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

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Approximately 25% of patients who are prescribed opioids for chronic pain misuse them, and 5 to 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 per 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.

addiction | nociceptin | GABA | amygdala | hyperalgesia

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, and Oxecta), and 500,000 are addicted to heroin (1). Over the past 15 y, 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 prostress and antistress 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, δ-opioid, or κ-opioid 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

Significance

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 and low 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.

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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 wk) and extended (12 h/d) 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. 1*A*. 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. 1*B*). 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. 1*C*) compared with pain thresholds during their naive state. To evaluate addiction-like behaviors in individual rats, we used an addiction

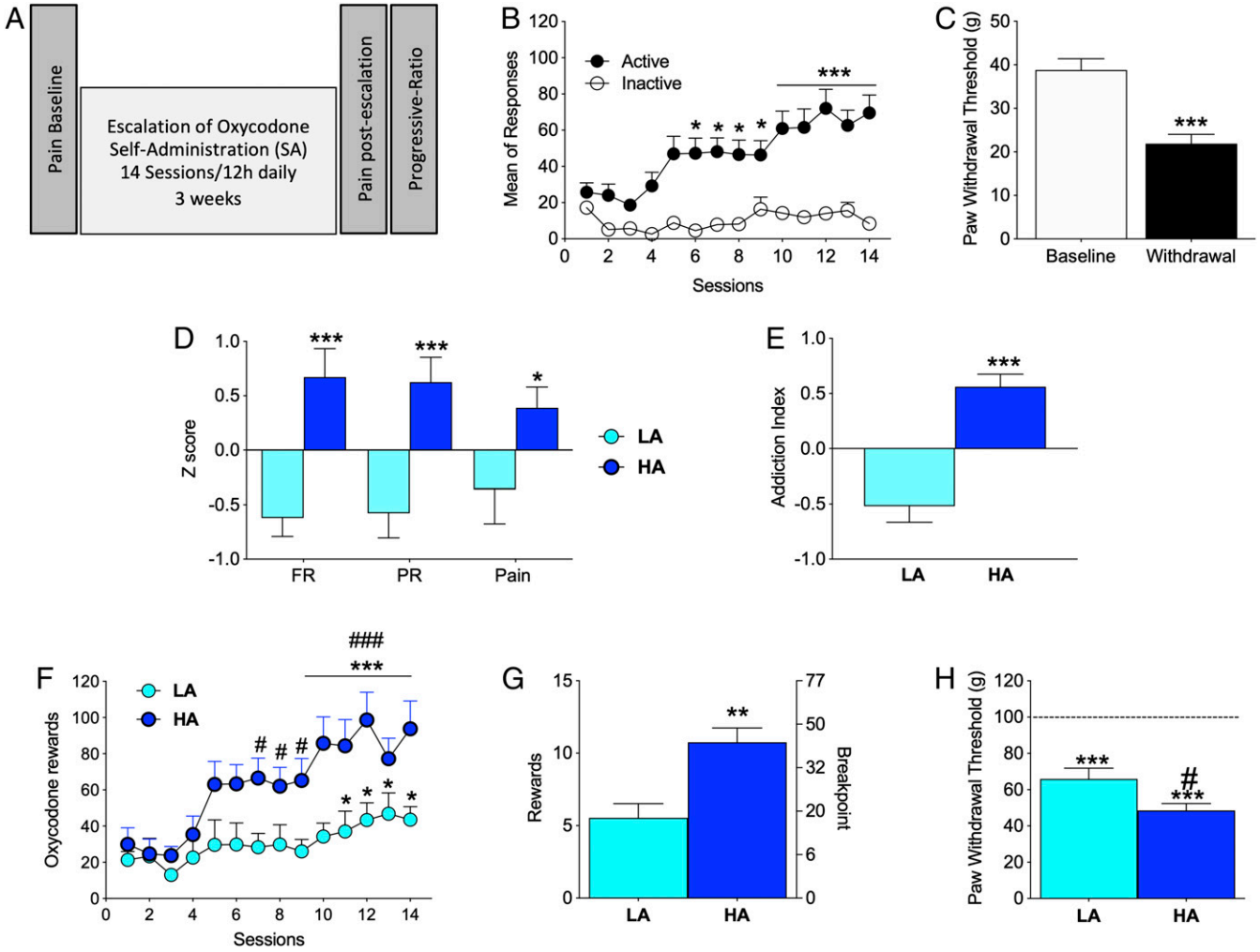

Fig. 1. Development of oxycodone dependence in two distinct populations of HS rats identified by the addiction index. (*A*) Schematic timeline of the experiments. (*B*) Escalation of oxycodone intake in HS rats. $^{*}P < 0.05$, $^{***}P < 0.01$, vs. day 1. (*C*) Oxycodone withdrawal-induced hyperalgesia 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 PR 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.

