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Nociceptin attenuates the escalation of oxycodone self-administration by normalizing CeA-GABA transmission in highly addicted rats

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Abstract

Approximately 25% of patients who are prescribed opioids for chronic pain misuse them, and 5-10% develop an opioid use disorder. Although the neurobiological target of opioids is well known, the molecular mechanisms that are responsible for the development of addiction-like behaviors in some but not all individuals are poorly known. To address this issue, we used a unique outbred rat population (Heterogeneous Stock) that better models the behavioral and genetic diversity that is found in humans. We characterized individual differences in addiction-like behaviors using an Addiction Index that incorporates the key criteria of opioid use disorder: escalated intake, highly motivated responding, and hyperalgesia. Using *in vitro* electrophysiological recordings in the central nucleus of the amygdala (CeA), we found that rats with high addiction-like behaviors (HA) exhibited a significant increase in γ-aminobutyric acid (GABA) transmission compared with rats with low addiction-like behaviors (LA) and naive rats. The superfusion of CeA slices with nociceptin/orphanin FQ peptide (N/OFQ; 500 nM), an endogenous opioid-like peptide, normalized GABA transmission in HA rats. Intra-CeA levels of N/OFQ were lower in HA rats than in LA rats. Intra-CeA infusions of N/OFQ (1 μg/site) reversed the escalation of oxycodone self-administration in HA rats but not in LA rats. These results demonstrate that the downregulation of N/OFQ levels in the CeA may be responsible for hyper-GABAergic tone in the CeA that is observed in individuals who develop addiction-like behaviors. Based on these results, we hypothesize that small molecules that target the N/OFQ system might be useful for the treatment of opioid use disorder.

Significance statement

More than 2 million individuals in the United States currently have a substance use disorder that is related to prescription opioid pain relievers. We identified individual differences in oxycodone addiction-like behaviors in outbred heterogenous stock rats with high (HA) and low (LA) addiction like-behaviors. We found that the downregulation of nociceptin levels in the central nucleus of the amygdala (CeA) may be responsible for hyper-γ-aminobutyric acid (GABA)ergic tone in the CeA that is observed in individuals who develop addiction-like behaviors. The development of small molecules that target the nociceptin system may have therapeutic efficacy for the treatment of opioid use disorder.

Introduction

More than 2 million individuals in the United States currently have a substance use disorder that is related to prescription opioid pain relievers, including oxycodone (Oxycontin®, Roxycodone®, Oxecta®), and 500,000 are addicted to heroin (1). Over the past 15 years, the consumption of oxycodone increased ~500%, and opioid-related overdose deaths quadrupled (2, 3). Although opioid medications effectively treat acute pain and help relieve chronic pain in some patients (4), the risk of addiction is a dilemma for healthcare providers who seek to relieve suffering while avoiding drug abuse and addiction. The misuse of and addiction to opioids— including prescription pain relievers, heroin, and synthetic opioids—is a serious national health crisis that affects public health and social and economic welfare. Opioid addiction is a complex disease that is characterized by phenotypic heterogeneity. One of the reasons for the lack of novel drug treatments for opioid use disorder is that the mechanisms that are responsible for individual differences in the propensity to develop mild to severe opioid use disorder are unknown. The better characterization of addiction-like behaviors using advanced models of extended access to oxycodone self-administration (5, 6) in animal strains that exhibit large individual differences, such as heterogeneous stock (HS) rats (7, 8), may provide new insights into medication development.

Opioid misuse and addiction develop through a spiral of positive reinforcement (reward) and negative reinforcement (withdrawal) (9). The rewarding effects of opioids decrease because of tolerance, whereas the negative affective state increases because of neuroadaptive changes that are hypothesized to occur in the central nucleus of the amygdala (CeA) (10-14), among several other key brain regions, such as the bed nucleus of the stria terminalis, ventral tegmental area, habenula, and ventral striatum (9). The CeA is the major output region of the amygdala, mainly

(95%) through long-range γ-aminobutyric acid (GABA)ergic output neurons that express high levels of pro-stress and anti-stress peptides (11).

Nociceptin/orphanin FQ (N/OFQ; hereinafter referred to as nociceptin) is a 17-amino-acid opioid-like peptide that binds with high affinity to the N/OFQ opioid (NOP) receptor but has no affinity for μ-opioid (MOP), δ-opioid (DOP), or κ-opioid (KOP) receptors. Both nociceptin and NOP receptors are expressed in the amygdala (15). Behavioral and neurochemical studies have suggested an important role for the nociceptin NOP receptor system in opioid tolerance and reward (16-19), addiction to alcohol (20-24) and other drugs of abuse (20, 25-27), and the modulation of stress (28-30). Interestingly, nociceptin, despite being an opioid-like peptide, blocks opioid-induced supraspinal analgesia (31) and morphine reward in the conditioned place preference paradigm (16, 17), morphine-induced dopamine release (32), and Fos expression in the nucleus accumbens (16). Additionally, pretreatment with a NOP receptor agonist modulated the subjective effects of oxycodone in mice (33). However, these previous studies were performed in nondependent animals with the goal of investigating the effect of nociceptin on the rewarding effects of opioids and elucidating the brain regions that are involved in the reward pathway (e.g., ventral tegmental area–nucleus accumbens pathway), and little was known about the role of nociceptin in opioid dependence and its role in brain pathways that are responsible for development of the negative affect state and motivation to take opioids during withdrawal. Interestingly, evidence of an association between two variants of the NOP receptor gene, *OPRL1*, and the vulnerability to opioid addiction in humans has been reported (34).

The present study investigated neuroadaptations of GABAergic and nociceptin transmission in the CeA in genetically diverse HS rats that exhibited high (HA) or low (LA) addiction-like behaviors after chronic (3 weeks) and extended (12 h/day) access to oxycodone self-

administration. We hypothesized that individual differences in addiction-like behaviors result from the dysregulation of GABAergic transmission by nociceptin in the CeA.

Results

Addiction Index: evaluation of individual differences in addiction-like behaviors

The timeline of the experiments is shown in Fig. 1A. The one-way analysis of variance (ANOVA) indicated that the animals significantly escalated their responses for oxycodone ($F_{14,308}$ = 9.934, $p < 0.001$; Fig. 1B). The Newman-Keuls *post hoc* test confirmed that significantly more active lever presses occurred from session 6 to session 9 ($p < 0.05$) and from session 10 to session 14 ($p < 0.001$) compared with session 1. Inactive lever responding remained low for the entire duration of the experiment, illustrating strong lever discrimination.

