditioning self-
administration session (Supplemental Figure S4). The light anesthetic regimen used maintains
neurovascular coupling and preserves odor-specific sensory processing (12, 13) (Supplemental
Figures S4, S5). The cues were presented in a counterbalanced, blocked design in sequential
scans, and respiration rate fluctuated in response to cue presentation (Supplemental Figure S4).

Using the percent change in the blood-oxygen-level dependent (BOLD) signal from
baseline in response to cue-only presentation as the dependent measure and respiration as the
covariate, a whole brain 2 (cues: saline- versus naloxone-paired) × 2 (heroin access: ShA versus
LgA) × 2 (cue presentation block: blocks 1 and 2 versus blocks 3 and 4) analysis of covariance
yielded a significant cue × heroin access interaction (Figure 1C, Supplemental Figure S4). The
large cluster was segregated into 19 anatomically defined regions of interest (ROIs) using a spatially aligned rat atlas (14) (Table 1).

We then evaluated the relationship between activation in each ROI and the number of heroin infusions during naloxone+cue conditioning sessions (shown in Figure 1B); the latter was used as an index of withdrawal severity. After correcting for multiple comparisons, BOLD activation was associated with withdrawal severity in two ROIs: (i) a hypothalamic cluster that comprised the paraventricular nucleus of the hypothalamus (PVN) and ventromedial hypothalamus (VM; Figure 2A) and (ii) amygdala nuclei that comprised the medial amygdala, central nucleus of the amygdala, central nucleus of the amygdala, and extended amygdala (Figure 2D). In both regions, the naloxone-paired cue increased the BOLD signal in LgA rats and decreased the BOLD signal in ShA rats (Figure 2B, E). Heroin intake during naloxone+cue conditioning (Figure 1B) was correlated with both the hypothalamic cluster (Figure 2C) and amygdala (Figure 2F) BOLD response to the naloxone-paired cue in both self-administration groups. Greater withdrawal severity during conditioning was associated with greater activation in these regions during conditioned withdrawal.

Opioid withdrawal activates extrahypothalamic (e.g., extended amygdala) emotional systems in opioid-dependent rats (11, 15) and opioid dependence alters amygdala connectivity in humans (16). Opioid withdrawal also is known to potently activate the hypothalamic-pituitary-adrenal (HPA) arousal/stress axis in opioid-dependent humans and rats (17, 18). Activation of the PVN during opioid withdrawal and subsequent driving of the HPA axis may be an early dysregulation that is associated with excessive opioid intake. We hypothesize that dysregulation of the HPA axis and sensitization of extended amygdala maintain negative emotional states via glucocorticoid signaling (19, 20). These results suggest that previously neutral stimuli gain
motivational value when paired with opioid withdrawal first by a hormonal stress-like response, which in turn activates extrahypothalamic brain stress systems in the extended amygdala, forming a pathway for negative emotional states that drive craving and relapse in humans (19–21).

Several other regions exhibited activation patterns that were similar to those seen in the hypothalamic cluster and amygdala (Table 1). Many of these regions have been implicated in emotional learning and are hypothesized to be dysregulated in addiction (2, 22, 23). These include the lateral hypothalamus, dorsomedial nucleus of the thalamus, ventrolateral thalamus and dorsal striatum. Along with the amygdala, these regions are activated by heroin cues in individuals with OUD (22, 23). The extended amygdala promotes both positive and negative emotional states via its downstream connections to such areas as the lateral hypothalamus (24). As part of a habit learning circuit, the thalamus and dorsal striatum are implicated in reward and incentive salience (2, 25). Additionally, the naloxone-paired cue activated the precommissural nucleus (PRC), periaqueductal gray/periventricular gray (PAG/PVG), and pretectal nucleus in LgA rats, whereas these same regions were deactivated in ShA rats. The saline-paired cue deactivated these regions in LgA rats. The PRC and PAG/PVG are anatomically connected to the EA, VM, and pretectal nucleus, and play a key role in negative emotional learning (5, 26).

Finally, both odor cues activated the anterior hypothalamus and a region consistent with the habenula in LgA rats, whereas opposite effects were observed in ShA rats. The habenula can inhibit the mesolimbic dopamine system and modulate emotional and motivational states (27). Together with the hypothalamus and amygdala, the habenula serves to maintain hedonic homeostasis (2, 19, 26). Although the anatomical resolution of fMRI and the small size of the rat
habenula limited our ability to confirm its activation, potential engagement of the habenula during negative conditioned responding warrants further investigation.