index that was adapted from Deroche-Gamonet et al. (35) and Belin et al. (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 ($Z = (x - \mu)/\sigma$), where x is the raw value, μ is the mean of the cohort, and σ is the SD of the cohort. We thus obtained an FR index, PR index, and pain index (Fig. 1*D*). 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 d 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. 1*E*). 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; *SI Appendix*, Tables S1 and S2). 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. 1 *D* 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 ($t_{23} = 2.304$, $P < 0.05$), and 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. 1*F*). 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 to 14 ($P < 0.001$), whereas LA rats received significantly more rewards on days 11 to 14 ($P < 0.05$). Moreover, HA rats exhibited significantly higher oxycodone intake than LA rats on days 7 to 9 ($P < 0.05$) and days 10 to 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. 1*G*). Finally, both HA and LA rats exhibited lower pain withdrawal thresholds compared with their preescalation 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. 1*H*).

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. 2*A*). HA and LA rats extinguished active lever pressing behavior similarly (Fig. 2*B*). 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. 2*B*), the two-way ANOVA, with group (HA vs. LA) as the between-subjects factor and experiment (average lever pressing during the last 3 d 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. 2*B*), but HA rats exhibited an increase in responding during S^D presentation compared with LA rats ($P < 0.001$; Fig. 2*B*). 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, positively correlated with their addiction index ($r = 0.56$, $P = 0.023$; Fig. 2*C*).

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. 3*A*). Output neurons in the CeA are mainly GABAergic; thus, we investigated GABAergic transmission by recording spontaneous inhibitory postsynaptic currents (sIPSCs) in CeA slices (Fig. 3*B*). 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. 3*C*). No differences in sIPSC amplitude ($F_{2,23} = 1.689$, $P > 0.05$; Fig. 3*D*), sIPSC rise ($F_{2,23} = 0.48$, $P > 0.05$; Fig. 3*E*), or sIPSC decay ($F_{2,23} = 0.58$, $P > 0.05$; Fig. 3*F*) 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. 4 *A*–*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. 4*D*). No differences in sIPSC amplitude were detected between groups upon the application of nociceptin (Fig. 4*E*).

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. 5*A*). 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. 5*B*). The two-way ANOVA, with group (HA vs. LA) as the between-subjects factor and