After the escalation phase, a significant increase in mechanical hypersensitivity was observed in rats that escalated their oxycodone intake, with a significant decrease in pain thresholds in animals during acute withdrawal (t_{24} = 8.654, $p < 0.001$; Fig. 1C) compared with pain thresholds during their naive state. To evaluate addiction-like behaviors in individual rats, we used an Addiction Index that was adapted from Deroche-Gamonet et al. (35, 36), which considers various addiction-like behaviors: escalation of oxycodone intake under a fixed-ratio (FR) schedule of reinforcement, motivation to maintain responding under a progressive-ratio (PR) schedule of reinforcement, and withdrawal-induced hyperalgesia. To combine these different behavioral outputs, each measure was normalized into an index using its Z-score ($Z = \frac{x-\mu}{\sigma}$), where χ is the raw value, μ is the mean of the cohort, and σ is the standard deviation of the cohort. We thus obtained an FR Index, PR Index, and Pain Index (Fig. 1D). For the FR Index, the final values were obtained by calculating a Z-score that was the average of the Z-scores of the last 3 days of

escalation. For the PR index, we calculated the Z-score of the breakpoint. For the Pain Index, the Z-score was calculated from the percent reduction of pain thresholds during withdrawal compared with naive baseline. Finally, we calculated the Addiction Index by averaging the Z-scores of the three dependent variables (FR Index, PR Index, and Pain Index; Fig 1E). Principal Component Analysis (PCA) was performed on the three addiction-like behaviors (escalation, motivation, and withdrawal-induced hyperalgesia) that comprise the Addiction Index to determine whether dimensionality could be reduced while maintaining variability. The PCA revealed only one component with an eigenvalue > 1, explaining 52% of variance and to which all three behaviors contributed in a valuable way ($r = 0.63$ to 0.83, Supporting Tables 1 and 2). This analysis supports the Addiction Index as a reflection of this first principal component and thus was a good approach to capture the variability in addiction-like behavior in one dimension.

We separated two groups of animals that were defined as high-addicted (HA; i.e., positive Addiction Index values) and low-addicted (LA; negative Addiction Index values) by a median split. As shown in Fig. 1D and E, significant differences in Z-scores were found for each individual variable after t-test comparisons when the groups were divided based on their Addiction Index: FR Index ($t_{23} = 4.203$, $p < 0.001$), PR Index ($t_{23} = 3.721$, $p < 0.001$), Pain Index ($t_{23} = 2.304$, $p < 0.05$), Addiction Index ($t_{23} = 5.778$, $p < 0.001$). Analyses of the raw data showed that both HA and LA rats escalated their oxycodone self-administration (Fig. 1F). The two-way ANOVA, with group (HA vs. LA) as the between-subjects factor and time (sessions) as the within-subjects factor, revealed significant effects of group ($F_{1,25} = 8.872$, $p < 0.01$) and time ($F_{13,325} = 8.933$, $p < 0.001$) and a significant group × time interaction ($F_{13,325} = 2.323$, $p < 0.01$). The Newman-Keuls $post\ hoc$ test showed that HA rats received significantly more drug infusions (rewards) on days 10-14 ($p < 0.001$), whereas LA rats received significantly more rewards on days 11-14 ($p < 0.05$). Moreover,

HA rats exhibited significantly higher oxycodone intake than LA rats on days 7-9 ($p < 0.05$) and days 10-14 ($p < 0.001$). The analysis of the PR data showed that HA rats reached higher breakpoints than LA rats (paired t-test: $t_{23} = 3.721$, $p < 0.01$; Fig. 1G). Finally, both HA and LA rats exhibited lower pain withdrawal thresholds compared with their pre-escalation baseline (LA: $t_{12} = 5.68$, $p < 0.001$; HA: $t_{11} = 13.29$, $p < 0.001$; paired t-tests), but HA rats exhibited an increase in hyperalgesia compared with LA rats (unpaired t-test: $t_{23} = 2.429$, $p < 0.05$; Fig. 1H).

Cue-induced reinstatement

We investigated the intensity of addiction-like behaviors in LA and HA rats by assessing their propensity to relapse to drug seeking (35). After training, escalation, and calculating the Addiction Index, 16 animals ($n = 8$ HA rats, $n = 8$ LA rats) were tested for cue-induced reinstatement (Fig. 2A). HA and LA rats extinguished active lever pressing behavior similarly (Fig. 2B). The two-way ANOVA, with group (HA *vs*. LA) as the between-subjects factor and time as the within-subjects factor, revealed a significant effect of time ($F_{9,126} = 7.8$, $p < 0.0001$) on active lever pressing but no significant effect of group ($F_{1,14} = 3.84$, $p = 0.07$) and no group × time interaction ($F_{9,126} = 0.75$, $p = 0.66$). For inactive lever presses, no significant effects of group ($F_{1,14} = 1.67$, $p = 0.22$) or time ($F_{9,126} = 1.40$, $p = 0.20$) were observed, with no group × time interaction ($F_{9,126} = 0.66$, $p = 0.75$). For reinstatement (Fig. 2B), the two-way ANOVA, with group (HA *vs*. LA) as the between-subjects factor and experiment (average lever pressing during the last 3 days of extinction, lever pressing during S^N presentation, and lever pressing during S^D presentation) as the within-subjects factor, revealed significant effects of group ($F_{1,14} = 4.60$, $p = 0.05$) and experiment ($F_{2,28} = 15.37$, $p < 0.0001$) and a significant group × experiment interaction ($F_{2,28} = 4.22$, $p = 0.025$). The Newman-Keuls *post hoc* test showed that reintroduction of the oxycodone-

discriminative cues (S^D) but not neutral stimuli (S^N) significantly reinstated extinguished oxycodone-seeking behavior in both groups ($p < 0.001$, *vs.* extinction for HA, and $p < 0.05$, *vs.* extinction for LA; Fig. 2B), but HA rats exhibited an increase in responding during S^D presentation compared with LA rats ($p < 0.001$; Fig. 2B). A significant effect of experiment on inactive lever presses was observed ($F_{2,28} = 9.24$, $p < 0.001$), with no effect of group ($F_{1,14} = 1.66$, $p = 0.22$) and no group × experiment interaction ($F_{2,28} = 1.38$, $p = 0.27$). The reinstatement score for individual animals, which was calculated from the Z-score of their active lever presses during S^D presentation, correlated with their Addiction Index ($r = 0.56$, $p = 0.023$; Fig 2C).

HA rats have higher basal GABA release in the CeA

We next investigated whether the behavioral differences between HA and LA rats reflected differences at the cellular level (Fig. 3A). Output neurons in the CeA are mainly GABAergic; thus, we investigated GABAergic transmission by recording spontaneous inhibitory postsynaptic currents (sIPSCs) in CeA slices (Fig. 3B). The resting membrane potential of the neuronal sample was -64 ± 0.5 mV ($n = 27$). The input resistance was 256 ± 8 MΩ ($n = 27$). The holding potential was -67 ± 0.6 mV ($n = 27$). We found differences in baseline sIPSC frequencies in the CeA between HA rats, LA rats, and naive rats, confirmed by one-way ANOVA ($F_{2,23} = 8.714$, $p < 0.01$). The Newman-Keuls *post hoc* test indicated an increase in the baseline frequency of sIPSCs in HA rats compared with LA rats ($p < 0.01$) and naive rats ($p < 0.05$; Fig. 3C). No differences in sIPSC amplitude ($F_{2,23} = 1.689$, $p > 0.05$; Fig. 3D), sIPSC rise ($F_{2,23} = 0.48$, $p > 0.05$; Fig. 3E), or sIPSC decay ($F_{2,23} = 0.58$, $p > 0.05$; Fig. 3F) were observed between groups.

