CRH^{CeA→VTA} inputs inhibit the positive ensembles to induce negative effect of opiate withdrawal

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Plasticity of neurons in the ventral tegmental area (VTA) is critical for establishment of drug dependence. However, the remodeling of the circuits mediating the transition between positive and negative effect remains unclear. Here, we used neuronal activity-dependent labeling technique to characterize and temporarily control the VTA neuronal ensembles recruited by the initial morphine exposure (morphine-positive ensembles, Mor-Ens). Mor-Ens preferentially projected to NAc, and induced dopamine-dependent positive reinforcement. Electrophysiology and rabies viral tracing revealed the preferential connections between the VTA-projective corticotropin-releasing hormone (CRH) neurons of central amygdala (CRH^{CeA→VTA}) and Mor-Ens, which was enhanced after escalating morphine exposure and mediated the negative effect during opiate withdrawal. Pharmacologic intervention or CRISPR-mediated repression of CRHR1 in Mor-Ens weakened the inhibitory CRH^{CeA→VTA} inputs, and alleviated the negative effect during opiate withdrawal. These data suggest that neurons encoding opioid reward experience are inhibited by enhanced CRH^{CeA→VTA} inputs during opiate withdrawal, leading to negative effect during opiate withdrawal, and provide new insight into the pathological changes in VTA plasticity after drug abuse and mechanism of opiate dependence.

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INTRODUCTION

Drugs of abuse (e.g., cocaine, opiates) trigger the initial acute-reward effect, lead to adaptive changes in the brain function following administration [1–3], and produce unpleasant physical and negative effects, including dysphoria, depression, irritability, and anxiety after the termination of use [4, 5]. Both the reward effect of the drug and the desire to avoid the unpleasant somatic symptoms or emotional feelings drive alternating rounds of positive and negative reinforcement for maintaining drug dependence, respectively [6]. For example, the clinical use of morphine, the most potent analgesic for chronic pain, has been limited due to severe withdrawal symptoms and a high risk of relapse [6, 7]. The transition to a drug-dependent state is accompanied by a series of changes in the plasticity of brain circuits [8], which triggers the pathological changes in the emotional processing.

Neurons in the ventral tegmental area (VTA) can be activated by emotional stimuli (i.e., positive/negative valence, high/low arousal) such as reward and aversion, and mediate the expression of the adaptively appropriate behavior [9–11]. Recent studies showed that synaptic inputs to the VTA from the laterodorsal tegmentum and the lateral habenula drive reward and aversion in mice, respectively [12, 13]. The establishment of an opiate-dependent state and aversive withdrawal symptoms is dependent on the plasticity of VTA [14, 15]. The diversity of GABAergic inputs modulates the activity of VTA dopaminergic neurons [16–19].

Dopaminergic neurons are activated by disinhibition of GABAergic projections from the RMTg during morphine withdrawal [20–22]. The transition from an opiate-naive to an opiate-dependent state is associated with a change, from an inhibitory to an excitatory response, of the GABA_A receptors on GABAergic neurons in the VTA [23, 24]. These studies indicate that heterogeneous inhibitory inputs to the VTA participate in the development of opioid dependence.

Drug dependence has been hypothesized to be driven by two relatively independent systems: the downregulation of the dopamine (DA)-reward system (DA function) in the VTA—a within-system neuronal adaptation that leads to anhedonia, and the upregulation of the stress system—a between-system neuronal adaptation that leads to stress disorders. Corticotropin-releasing hormone (CRH) system has been shown to be involved in neuroplasticity changes evoked during drug withdrawal [25, 26]; however, how CRH neurons participate in the synaptic inhibition of the reward system following chronic opiate exposure remains undetermined.

In this study, we specifically labeled neuronal ensembles recruited by initial morphine exposure in the VTA, taking the advantage of the immediate early gene-based, synaptic and neuronal activity-responsive systems. Our data reveal that chronic morphine administration preferentially enhanced the GABAergic inputs of CRH neurons in the central amygdala (CeA) to VTA dopaminergic morphine ensembles, which was essential for the development of the negative effect during opiate withdrawal.
Our results highlight the importance of CRH neuron-mediated functional connectivity between CeA and VTA, which reduces morphine euphoria and drives the negative effect via enhanced GABAergic transmission onto the VTA ensembles encoding a drug-reward experience.