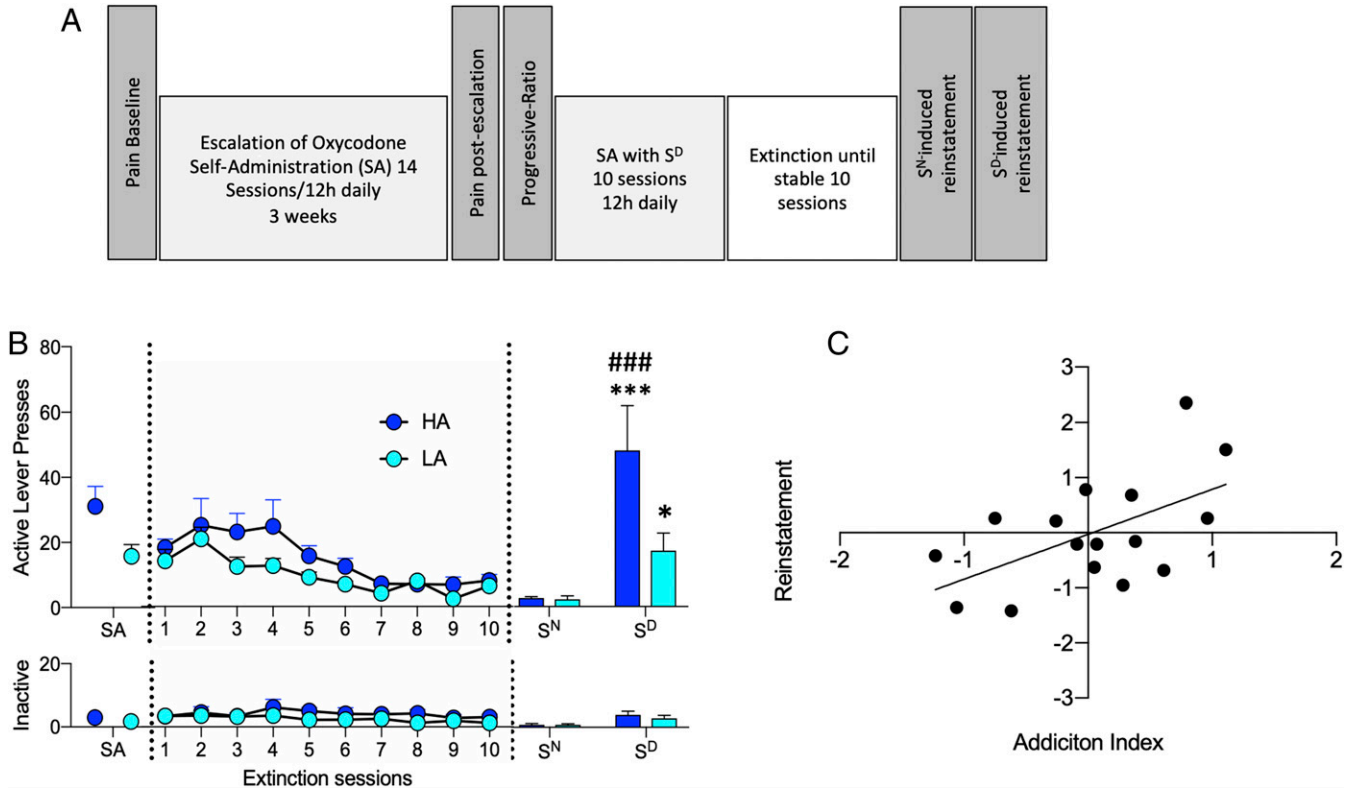

Fig. 2. Extinction and cue-induced reinstatement in HA and LA rats. (*A*) Schematic timeline of the experiments. (*B*) HA rats exhibited significant reinstatement of oxycodone intake compared with LA rats. The figure shows the baselines at the end of the self-administration phase (SA), lever pressing 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 per group]). (*C*) Correlation between addiction index and Z-score of the number of active lever presses during cue-induced reinstatement. $^{*}P < 0.05$.

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. 5*C*). Data from these two rats were