Nociceptin-mediated modulation of GABAergic transmission in the CeA in HA rats vs. LA rats

We next investigated whether nociceptin prevents hyper-GABAergic transmission in the CeA. The acute (10 min) application of nociceptin (500 nM) (28) on CeA slices significantly decreased sIPSC frequencies compared with baseline frequencies in oxycodone-treated rats but not in naive rats (naive: $t_7 = 1.855$, $p > 0.05$; LA: $t_7 = 3.998$, $p < 0.05$; HA: $t_9 = 6.367$, $p < 0.01$; Fig. 4A-C). The one-way ANOVA showed that the effect of nociceptin was more pronounced in HA rats ($F_{2,23} = 4.415$, $p < 0.05$) than in naive and LA rats. The Newman-Keuls *post hoc* test showed a significant difference in sIPSC frequency between HA and LA rats ($p < 0.05$; Fig. 4D). No differences in sIPSC amplitude were detected between groups upon the application of nociceptin (Fig. 4E).

Nociceptin in the CeA selectively reduces oxycodone self-administration in HA rats

To further investigate the effects of nociceptin in the CeA on addiction-like behaviors, two cohorts of rats were trained to self-administer oxycodone and then underwent bilateral cannulation in the CeA for localized nociceptin administration (Fig. 5A). After recovery and reestablishing baseline self-administration in one cohort, nociceptin and vehicle were injected in a Latin-square design, and the effects of nociceptin on oxycodone self-administration were evaluated in HA and LA rats ($n = 8$ HA rats, $n = 7$ LA rats; Fig. 5B). The two-way ANOVA, with group (HA *vs.* LA) as the between-subjects factor and treatment as the within-subjects factor, revealed significant effects of group ($F_{1,11} = 7.78$, $p < 0.05$) and treatment ($F_{1,11} = 15.68$, $p < 0.01$) and a significant group × treatment interaction ($F_{1,11} = 10.66$, $p < 0.01$). The Newman-Keuls *post hoc* test showed that HA rats self-administered significantly more oxycodone compared with LA rats when the animals received vehicle ($p < 0.01$). Nociceptin administration selectively reduced oxycodone intake in HA rats ($p < 0.01$) but was ineffective in LA rats. The histological verification of cannula

placements identified two animals with misplaced cannulas (Fig. 5C). Data from these two rats were excluded from the main data analysis. Importantly, the behavioral data from these two rats showed that the injection of nociceptin outside the CeA did not produce any behavioral effects, suggesting that the reduction of oxycodone intake was CeA-specific.

In the second cohort, the effect of nociceptin on withdrawal-induced hyperalgesia was evaluated in HA and LA rats ($n = 7$ HA rats, $n = 7$ LA rats) that received intra-CeA injections of nociceptin or vehicle in a Latin-square design (Fig. 5D). The two-way ANOVA, with group (HA *vs*. LA) as the between-subjects factor and treatment as the within-subjects factor, revealed a significant effect of group ($F_{1,12} = 9.095$, $p = 0.01$) but no effect of treatment ($F_{1,12} = 2.99$, $p = 0.1$) and no group × treatment interaction ($F_{1,12} = 0.067$, $p = 0.8$). Nociceptin treatment showed only a nonsignificant trend toward a reduction of pain withdrawal thresholds in both groups.

HA rats have low levels of nociceptin in the CeA

Oxycodone-dependent rats ($n = 7$ HA rats, $n = 7$ LA rats) were euthanized 12 h into withdrawal, and their brains were rapidly removed (Fig. 5A). Relative nociceptin levels were determined between HA and LA rats and compared with their naive littermates (Fig. 5E). The one-way ANOVA showed a significant effect of group ($F_{2,18} = 5.034$, $p = 0.02$). The Newman-Keuls *post hoc* test showed that nociceptin levels significantly decreased in HA rats compared with naive rats ($p = 0.02$; Fig. 5F). The Z-score of individual nociceptin levels in the CeA correlated with individual animals' Addiction Index ($r = -0.62$, $p = 0.017$, Fig. 5G).

Discussion

The present study identified individual differences in oxycodone addiction-like behaviors in a population of genetically diverse HS rats with high (HA) and low (LA) addiction like-behaviors. HA rats exhibited an increase in CeA GABAergic transmission. Nociceptin decreased CeA GABAergic transmission in oxycodone-dependent rats, with a more pronounced effect in HA rats. HA rats also exhibited lower levels of nociceptin in the CeA. Intra-CeA nociceptin administration reduced oxycodone self-administration selectively in HA rats, without affecting intake in LA rats.

A critical step in the identification of novel targets for medication development is the use of animal models that incorporate key behavioral endpoints that are used in the diagnosis of opioid use disorder in humans. Such an analysis should be performed in a genetically diverse outbred population of rodents to better mimic genetic diversity among humans. We used an animal model of extended access to oxycodone self-administration combined with advanced behavioral analysis of the transition from controlled to escalated oxycodone self-administration (5, 6) in outbred HS rats. We measured the escalation of oxycodone intake using an FR schedule of reinforcement and the motivation for oxycodone intake using a PR schedule of reinforcement (35, 37). We also longitudinally assessed mechanical pain thresholds to evaluate hyperalgesia during oxycodone withdrawal (38). This advanced behavioral analysis provided dependent measures that we used to calculate the Addiction Index and identify animals with high (HA) and low (LA) addiction-like behaviors. Importantly, both HA rats and LA rats developed oxycodone dependence, in which they both significantly escalated their oxycodone intake and exhibited lower pain thresholds during withdrawal. However, we found significant differences between HA and LA rats in all three behavioral measures. HA rats exhibited higher escalation of oxycodone intake, higher breakpoints on the PR schedule, and lower pain thresholds during withdrawal compared with LA rats (Fig. 1).

A cue-induced reinstatement test was also performed in these rats. No difference in responding for the lever that was previously paired with oxycodone was observed between HA and LA rats during extinction, but HA rats were more vulnerable to cue-induced relapse than LA rats, which is another major criteria for opioid use disorder (Fig. 2).

We next investigated whether the behavioral differences between HA and LA rats reflected differences at the cellular level by examining withdrawal-induced neuroadaptations in the CeA during extended access to oxycodone self-administration. HA rats exhibited an increase in baseline spontaneous GABAergic transmission, suggesting an increase in tonic GABA levels in HA rats (Fig. 3). This is consistent with previous studies that reported CeA neuroadaptations after extended access to cocaine self-administration (39) and after chronic intermittent exposure to alcohol vapor (40, 41). The lack of an effect on sIPSC amplitude, rise, and decay suggests that the increase in sIPSC frequency mostly reflected an increase in GABA release rather than postsynaptic adaptation. Additionally, extended access to oxycodone self-administration altered the sensitivity of CeA GABAergic transmission in response to nociceptin. Indeed, the acute bath application of nociceptin (500 nM) on CeA slides significantly decreased sIPSC frequencies compared with baseline frequencies in oxycodone-exposed rats but not in naive rats. The lack of an effect of nociceptin in naive animals contrasts with previous findings (28, 42) but may be related to the different strains of rats that were used in these studies. However, the nociceptin-induced decreases in GABAergic transmission were significantly higher in HA rats than in LA rats. These results are similar to findings in alcohol-dependent rats, in which nociceptin decreased basal GABAergic transmission and blocked the CRF-induced increase in GABA release to a greater extent than in naive controls (42). In the present study, all of the electrophysiological recordings were performed in the medial part of the CeA (CeM) because this area is a major output region that has been

repeatedly implicated in the addiction process (43). However, the laterocapsular CeA has been referred to as the nociceptive amygdala, and neuroadaptations may also have occurred in this region (44-46). Our results suggest a presynaptic effect on GABA release onto the recorded CeM neurons. The precise location of the GABA-releasing neurons is unknown but may have originated from laterocapsular CeA or local CeM GABA neurons. This should be investigated in future studies.