RESULTS
The activation of VTA neuronal ensembles recruited by morphine induces DA releasing in NAc and positive reinforcement

We used adeno-associated virus (AAV) expressing CreERT2 driven by enhanced synaptic activity-responsive element (AAV-E-SARE-CreERT2) and AAV-DIO-mCherry to label VTA neuronal ensembles activated by saline or morphine exposure. Mice were given a single intraperitoneal injection (i.p.) of saline or 10 mg/kg morphine at various times after tamofoxen (TAM) induction in the home cage. Ensembles were efficiently labeled 24-36 h after TAM induction (Supplementary Fig. 1a-d). The specific recruitment of the Sal-Ens and Mor-Ens by ESARE-triggered approach was verified by c-Fos staining following reexposure to saline or morphine, respectively (Supplementary Fig. 1e, f). Quantification of the ensembles labeled by saline (Sal-Ens) and morphine (Mor-Ens) across the anterior–posterior axis of the VTA (Fig. 1a, b and Supplementary Fig. 2a-d) showed that Sal-Ens was enriched in rostral VTA, and Mor-Ens was relatively enriched in medium VTA (Fig. 1b). Both Mor-Ens and Sal-Ens sent the majority of collaterals to the nucleus accumbens (NAc), lateral habenula (LHb), amygdala, and medial prefrontal cortex (mPFC). The normalized terminal intensity of the ensembles indicated that Mor-Ens terminals were more abundant in the NAc, while they showed no difference in the LHb, mPFC and amygdala compared with that of the Sal-Ens (Supplementary Fig. 2e, f). To validate and quantify the NAC-projecting ensembles in the VTA, the green retrograde tracer (cholera toxin B subunit, CTB-488) was injected into the NAC of mice, and the tagged ensembles in the VTA were analyzed (Fig. 1c, d). The percentage of retrogradely labeled cells in Mor-Ens was higher than that in Sal-Ens (Fig. 1e). Triple labeling with tyrosine hydroxylase (TH) staining indicated that the percentage of dopaminergic NAc-projecting Mor-Ens was greater than that in Sal-Ens (Fig. 1f). These results show that VTA Mor-Ens form more dopaminergic connections to NAc, as compared with Sal-Ens.

Instrumental reinforcement procedures combined with chemogenetic activation of the ensembles were used to evaluate the reinforcement effect of the morphine ensembles. Mice infected with AAV-E-SARE-CreERT2 and AAV-DIO-Hm3Dq-mCherry or AAV-DIO-mCherry in the VTA wereconditioned in a place-preference chamber after clozapine N-oxide (CNO) injection to specifically activate VTA Sal- or Mor-Ens in vivo (Supplementary Fig. 3a, b). Mice in Mor-Ens group showed significant preference for the CNO-paired chamber, more time spent in open arm, without affecting locomotor activity and saccharin preference, compared with the Sal-Ens group (Supplementary Fig. 3c-f).

Virus infected mice implanted with an optic fiber above the VTA were trained to intracranial self-stimulation (ICSS) with 473 nm laser (20 Hz, 5 ms) by nose pokes after ensemble labeling (Fig. 1g, h). The number of nose pokes coupled to optical stimulation in mice of Mor-Ens group increased during training trials, and reached to a high level at training days 5-6, in contrast to unchanged nose pokes throughout training trials in Sal-Ens group or control mice lacking ChR2 (Fig. 1l). Pretreatment with D2 receptor antagonist flupenthixol (0.5 mg/kg, i.p.) [27] significantly attenuated nose pokes associated with laser stimulation of Mor-Ens (Fig. 1j). Dopamine (DA) release was determined by recording fluorescence dynamics of DA4.4, a DA sensor [28, 29] (Fig. 1k, l). We observed a concordant increase of DA sensor fluorescence in the NAC when activating Mor-Ens, while activating Sal-Ens induced DA release at a much lower level (Fig. 1m-o). These data show that DA release in NAC from terminals of the activated Mor-Ens is crucial for positive reinforcement.