We found that conditioned heroin withdrawal motivated heroin intake and engaged brain regions that are associated with negative emotional learning, particularly extrahypothalamic and hypothalamic stress/arousal circuitries. These circuits are consistent with and extend fMRI findings in individuals with OUD on drug cue reactivity tasks (22, 23). Thus, we argue that conditioned cues can maintain compulsive drug use by removing aversive states (conditioned negative reinforcement) as well as by producing positive incentive states (conditioned positive reinforcement) (2, 22, 28) and that both forms of learning contribute to allostatic changes in emotional processes that perpetuate opioid addiction.

Exposure to conditioned withdrawal stimuli may drive craving and provoke relapse in individuals with OUD by inducing a powerful aversive stress state that is relieved by opioid use (7, 29). The stimuli that trigger conditioned withdrawal are likely the same as those that convey learned tolerance (30). Thus, individuals with OUD are especially susceptible to overdose death when they use opioids in unfamiliar contexts or with unfamiliar cues (e.g., different administration procedure or opioid) and their bodies fail to engage learned compensatory mechanisms (30), or when they encounter conditioned withdrawal stimuli and their drug tolerance is reduced, such as following detoxification in a treatment facility or release from incarceration (31). Critically, the three United States Food and Drug Administration-approved medications for OUD, which target opioid receptors (8), may not fully alleviate cue-conditioned withdrawal.

Therefore, we propose that understanding and targeting the brain circuits that underlie conditioned withdrawal and downstream emotional circuitries (e.g., brain stress circuits),
provides an innovative conceptual framework for novel treatment and the prevention of opioid overdose deaths. Examining fMRI, and in parallel, psychological responses to drug-related cues provides a potentially powerful approach to understanding individual differences leading to and maintaining compulsive opioid use. Indeed, dysfunction in brain circuits of negative emotional learning is a potential biomarker for tracking progression and remission of opioid use disorder.
Methods

Further information can be found in Supplemental Methods and Supplemental Figures 1-5.

Subjects

Adult male Long-Evans rats (Charles River Laboratories, Kingston, NY, USA) were group-housed (2-3/cage) on a 12 h/12 h light/dark cycle at the National Institute on Drug Abuse (NIDA) animal facilities in the Biomedical Research Center (Baltimore, MD, USA). At approximately 6 weeks of age (250-275 g), rats were implanted with chronic indwelling intravenous catheters in the right jugular vein under isoflurane anesthesia as previously described (9). Rats underwent MRI scanning between 3 and 3.5 months of age. Standard rat chow and water were available ad libitum in home cages and throughout the self-administration experiments, but not during the other experimental procedures. The plurality of experimental procedures was performed in the dark cycle, with few extending into the light cycle.

Drugs

Heroin hydrochloride was obtained from NIDA and dissolved in 0.9% sterile saline for intravenous infusions (60 µg/kg/0.1 ml). Naloxone hydrochloride was obtained from Hospira (Lake Forest, IL, USA) and dissolved in 0.9% sterile saline for a subcutaneous injection in a volume of 1 ml/kg and concentration of 120 µg/kg.

Statistics

Heroin self-administration data were analyzed using two-way analysis of variance (ANOVA), with heroin access (ShA versus LgA) as a between-subjects factor and session or cue (saline- versus naloxone-paired) as the within-subjects factor. Statistical significance was set at \( \alpha = 0.05 \). Post hoc comparisons were conducted when appropriate and \( P \) values were corrected for multiple comparisons using the Bonferroni method. The statistical analyses for behavioral
experiments were performed using GraphPad Prism 7 software. One LgA rat was excluded from the MRI study for failed catheter patency.

For fMRI data analysis, the averaged signal from white matter and the ventricles was removed by multiple regression analysis. We conducted a whole brain 2 (heroin access: ShA versus LgA) × 2 (cue: saline- versus naloxone-paired odor) × 2 (cue presentation block: blocks 1 and 2 versus blocks 3 and 4) mixed-design ANCOVA of the percent BOLD signal change, using average respiration rate during the block as a covariate. No a priori regional hypotheses were tested. The 3dClustSim program in AFNI was used to estimate the probability of false positive clusters, which was used to estimate the cluster size threshold for a given voxel-wise $P$ value threshold to correct for multiple comparisons. A cluster size of 13 with a corrected $P<0.01$ (uncorrected $P<0.01$) was considered significant. The correlation between behavior (withdrawal severity as the number of heroin infusions during naloxone conditioning) and BOLD signal (in response to odor cues in each of the 19 ROIs that were extracted from the activation cluster, defined by a significant cue × heroin access interaction) was evaluated using the Pearson test. The $P$ values were corrected for multiple comparisons using False Discovery Rate ($q=0.05$).