This mechanism was then confirmed *in vivo*. Significantly lower levels of nociceptin were detected by Western blot in the CeA in HA rats compared with naive rats. The restoration of nociceptin levels by site-specific microinjections of nociceptin in the CeA before the self-administration session selectively reduced operant responding for oxycodone in HA rats (Fig. 5), without affecting oxycodone intake in LA rats. Intra-CeA nociceptin administration did not selectively reduce withdrawal-induced hyperalgesia in HA rats. This may indicate that withdrawal-induced hyperalgesia was regulated by a different pathway.

In summary, the present study found that the CeA nociceptin-GABA system is involved in the transition from controlled to escalated oxycodone intake. We hypothesize that high oxycodone intake in HA rats may lead to downregulation of the nociceptin system in the CeA and consequently the upregulation of CeA GABAergic transmission, which in turn may promote addiction-like behaviors. The disinhibition of GABAergic transmission in the CeA was normalized by the exogenous application of nociceptin. Intra-CeA nociceptin administration also attenuated addiction-like behaviors in HA rats. These results are consistent with a previous study in alcohol-dependent animals (42) and suggest that the dysregulation of nociceptin may be a critical step in the transition to addiction-like behaviors. These findings suggest that the nociceptin system may be a promising target for the treatment of opioid use disorder. The direct effects of oxycodone on

nociceptin and associations with the underlying diverse individual genotype of HS rats will be the topic of future genome-wide association studies.

Materials and Methods

Animals

Male HS rats were created to have as much genetic diversity as possible at the National Institutes of Health in the 1980s by outbreeding eight inbred rat strains (ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N, and WN/N) (7). The HS rats ($n = 50$) were provided by Dr. Leah Solberg Woods (Medical College of Wisconsin, now at Wake Forest University School of Medicine). The animals were housed two per cage on a reverse 12 h/12 h light/dark cycle (lights off at 8:00 AM) in a temperature (20-22°C) and humidity (45-55%) controlled vivarium with *ad libitum* access to tap water and food pellets (PJ Noyes Company, Lancaster, NH, USA). All of the procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. At the time of testing, the rats' body weights ranged between 350 and 400 g.

Intravenous catheterization and intracranial surgery

The animals were anesthetized by isoflurane inhalation, and intravenous catheters were aseptically inserted in the right jugular vein using a modified version of a procedure that was described previously (47, 48). The vein was punctured with a 22-gauge needle, and the tubing was inserted and secured inside the vein by tying the vein with suture thread. The catheter assembly consisted of an 18 cm length of Micro-Renathane tubing (0.023 inch inner diameter, 0.037 inch

outer diameter; Braintree Scientific, Braintree, MA, USA) that was attached to a guide cannula (Plastics One, Roanoke, VA, USA). The guide cannula was bent at a near right angle, embedded in dental acrylic, and anchored with mesh (2 cm square). The catheter exited through a small incision on the back, and the base was sealed with a small plastic cap and metal cover cap. This design helped maintain the catheter base sterile and protected. The catheters were flushed daily with heparinized saline (10 U/ml of heparin sodium; American Pharmaceutical Partners, Schaumburg, IL, USA) in 0.9% bacteriostatic sodium chloride (Hospira, Lake Forest, IL, USA) that contained 20 mg/0.2 ml of the antibiotic Timentin (GlaxoSmithKline).

A subset of the animals that received intra-CeA injections for behavioral analysis underwent stereotaxic surgery. To reach the CeA, guide cannulas were bilaterally implanted using the following coordinates with reference to bregma: anterior/posterior, -2.6 mm; medial/lateral, ±4.2 mm; dorsal/ventral, -6.1 mm. The drug infusion was performed using a stainless-steel injector that protruded 2 mm from the guide cannula into the CeA. The animals were allowed to recover for 1 week after surgery.

Drugs

Oxycodone (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% sodium chloride (Hospira, Lake Forest, IL, USA) and administered at a dose 150 µg/0.1 ml/kg. For the intracranial injections, nociceptin (Tocris, Minneapolis, MN, USA) was dissolved in 0.9% sodium chloride and injected bilaterally in the CeA at a concentration of 1 µg/0.5 µl/site. For electrophysiology, nociceptin (500 nmol/ml) was dissolved in the superfusate 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 CGP55845A (1 µM).

Operant training

Self-administration was performed in operant conditioning chambers (Med Associates, St. Albans, VT, USA) that were enclosed in sound-attenuating, ventilated environmental cubicles. The front door and back wall of the chambers were constructed of transparent plastic, and the other walls were opaque metal. Each chamber was equipped with two retractable levers that were located on the front panel. Oxycodone was delivered through plastic catheter tubing that was connected to an infusion pump. The infusion pump and a cue light were activated by responses on the right (active) lever. Responses on the left (inactive) lever were recorded but did not have any scheduled consequences. Activation of the pump resulted in the delivery of 0.1 ml of the fluid. A computer controlled fluid delivery and behavioral data recording.

Oxycodone self-administration

Each session was initiated by the extension of two retractable levers into the operant chamber (29 cm × 24 cm × 19.5 cm; Med Associates, St. Albans, VT, USA). Responses on the right active lever were reinforced on an FR1 schedule by intravenous (IV) oxycodone (150 μg/0.1 ml/kg/infusion) administration that was infused over 6 s followed by a 20 s timeout (TO20 s) period that was signaled by the illumination of a cue light above the active lever for 14 days in 12-h daily sessions (5 sessions/week). Responses on the left inactive lever were recorded but had no scheduled consequences. On day 15, the rats were tested on a PR schedule of reinforcement, in which the response requirements for receiving a single reinforcement increased according to the following equation: $[5e^{(\text{injection number} \times 0.2)}] - 5$. This resulted in the following progression of response requirements: 1, 1, 2, 2, 3, 3, 4, 4, 6, 6, 8, 8, 10, 11, 12, 13, 14,

15, 16, 17, etc. (+1) until 50, 60, 70, 80. 90, 100. The breakpoint was defined as the last ratio attained by the rat prior to a 60 min period during which a ratio was not completed.

Mechanical nociceptive von Frey test

Hindpaw withdrawal thresholds were determined using von Frey filaments, ranging from 3.63 to 125.89 g. A test session began after 10 min of habituation to the testing environment. A series of von Frey filaments was applied from below the wire mesh to the central region of the plantar surface of the left hindpaw in ascending order, beginning with the smallest filament (3.63 g). The filament was applied until buckling of the hair occurred, and it remained in place for approximately 2 s. A sudden withdrawal of the hindpaw indicated a positive response. The stimulus was incrementally increased until a positive response was observed and then decreased until a negative response was observed to determine a pattern of responses to apply to the statistical method of Dixon (49). The 50% paw withdrawal threshold was determined by the formula $Xf + k\delta$, where Xf is the last von Frey filament applied, k is the Dixon value that corresponded to the response pattern, and δ is the mean difference between stimuli. Once the threshold was determined for the left hindpaw, the same testing procedure was repeated for the right hindpaw after 5 min. Paw withdrawal thresholds were recorded before beginning the oxycodone self-administration sessions (i.e., when the animal was still naive to oxycodone) and after the last self-administration session (i.e., the morning of day 15, 12 h into withdrawal, immediately before PR testing). Paw withdrawal thresholds were also recorded for one cohort ($n = 14$) 10 min after the intra-CeA injection of nociceptin or saline (12 h after the last self-administration session).