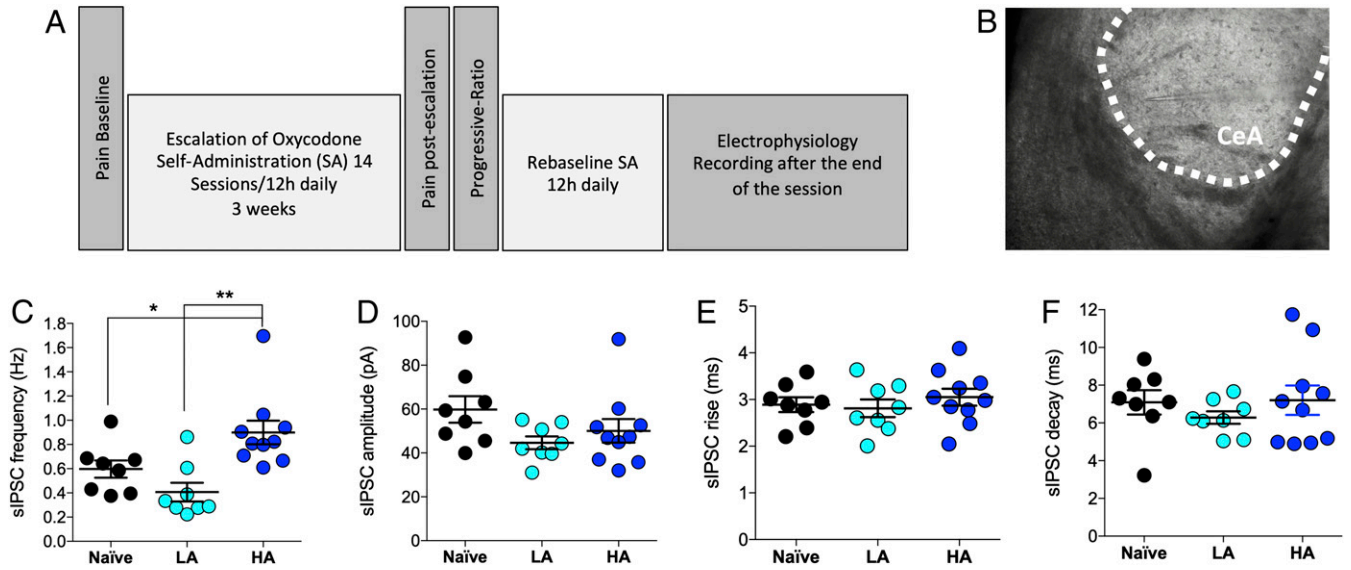

Fig. 3. HA rats exhibited an increase in GABAergic transmission in the CeA. (*A*) Schematic timeline of the experiments. (*B*) Central nucleus of the amygdala slice that shows placement of the recording electrode (infrared optics, 4× objective). (*C*) HA rats exhibited an increase in sIPSC frequency 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.