**Study Approval**

All procedures were reviewed and approved by the NIDA Intramural Research Program’s Institutional Animal Care and Use Committee (Baltimore, MD, USA), in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.
Author contributions

SAC, JCMV, and HL conducted the experiments. SAC, RJK, and EGLG analyzed the data. EAS and HL supervised the fMRI experiments. LFV and GFK supervised the behavioral experiments. SAC, GFK and LFV conceptualized and designed the study and wrote the manuscript. All authors contributed to the manuscript writing.

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References


Figure 1. Conditioned heroin withdrawal engages negative emotional learning neurocircuits. (A) Heroin intake during short (ShA) or long (LgA) access self-administration sessions. A significant heroin access × session interaction was observed (F_{9,171}=4.25, p=0.002; two-way repeated-measures ANOVA). (B) Heroin intake following saline (0 µg/kg, s.c.) or naloxone (Nx; 120 µg/kg, s.c.) treatments during cue pairings, presented as the average of the four cue pairings per treatment. Significant main effects of treatment (F_{1,19}=35.5, P<0.0001) and heroin access (F_{1,19}=4.215, P=0.05) were found (two-way repeated-measures ANOVA). (C) Statistical map (F-values) of the cue × heroin access BOLD signal interaction following a whole brain three-way ANCOVA, with respiration as the covariate (P<0.01; 233 voxels, corrected for multiple comparisons). The up-sampled (to anatomical images) statistical map is superimposed on anatomical coronal images from a representative subject. Below each section is the anterior-posterior distance from bregma (in millimeters). Data represent the means ± SEM in panels A and B. *P≤0.05, **P<0.01, ***P<0.001 (different from Session 1 and corrected for multiple comparisons in A). n = 11 for ShA rats and n = 10 for LgA rats.
Figure 2. Withdrawal severity during conditioning is associated with changes in the hypothalamic and amygdala nuclei BOLD signal change in response to the naloxone-paired cue. (A) Hypothalamic cluster extracted from the BOLD signal cue × heroin access interaction shown in Figure 1C, with a 3-D rendered whole brain underlay. (B) Mean percent BOLD signal change from baseline in the hypothalamic cluster to the cue only presentations. Dot plot displays individual data for each condition. (C) Scatter plot of the relationship between heroin intake during naloxone conditioning and hypothalamic percent BOLD signal change from baseline in response to the naloxone-paired cue across both heroin access groups (Pearson correlation). (D) Amygdala nuclei extracted from the BOLD signal cue × heroin access interaction shown in Figure 1C, with a 3-D rendered whole brain underlay. (E) Mean percent BOLD signal change from baseline in amygdala nuclei to the cue only presentations. Dot plot displays individual data for each condition. (F) Scatter plot of the relationship between heroin intake during naloxone conditioning and amygdala nuclei percent BOLD signal change from baseline to the naloxone-paired cue across both heroin access groups (Pearson correlation). \( n = 11 \) for ShA rats and \( n = 10 \) for LgA rats.
Table 1. Mean percent change in BOLD signal from baseline in response to saline and naloxone-paired cue-only presentation for short-access (ShA) and long-access (LgA) rats in regions of interest (ROIs)\textsuperscript{A}.

<table>
<thead>
<tr>
<th>ROI</th>
<th>ShA Saline Cue</th>
<th>ShA Naloxone Cue</th>
<th>LgA Saline Cue</th>
<th>LgA Naloxone Cue</th>
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<td>0.1</td>
<td>0.9</td>
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<td>-0.1</td>
<td>0.4</td>
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<td>-1.1</td>
<td>-1.1</td>
<td>0.6</td>
</tr>
<tr>
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<td>0.2</td>
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\textsuperscript{A}We anatomically segregated the cluster defined by the statistically significant heroin access (ShA versus LgA) × cue (saline-paired versus naloxone-paired) interaction (shown in Figure 1C) into 19 ROIs using a standard rat atlas (14).