Cue-induced reinstatement

Once the escalation of oxycodone self-administration was established and after the rats were characterized as HA or LA, one cohort of male HS rats ($n = 14$) underwent 10 additional 12-h self-administration sessions in the presence of an intermittent tone that served as a contextual/discriminative stimulus (S^D) that signaled availability of the reinforcer throughout the session. Following completion of the training procedure, the rats underwent daily 2-h extinction sessions, in which responses on the previously active lever had no programmed consequences (i.e., no oxycodone delivery and no light or sound cue presentation). This phase lasted until responding was extinguished (< 10 responses per session for 3 consecutive days). Twenty-four hours after the last extinction session, all of the rats were presented with a neutral stimulus (S^N) in a 2-h session to control for specificity of the S^D to reinstate extinguished oxycodone-seeking behavior. During the S^N session, continuous 70 dB white noise and illumination of a 2.8 W house light that was located at the top of the chamber's front panel signaled non-availability of the reinforcer. The next day, the rats were presented with the light and sound S^D.

Electrophysiology

Slices of the CeA were prepared from naive and oxycodone-dependent (HA and LA) rats ($n = 15$ rats total, $n = 5$/group). The naive rats were age-matched to the rats that were used in the behavioral experiments. Sham IV surgery was performed to equalize post-surgical stress in both groups. The rats were deeply anesthetized with isoflurane, and brains were rapidly removed and placed in oxygenated (95% O_2, 5% CO_2) ice-cold cutting solution that contained 206 mM sucrose, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 5 mM glucose, and 5 mM HEPES. Transverse slices (300 μm thick) were cut on a Vibratome (Leica VT1000S, Leica Microsystems, Buffalo Grove, IL, USA) and transferred to

oxygenated artificial cerebrospinal fluid (aCSF) that contained 130 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.0 mM $MgSO_4 \cdot 7H_2O$, 2.0 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose. The slices were first incubated for 30 min at 35°C and then kept at room temperature for the remainder of the experiment. Individual slices were transferred to a recording chamber that was mounted on the stage of an upright microscope (Olympus BX50WI). The slices were continuously perfused with oxygenated aCSF at a rate of 2-3 ml/min. Neurons were visualized with a 60× water immersion objective (Olympus), infrared differential interference contrast optics, and a charge-coupled device camera (EXi Blue, QImaging, Surrey, Canada). Whole-cell recordings were performed using a Multiclamp 700B amplifier (10 kHz sampling rate, 10 kHz low-pass filter) and Digidata 1440A and pClamp 10 software (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes (4-7 MΩ) were pulled from borosilicate glass (Warner Instruments, Hamden, CT, USA) and filled with 70 mM $KMeSO_4$, 55 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, 2 mM Na-ATP, and 0.2 mM Na-GTP. Liquid junction potential corrections were performed offline. Pharmacologically isolated sIPSCs were recorded in the presence of the glutamate receptor blockers CNQX and APV and the $GABA_B$ receptor antagonist CGP55845. Experiments with a series resistance of > 15 MΩ or > 20% change in series resistance were excluded from the final dataset. The frequency, amplitude, and kinetics of sIPSCs were analyzed using semi-automated threshold-based mini detection software (MiniAnalysis, Synaptosoft, Fort Lee, NJ, USA) and visually confirmed.

Effect of intra-CeA nociceptin on oxycodone self-administration and withdrawal-induced hyperalgesia

Once the escalation of oxycodone self-administration was established, the animals ($n = 15$)

underwent stereotaxic surgery for bilateral cannula implantation in the CeA. After 1 week of post-surgical recovery, the rats were returned to the self-administration chambers to restore their oxycodone self-administration within 10% of previous baseline levels. At this point, treatment began. One cohort of rats received bilateral injections of nociceptin (1 µg/0.5 µl/site) or vehicle 5 min before beginning the self-administration session in a counterbalanced order. A session of baseline oxycodone self-administration was performed between the two rounds of the Latin-square design. At the end of the experiments, the animals were re-baselined for oxycodone self-administration and euthanized 4 days later for histology and Western blot analysis.

After cannulation surgery, recovery, and re-baselining, another cohort of rats ($n = 16$) received bilateral injections of nociceptin (1 µg/0.5 µl/site) or vehicle 10 min before testing hyperalgesia using the mechanical nociceptive von Frey test 12 h into withdrawal (i.e., when the animal usually starts a session). A session of baseline oxycodone self-administration was performed between the two rounds of the Latin-square design. At the end of the experiments, the animals were re-baselined for oxycodone self-administration and euthanized the next day for Western blot analysis.

Western blot

The rats were euthanized by decapitation 12 h into withdrawal (i.e., the time point that corresponded to initiation of the daily self-administration sessions). The brains were rapidly removed, snap frozen, and entirely sliced (300-µm-thick slices). The CeA was dissected by punching (16 gauge needle), and the tissue was kept frozen until the analysis. The samples (1 punch/20 µl) were homogenized in cold RIPA lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) that

contained Halt protease and phosphatase inhibitor cocktail (catalog no. 78441, Thermo Fisher Scientific). The homogenates were centrifuged at 10,000 × g for 10 min at 4°C, and the protein concentration of the supernatant was determined using the Pierce PCA protein assay (catalog no. 23227, Thermo Fisher Scientific). The samples (15 μl + 5 μl 4× Laemmli sample buffer with 2-mercaptoethanol, Bio-Rad) were heated at 95°C for 5 min and loaded on a 4-15% Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-Rad) with 1× running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Electrophoretic separation was performed on gel at 200 V for 30 min, which was then transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad) by wet transfer at 25 V for 45 min. After transfer, the membrane was blocked with 5% milk in 0.05% Tween-20 in PBS (PBST). Immunoblot analysis was performed by overnight incubation at 4°C with primary antibodies for prepronociceptin (1:500, catalog no. NB100-1619, Novus Biologicals) and β-tubulin (1:1000, catalog no. NB600-936, Novus Biologicals) in 3% bovine serum albumin in PBST and secondary antibodies conjugated to horseradish peroxidase (rabbit, 1:10000, Cell Signaling Technology) in 5% milk in PBST at room temperature for 45 min. Antigen-antibody complexes were detected by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and analyzed using a ChemiDoc MP (Bio-Rad) with Image Lab and ImageJ software. Prepronociceptin band intensities were normalized to β-tubulin band intensities to account for sample differences and are expressed relative to one of the samples from the naive group that was loaded on the same blot to account for differences in transfer efficiency between blots.