Chronic morphine enhances inhibitory synaptic transmission onto Mor-Ens, and this is involved in the development of the negative effect during opiate withdrawal

Chronic opiate administration causes pathological changes of VTA plasticity. Therefore, whole-cell patch-clamp recordings were performed to evaluate synaptic remodeling of the ensembles after chronic morphine exposure (Fig. 2a-c). The mIPSC and mEPSC of Sal-Ens and Mor-Ens were not different before chronic morphine treatment (day 1) (Supplementary Fig. 4a-d). After the escalating dose of morphine administration (day 7), the mIPSC amplitude and frequency were increased significantly in TH+ population of Mor-Ens, compared with that in Sal-Ens (Fig. 2d-f), while no differences in mEPSC were observed (Fig. 2g-i). In TH+ population, mIPSC amplitude exhibited a minor decrease in Mor-Ens, compared with that in Sal-Ens (Supplementary Fig. 4e-j). These data indicate that the dopaminergic Mor-Ens receives more inhibitory inputs than Sal-Ens after chronic morphine exposure.

To assess whether the activity of Mor-Ens is critical for the negative effects, hM4Di was expressed in labeled Mor-Ens and Sal-Ens. In unlabeled mice, CNO treatment did not affect the development of conditioned place aversion (CPA), locomotor activity, the time spent in the open arm, and saccharin preference following morphine withdrawal (Supplementary Fig. 5a-e). In the groups with or without escalating dose of morphine treatment, inhibition of VTA Mor-Ens by CNO significantly increased the CPA score, and decreased the time spent in the open arms of the elevated-plus maze (EPM) (Figs. 2j-l, m-p), while having no effects on locomotor activity and saccharin preference (Supplementary Fig. 6d, e). Instead, when expressing hM3Dq in labeled Mor-Ens or Sal-Ens, activation of Mor-Ens by CNO during the place-aversion conditioning reduced the CPA score, increased the time spent in the open arms in EPM (Fig. 2q-s), while having no effects on locomotor activity and saccharin preference (Supplementary Fig. 6a-c). These results suggest that the activity of VTA Mor-Ens is required for the formation of conditioned aversion and anxiety.

Spontaneous morphine withdrawal evokes negative somatic symptoms. Behavioral patterns were assessed 9 hrs after the last injection of escalating dose of morphine (Fig. 2t). There were significant differences in walking, sniffing, rearing, and tremoring between morphine and saline treatment groups (Supplementary Fig. 6f, g). Analysis of the individual behaviors showed mild differences between activation of Mor- and Sal-Ens by CNO during spontaneous withdrawal (Fig. 2u and Supplementary Fig. 6h). However, evaluation of the behavior spectrum using t-distributed stochastic neighbor embedding (t-SNE) revealed three separated clusters: a negative cluster (consisting mostly of opiate-withdrawal individuals), a neutral cluster (consisting mostly of naive individuals, which did not undergo withdrawal and CNO activation), and a chemo-activated cluster, which was composed of individuals in which Sal- or Mor-Ens were activated by CNO during spontaneous withdrawal (Fig. 2v). Activation of Mor-Ens in mice withdrawal from morphine increased the percentage of individuals clustered into the neutral cluster (Fig. 2w). Taken together, these results suggest that activation of the ensembles recruited by the initial morphine exposure reduces conditioned aversion, alleviates negative somatic symptoms, and anxiety during opiate withdrawal.

The activity of CRH-CeA–VTA neurons is increased following chronic morphine administration

Upregulation of the brain-stress system and downregulation of the reward system have been found, and corticotrophin-releasing hormone (CRH) has been implicated in behavioral responses during acute and chronic withdrawal [26, 30, 31]. AAV-Retro-EF1a-DIO-EYFP was infected into the VTA of CRH-ires-Cre mice, and
VTA-projecting CRH neurons tagged with EYFP in the CeA, the bed nucleus of stria terminals (BNST), and the paraventricular nucleus of the hypothalamus (PVN) were detected. c-Fos expression was increased in CRH CeA→VTA and CRH BNST→VTA neurons, while unchanged in CRH PVN→VTA neurons after chronic morphine treatment (Fig. 3a–c), indicating that CRH CeA→VTA and CRH BNST→VTA neurons are activated during opiate withdrawal.

CeA contains primarily inhibitory outputs and orchestrates adaptive responses to emotional events [32–34]. To assess whether the inhibitory CRH CeA→VTA inputs changes following chronic morphine administration, CRH-ires-Cre mice were infected with AAV-Retro-EF1a-DIO-EYFP in the VTA to label CRH CeA→VTA CRH neurons. mEPSC, mIPSC and evoked action potential (AP) in CRH CeA→VTA neurons were recorded (Fig. 3d, e). The frequency and
amplitude of mEPSC in CRH<sup>CeA-VTA</sup> neurons were increased following escalating dose of morphine administration, while mIPSC was not changed (Fig. 3f–h and Supplementary Fig. 7a–c).