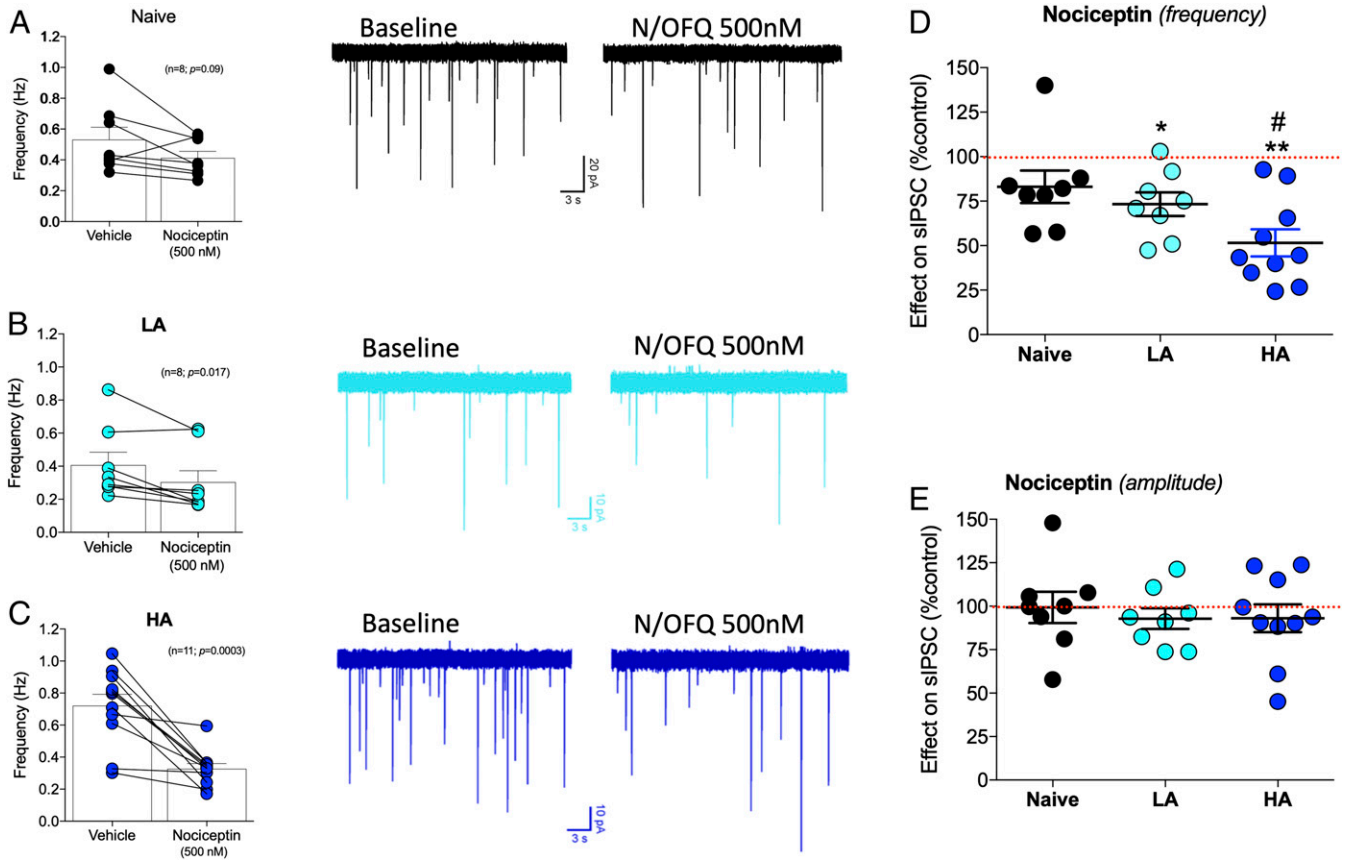

Fig. 4. Nociceptin decreased GABAergic transmission in CeA neurons in oxycodone-dependent rats. (*A*, *Left*) Nociceptin did not significantly affect sIPSC frequency in naive rats. (*A*, *Right*) Representative whole-cell recordings and the effect of nociceptin on sIPSCs in naive rats. (*B*, *Left*) Nociceptin reduced sIPSC frequency in LA rats ($P < 0.05$). (*B*, *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. (*C*, *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.

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. 5*D*). 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 sacrificed 12 h into withdrawal, and their brains were rapidly removed (Fig. 5*A*). Relative nociceptin levels were determined between HA and LA rats and compared with their naive littermates (Fig. 5*E*). 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. 5*F*). The Z-score of individual nociceptin levels in the CeA correlated with individual animals' addiction index ($r = -0.62$, $P = 0.017$; Fig. 5*G*).