Statistical analysis

The self-administration data were analyzed using repeated-measures ANOVA of the number of infusions that were earned during the escalation interval. Data from the mechanical

nociception von Frey test during withdrawal were analyzed using Student's *t*-test by comparing the withdrawal results with baselines during the animals' naive state or comparing the HA and LA groups. Data from the cue-induced reinstatement and intra-CeA nociceptin experiments were analyzed using mixed factorial ANOVA, with group as the between-subjects factor and treatment as the within-subjects factor. Significant main effects were followed by the Newman-Keuls multiple-comparison *post hoc* test. Locally evoked IPSP amplitudes were analyzed using Clampfit 10 software (Molecular Devices, Sunnyvale, CA, USA). The electrophysiological data were analyzed using *t*-tests or ANOVAs followed by the Newman-Keuls *post hoc* test when appropriate. Nociceptin levels between groups were analyzed using one-way ANOVA. Correlation analysis was performed by calculating Pearson correlation coefficients. For the Principal Component Analysis, three variables were considered: FR (measured as the average of number of rewards in the last 3 days), PR (measured as breakpoints during the PR schedule), and pain thresholds (measured as hyperalgesia during withdrawal). The principal component was selected according to an eigenvalue > 1 (see Supporting Table 1). The data are expressed as the mean ± SEM. In cases of ≤ 10 data points, individual data points are represented in the graphs. The statistical analyses were performed using GraphPad Prism, SPSS, and Statistica 7 software. Values of $p < 0.05$ were considered statistically significant.

References

1. Administration SAaMHS (2013) *Results from the 2012 National Survey on Drug Use and Health: Summary of National Findings* (Rockville, MD), (Administration SAaMHS).

2. Kolodny A*, et al.* (2015) The prescription opioid and heroin crisis: a public health approach to an epidemic of addiction. *Annual review of public health* 36:559-574.

3. Compton WM, Jones CM, & Baldwin GT (2016) Relationship between Nonmedical Prescription-Opioid Use and Heroin Use. *New England Journal of Medicine* 374(2):154-163.

4. Moore A, Derry S, Eccleston C, & Kalso E (2013) Expect analgesic failure; pursue analgesic success. *BMJ (Clinical research ed.)* 346:f2690.

5. Zhang Y*, et al.* (2014) Extended access oxycodone self-administration and neurotransmitter receptor gene expression in the dorsal striatum of adult C57BL/6J mice. *Psychopharmacology* 231(7):1277-1287.

6. Wade CL, Vendruscolo LF, Schlosburg JE, Hernandez DO, & Koob GF (2015) Compulsive-Like Responding for Opioid Analgesics in Rats with Extended Access. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 40(2):421-428.

7. Hansen C & Spuhler K (1984) Development of the National Institutes of Health genetically heterogeneous rat stock. *Alcohol Clin Exp Res* 8(5):477-479.

8. Woods LC & Mott R (2017) Heterogeneous Stock Populations for Analysis of Complex Traits. *Methods Mol Biol* 1488:31-44.

9. Koob GF (2019) Neurobiology of Opioid Addiction: Opponent Process, Hyperkatifeia, and Negative Reinforcement. *Biological psychiatry*.

10. Cohen A*, et al.* (2015) Extended access to nicotine leads to a CRF1 receptor dependent increase in anxiety-like behavior and hyperalgesia in rats. *Addict Biol* 20(1):56-68.

11. Gilpin NW, Herman MA, & Roberto M (2015) The central amygdala as an integrative hub for anxiety and alcohol use disorders. *Biological psychiatry* 77(10):859-869.

12. Koob GF (2008) A role for brain stress systems in addiction. *Neuron* 59(1):11-34.

13. Schmeichel BE, Herman MA, Roberto M, & Koob GF (2017) Hypocretin Neurotransmission Within the Central Amygdala Mediates Escalated Cocaine Self-administration and Stress-Induced Reinstatement in Rats. *Biological psychiatry* 81(7):606-615.

14. Li X, Zeric T, Kambhampati S, Bossert JM, & Shaham Y (2015) The central amygdala nucleus is critical for incubation of methamphetamine craving. *Neuropsychopharmacology* 40(5):1297-1306.

15. Neal CR, Jr.*, et al.* (1999) Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. *J Comp Neurol* 406(4):503-547.

16. Ciccocioppo R, Angeletti S, Sanna PP, Weiss F, & Massi M (2000) Effect of nociceptin/orphanin FQ on the rewarding properties of morphine. *Eur J Pharmacol* 404(1-2):153-159.

17. Murphy NP, Lee Y, & Maidment NT (1999) Orphanin FQ/nociceptin blocks acquisition of morphine place preference. *Brain Res* 832(1-2):168-170.

18. Scoto GM, Arico G, Iemolo A, Ronsisvalle G, & Parenti C (2010) Selective inhibition of the NOP receptor in the ventrolateral periaqueductal gray attenuates the development and

the expression of tolerance to morphine-induced antinociception in rats. *Peptides* 31(4):696-700.

19.   Yuan L, Han Z, Chang JK, & Han JS (1999) Accelerated release and production of orphanin FQ in brain of chronic morphine tolerant rats. *Brain Res* 826(2):330-334.

20.   Ciccocioppo R, Panocka I, Polidori C, Regoli D, & Massi M (1999) Effect of nociceptin on alcohol intake in alcohol-preferring rats. *Psychopharmacology (Berl)* 141(2):220-224.

21.   Ciccocioppo R*, et al.* (2002) Pharmacological characterization of the nociceptin receptor which mediates reduction of alcohol drinking in rats. *Peptides* 23(1):117-125.

22.   Ciccocioppo R*, et al.* (2014) Chronic treatment with novel brain-penetrating selective NOP receptor agonist MT-7716 reduces alcohol drinking and seeking in the rat. *Neuropsychopharmacology* 39(11):2601-2610.

23.   de Guglielmo G, Martin-Fardon R, Teshima K, Ciccocioppo R, & Weiss F (2015) MT-7716, a potent NOP receptor agonist, preferentially reduces ethanol seeking and reinforcement in post-dependent rats. *Addict Biol* 20(4):643-651.

24.   Martin-Fardon R, Ciccocioppo R, Massi M, & Weiss F (2000) Nociceptin prevents stress-induced ethanol- but not cocaine-seeking behavior in rats. *Neuroreport* 11(9):1939-1943.

25.   Zhao RJ*, et al.* (2003) Orphanin FQ/nociceptin blocks methamphetamine place preference in rats. *Neuroreport* 14(18):2383-2385.

26.   Kotlinska J*, et al.* (2003) Nociceptin inhibits acquisition of amphetamine-induced place preference and sensitization to stereotypy in rats. *Eur J Pharmacol* 474(2-3):233-239.

27.   Kuzmin A, Sandin J, Terenius L, & Ogren SO (2003) Acquisition, expression, and reinstatement of ethanol-induced conditioned place preference in mice: effects of opioid

receptor-like 1 receptor agonists and naloxone. *The Journal of pharmacology and experimental therapeutics* 304(1):310-318.

28. Ciccocioppo R*, et al.* (2014) Restraint stress alters nociceptin/orphanin FQ and CRF systems in the rat central amygdala: significance for anxiety-like behaviors. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 34(2):363-372.

29. Devine DP, Hoversten MT, Ueda Y, & Akil H (2003) Nociceptin/orphanin FQ content is decreased in forebrain neurones during acute stress. *J Neuroendocrinol* 15(1):69-74.

30. Devine DP, Watson SJ, & Akil H (2001) Nociceptin/orphanin FQ regulates neuroendocrine function of the limbic-hypothalamic-pituitary-adrenal axis. *Neuroscience* 102(3):541-553.

31. Mogil JS*, et al.* (1996) Orphanin FQ is a functional anti-opioid peptide. *Neuroscience* 75(2):333-337.

32. Di Giannuario A & Pieretti S (2000) Nociceptin differentially affects morphine-induced dopamine release from the nucleus accumbens and nucleus caudate in rats. *Peptides* 21(7):1125-1130.

33. Walentiny DM, Wiebelhaus JM, & Beardsley PM (2018) Nociceptin/orphanin FQ receptors modulate the discriminative stimulus effects of oxycodone in C57BL/6 mice. *Drug Alcohol Depend* 187:335-342.

34. Briant JA*, et al.* (2010) Evidence for association of two variants of the nociceptin/orphanin FQ receptor gene OPRL1 with vulnerability to develop opiate addiction in Caucasians. *Psychiatr Genet* 20(2):65-72.

35. Deroche-Gamonet V, Belin D, & Piazza PV (2004) Evidence for addiction-like behavior in the rat. *Science* 305(5686):1014-1017.

36. Belin D, Balado E, Piazza PV, & Deroche-Gamonet V (2009) Pattern of intake and drug craving predict the development of cocaine addiction-like behavior in rats. *Biological psychiatry* 65(10):863-868.

37. Chen BT, *et al.* (2013) Rescuing cocaine-induced prefrontal cortex hypoactivity prevents compulsive cocaine seeking. *Nature* 496(7445):359-362.

38. Edwards S, *et al.* (2012) Development of mechanical hypersensitivity in rats during heroin and ethanol dependence: alleviation by CRF(1) receptor antagonism. *Neuropharmacology* 62(2):1142-1151.

39. Kallupi M, *et al.* (2013) Kappa opioid receptor-mediated dysregulation of gamma-aminobutyric acidergic transmission in the central amygdala in cocaine addiction. *Biological psychiatry* 74(7):520-528.

40. Repunte-Canonigo V, *et al.* (2015) Nf1 regulates alcohol dependence-associated excessive drinking and gamma-aminobutyric acid release in the central amygdala in mice and is associated with alcohol dependence in humans. *Biological psychiatry* 77(10):870-879.

41. Roberto M, Madamba SG, Stouffer DG, Parsons LH, & Siggins GR (2004) Increased GABA release in the central amygdala of ethanol-dependent rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 24(45):10159-10166.

42. Cruz MT, Herman MA, Kallupi M, & Roberto M (2012) Nociceptin/orphanin FQ blockade of corticotropin-releasing factor-induced gamma-aminobutyric acid release in central amygdala is enhanced after chronic ethanol exposure. *Biological psychiatry* 71(8):666-676.

43. Koob GF (2009) Brain stress systems in the amygdala and addiction. *Brain Res* 1293:61-75.

44.   Bourgeais L, Gauriau C, & Bernard JF (2001) Projections from the nociceptive area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. *The European journal of neuroscience* 14(2):229-255.

45.   Gauriau C & Bernard JF (2002) Pain pathways and parabrachial circuits in the rat. *Exp Physiol* 87(2):251-258.

46.   Li W & Neugebauer V (2004) Differential roles of mGluR1 and mGluR5 in brief and prolonged nociceptive processing in central amygdala neurons. *J Neurophysiol* 91(1):13-24.

47.   Caine SB & Koob GF (1993) Modulation of cocaine self-administration in the rat through D-3 dopamine receptors. *Science* 260(5115):1814-1816.

48.   de Guglielmo G*, et al.* (2013) Pregabalin reduces cocaine self-administration and relapse to cocaine seeking in the rat. *Addict Biol* 18(4):644-653.

49.   Dixon WJ (1980) Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 20:441-462.

Figure Legends

Figure 1. Development of oxycodone dependence in two distinct populations of HS rats identified by the Addiction Index. (**A**) Schematic timeline of the experiments. (**B**) Escalation of oxycodone intake in HS rats. *$p < 0.05$, ***$p < 0.01$, *vs.* day 1. (**C**) Oxycodone withdrawal-induced hyperalgesia in HS rats. Bars represent the paw withdrawal threshold. ***$p < 0.01$, *vs.* baseline. (**D**) Z-scores for FR Index, PR Index, and Pain Index in LA rats (light blue) and HA rats (dark blue). *$p < 0.05$, ***$p < 0.001$, *vs.* LA. (**E**) Addiction Index in LA rats (light blue) and HA rats (dark blue). ***$p < 0.001$, *vs.* LA. (**F**) Escalation of oxycodone intake in HA and LA rats. *$p < 0.05$, ***$p < 0.001$, *vs.* day 1; #$p < 0.05$, ###$p < 0.001$, *vs.* LA. (**G**) Oxycodone self-administration on a progressive-ratio schedule of reinforcement in HA and LA rats. **$p < 0.01$, *vs.* LA. (**H**) Oxycodone withdrawal-induced hyperalgesia in HA and LA rats. ***$p < 0.001$, *vs.* baseline; #$p < 0.05$, *vs.* LA. The dotted line indicates the naive baseline threshold.

Figure 2. Extinction and cue-induced reinstatement in HA and LA rats. (**A**) Schematic timeline of the experiments. (**B**) HA rats exhibited significant reinstatement of oxycodone intake compared with LA rats. The figure shows the baselines at the end of the self-administration phase (SA), lever pressing during extinction, and responses during the neutral stimulus (S^N) session and contextual/discriminative stimulus (S^D) session. ***$p < 0.001$, *$p < 0.05$, *vs.* Extinction; ##$p < 0.001$, *vs.* LA ($n = 16$ [$n = 8$/group]). (**C**) Correlation between Addiction Index and Z-score of the number of active lever presses during cue-induced reinstatement. *$p < 0.05$.
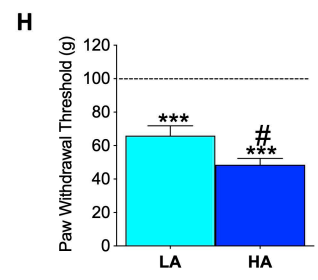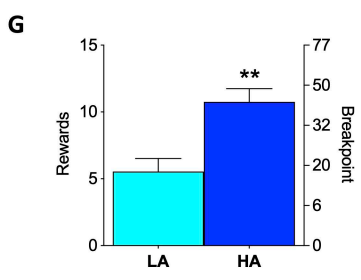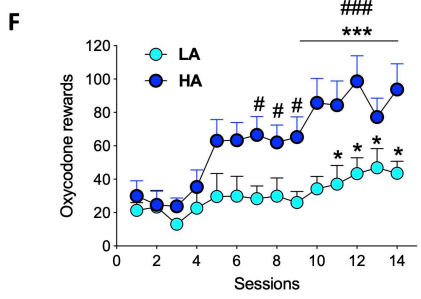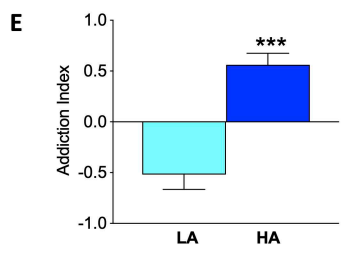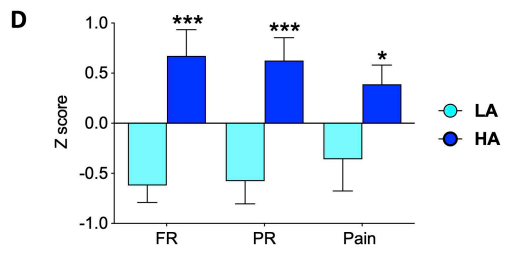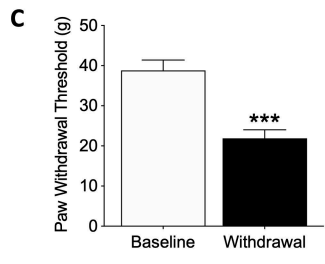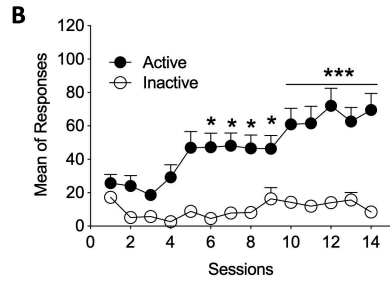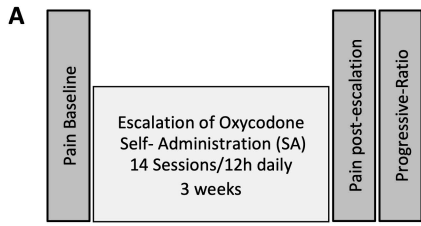
Figure 3. HA rats exhibited an increase in GABAergic transmission in the CeA. (**A**) Schematic timeline of the experiments. (**B**) Central nucleus of the amygdala slice that shows placement of the