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

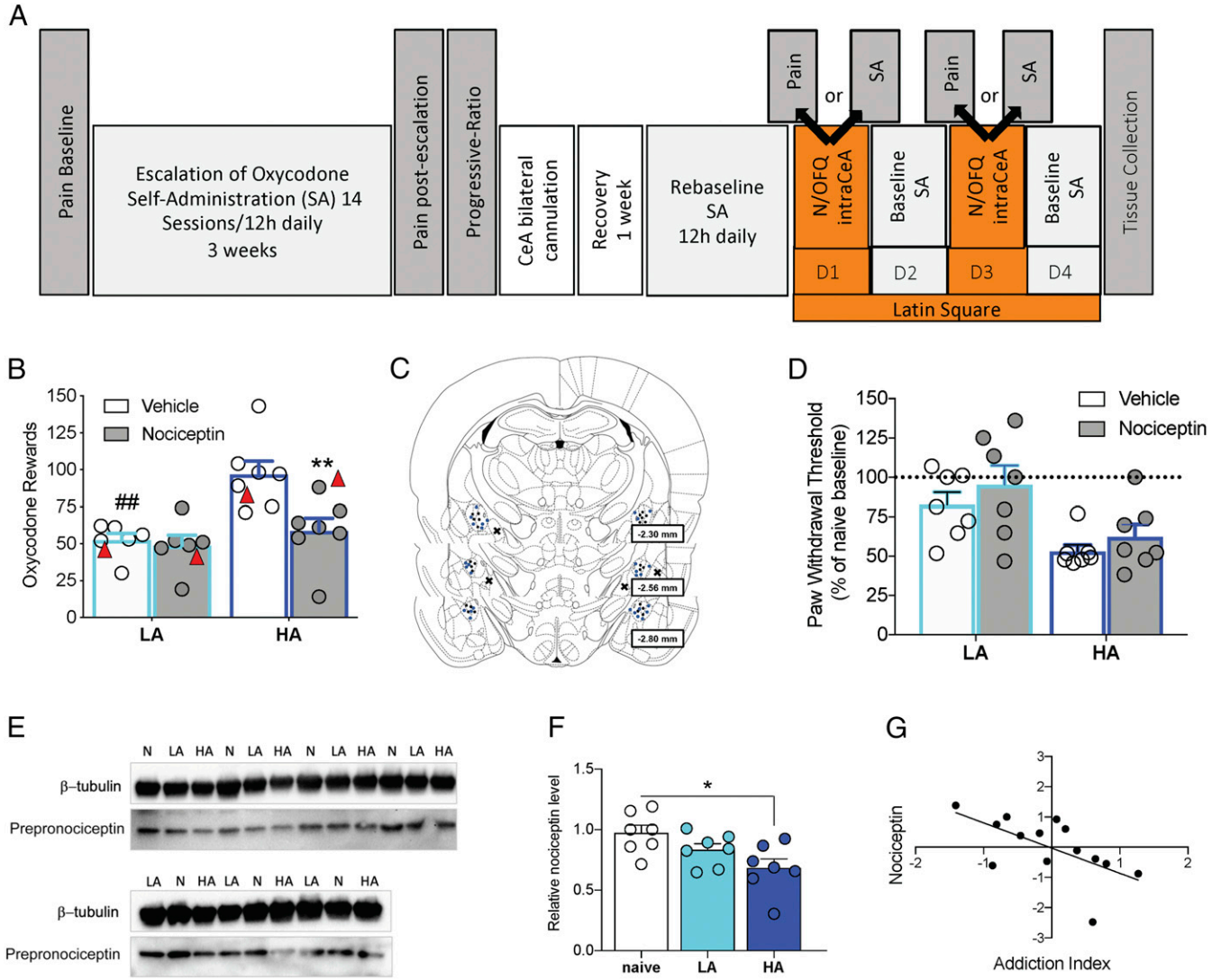

Fig. 5. Intra-CeA nociceptin administration reversed the escalation of oxycodone intake selectively in HA rats. (*A*) Schematic timeline of the experiments. Two cohorts underwent this procedure. One cohort was tested for the effect of intra-CeA nociceptin administration on self-administration, and the 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. $n = 13$ ($n = 6$ or 7 per group). (*E*) Immunoluminescent Western blots that show nociceptin levels in naive, LA, and HA rats. $n = 21$ ($n = 7$ per group). (*F*) Western blot revealed a significant decrease in nociceptin levels (internally normalized to β-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. $^{*}P < 0.02$.

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 criterion 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 corticotropin-releasing factor–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 L.C.S.W. 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 to 22 °C) and humidity- (45 to 55%) controlled vivarium with ad libitum access to tap water and food pellets (P. J. Noyes Company). All of the procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (47) 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 (i.v.) catheters were aseptically inserted in the right jugular vein using a modified version of a procedure that was described previously (48, 49). 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) that was attached to a guide cannula (Plastics One). 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) in 0.9% bacteriostatic sodium chloride (Hospira) 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 wk after surgery.

Drugs. Oxycodone (Sigma-Aldrich) was dissolved in 0.9% sodium chloride (Hospira) and administered at a dose of 150 μg/0.1 mL/kg. For the intracranial injections, nociceptin (Tocris) 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) 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). Responses on the right active lever were reinforced on an FR1 schedule by i.v. oxycodone (150 μg/0.1 mL/kg per infusion) administration that was infused over 6 s followed by a 20-s timeout period that was signaled by the illumination of a cue light above the active lever for 14 d in 12-h daily sessions (five sessions per wk). 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, and so on (+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 ∼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 (50). 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 three 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 nonavailability 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, $n = 5$ per group). The naive rats were age-matched to the rats that were used in the behavioral experiments. Sham i.v. surgery was performed to equalize postsurgical 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) 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 to 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). 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). Patch pipettes (4–7 MΩ) were pulled from borosilicate glass (Warner Instruments) 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 semiautomated threshold-based mini detection software (MiniAnalysis; Synaptosoft) 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 wk of postsurgical 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 per 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 rebaselined for oxycodone self-administration and sacrificed 4 d later for histology and Western blot analysis.

After cannulation surgery, recovery, and rebaselining, another cohort of rats ($n = 16$) received bilateral injections of nociceptin (1 μg/0.5 μL per 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 rebaselined 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 per 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 mixture (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 (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 to 15% Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-Rad) with 1× running buffer (25 mM Tris, 192 mM glycine, and 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 phosphate-buffered saline (PBST). Immunoblot analysis was performed by overnight incubation at 4 °C with primary antibodies for prepronociceptin (1:500, NB100-1619; Novus Biologicals) and β-tubulin (1:1,000, NB600-936; Novus Biologicals) in 3% bovine serum albumin in PBST and secondary antibodies conjugated to horseradish peroxidase (rabbit, 1:10,000; 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 inhibitory postsynaptic potential amplitudes were analyzed using Clampfit 10 software (Molecular Devices). 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 PCA, three variables were considered: FR (measured as the average of number of rewards in the last 3 d), 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 (*SI Appendix*, Table S1). 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.

Data Availability. The datasets presented in the current study are available from the corresponding author upon request.

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