recording electrode (infrared optics, 4× objective). (**C**) HA rats exhibited an increase in sIPSC frequency compared with naive rats (*$p < 0.05$) and LA rats (**$p < 0.01$). No differences were observed in (**D**) sIPSC amplitude, (**E**) sIPSC rise, or (**F**) sIPSC decay.

Figure 4. Nociceptin decreased GABAergic transmission in CeA neurons in oxycodone-dependent rats. (**A**) (*Left*) Nociceptin did not significantly affect sIPSC frequency in naive rats. (*Right*) Representative whole-cell recordings and the effect of nociceptin on sIPSCs in naive rats. (**B**) (*Left*) Nociceptin reduced sIPSC frequency in LA rats ($p < 0.05$). (*Right*) Representative whole-cell recordings and the effect of 500 nM nociceptin on sIPSCs in LA rats. (**C**) (*Left*) Nociceptin reduced sIPSC frequency in HA rats. (*Right*) Representative whole-cell recordings and the effect of nociceptin on sIPSCs in HA rats. (**D**) Nociceptin more effectively reduced GABAergic transmission in HA rats than in LA rats. *$p < 0.05$, **$p < 0.01$, *vs*. baseline; [#]$p < 0.05$, *vs*. LA rats. (E) Nociceptin did not affect the sIPSC amplitude in any of the three groups.

Figure 5. Intra-CeA nociceptin administration reversed the escalation of oxycodone intake selectively in HA rats. (**A**) Schematic timeline of the experiments. Two cohorts underwent this procedure: one cohort was tested for the effect of intra-CeA nociceptin administration on self-administration, and the other cohort was tested for the effect of intra-CeA nociceptin administration on pain thresholds. (**B**) Effect of intra-CeA nociceptin administration on oxycodone self-administration in HA and LA rats. *$p < 0.05$, *vs*. vehicle; [##]$p < 0.01$, *vs*. HA rats. Red triangles represent data from animals with cannula misplacements. (**C**) Schematic diagram of intra-CeA cannula placements for the self-administration experiment (black dots) and pain threshold experiment (blue dots). (**D**) Effect of intra-CeA nociceptin administration on

oxycodone withdrawal-induced hyperalgesia in HA and LA rats. The dotted line indicates the naive baseline threshold. $n = 13$ ($n = 6$-7/group). (**E**) Immunoluminescent Western blots that show nociceptin levels in naive, LA, and HA rats. $n = 21$ ($n = 7$/group). (**F**) Western blot revealed a significant decrease in nociceptin levels (internally normalized to tubulin and a naive rat on the blot) in HA rats compared with naive rats. HA rats exhibited a significant decrease in the normalized signal intensity of nociceptin compared with naive rats. *$p = 0.02$. (**G**) Correlation between Addiction Index and the Z-score of nociceptin levels in the CeA. *$p < 0.02$.

A Pain Baseline | Escalation of Oxycodone Self-Administration (SA) 14 Sessions/12h daily 3 weeks | Pain post-escalation | Progressive-Ratio

B

Mean of Responses vs Sessions

- Active (●)
- Inactive (○)

***

C

Paw Withdrawal Threshold (g)

Baseline | Withdrawal ***

D

Z score

FR *** | PR *** | Pain *

- LA (cyan)
- HA (blue)

E

Addiction Index

LA | HA ***

F

Oxycodone rewards vs Sessions

- LA (cyan)
- HA (blue)

/ *** / # #

G

Rewards / Breakpoint

LA | HA **

H

Paw Withdrawal Threshold (g)

LA *** | HA # / ***

A

| Pain Baseline | Escalation of Oxycodone Self- Administration (SA) 14 Sessions/12h daily 3 weeks | Pain post-escalation | Progressive-Ratio | SA with S^D 10 sessions 12h daily | Extinction till stable 10 sessions | S^N-induced reinstatement | S^D-induced reinstatement |

B

C

A

| Pain Baseline | Escalation of Oxycodone Self- Administration (SA) 14 Sessions/12h daily 3 weeks | Pain post-escalation | Progressive-Ratio | Rebaseline SA 12h daily | Electrophysiology Recording after the end of the session |

B

CeA

C sIPSC frequency (Hz) — Naïve, LA, HA; * **

D sIPSC amplitude (pA) — Naïve, LA, HA

E sIPSC rise (ms) — Naïve, LA, HA

F sIPSC decay (ms) — Naïve, LA, HA

A Naive

Frequency (Hz)

(n=8; *p*=0.09)

Vehicle | Nociceptin (500 nM)

Baseline | N/OFQ 500nM

20 pA
3 s

B LA

Frequency (Hz)

(n=8; *p*=0.017)

Vehicle | Nociceptin (500 nM)

Baseline | N/OFQ 500nM

10 pA
3 s

C HA

Frequency (Hz)

(n=11; *p*=0.0003)

Vehicle | Nociceptin (500 nM)

Baseline | N/OFQ 500nM

10 pA
3 s

D **Nociceptin** *(frequency)*

Effect on sIPSC (%control)

*

#
**

Naive | LA | HA

E **Nociceptin** *(amplitude)*

Effect on sIPSC (%control)

Naive | LA | HA

A

Pain Baseline | Escalation of Oxycodone Self-Administration (SA) 14 Sessions/12h daily 3 weeks | Pain post-escalation | Progressive-Ratio | CeA bilateral cannulation | Recovery 1 week | Rebaseline SA 12h daily | Pain or SA — N/OFQ intraCeA | Baseline SA | Pain or SA — N/OFQ intraCeA | Baseline SA | Tissue Collection

D1 | D2 | D3 | D4

Latin Square

B

Oxycodone Rewards

C

-2.30 mm
-2.56 mm
-2.80 mm

D

Paw Withdrawal Threshold (% of naive baseline)

E

β–tubulin
Prepronociceptin

β–tubulin
Prepronociceptin

F

Relative nociceptin level

naive   LA   HA

G

Nociceptin

Addiction Index