Additionally, chronic morphine treatment increased excitability (Fig. 3i, j), reduced AP threshold, and increased input resistance of the CRH<sup>CeA-VTA</sup> neurons (Supplementary Fig. 7d, e), but had no effect on after-hyperpolarization potential, amplitude, and half width of APs in these neurons (Supplementary Fig. 7f–h). These results suggest that the activation, excitatory transmission and membrane excitability of CRH<sup>CeA-VTA</sup> neurons are enhanced following chronic morphine administration.

The activation of CRH<sup>CeA-VTA</sup> terminals increases anxiety and drives conditioned-place aversion induced by opiate withdrawal

The effect of activation of CRH<sup>CeA-VTA</sup> projections on morphine-withdrawal-induced CPA and anxiety was then examined. CRH<sup>ires-Cre</sup> mice were infected with AAV-DIO-eNhPr3.0-EYFP in the CeA (Fig. 3k, l and Supplementary Fig. 8a, b). Optogenetic inhibition of CRH<sup>CeA-VTA</sup> terminals by 594-nm laser stimulation during conditioning decreased conditioned aversion induced by opiate withdrawal (Fig. 3m–o). Optogenetic inhibition of CRH<sup>CeA-VTA</sup> terminals increased the activity of VTA neuron by chronic morphine administration (Fig. 3p), while it did not affect the locomotor activity (Supplementary Fig. 8d). The above data suggest that the activation of CRH<sup>CeA-VTA</sup> terminals promotes anxiety level and the formation of conditioned aversion induced by opiate withdrawal.

To examine the role of CRH<sup>CeA-VTA</sup> projections in the development of negative effect, CRH<sup>ires-Cre</sup> mice were briefly infected with AAV-DIO-hChR2-mCherry in the CeA and implanted optic fibers above the VTA to activate CRH<sup>CeA-VTA</sup> terminals (Fig. 3q, r and Supplementary Fig. 8c). Optical activation of CRH<sup>CeA-VTA</sup> terminals by 473-nm laser induced a place aversion (Fig. 3s–u) and decreased the time spent in the open arms in the EPM test (Fig. 3v), while it had no effect on locomotor activity (Supplementary Fig. 8e). These experiments indicate that the increased activity of CRH<sup>CeA-VTA</sup> projection neurons contributes to the development of CPA and anxiety during opiate withdrawal.

Specific remodeling of the circuits of CRH<sup>CeA-VTA</sup> neurons and Mor-Enss by chronic morphine administration

CRH<sup>ires-Cre</sup> mouse was infected with AAV-Retro-EF1a-DIO-EYFP and AAV-Retro-hSyn-tdTomato in the VTA to label VTA-innervating neurons. c-Fos expression in CRH<sup>+</sup> (EYFP<sup>+</sup>) and CRH<sup>+</sup> (EYFP<sup>-</sup>tdTomato<sup>-</sup>) neurons in the CeA was assessed (Fig. 4a, b). The proportion of c-Fos<sup>+</sup> CRH<sup>+</sup> neurons was increased after chronic morphine administration, while the proportion of c-Fos<sup>+</sup> CRH<sup>+</sup> neurons was not changed (Fig. 4c), indicating the specific regulation of CRH<sup>CeA-VTA</sup> neuron activity by chronic morphine administration.

To investigate the monosynaptic inputs from the CeA to Sal-Ens or Mor-Enss, C57BL/6 mice were infected with AAV-V-SARE-Cre<sup>+</sup> and Cre-dependent helper viruses (AAV-DIO-H2B-EYFP-VTA and AAV-DIO-RVG) in the VTA. The rabies virus RV-<sup>Envs</sup>dsRed was injected one day after the ensemble labeling (Fig. 4d). The starter ensembles expressing EGFP and dsRed were restricted in the VTA (Fig. 4e) and co-estimated with anti-TH antibody to identify the cell type composition (Supplementary Fig. 9a, b). The distribution of dsRed<sup>+</sup> neurons showed that inputs cells were concentrated in the NAc, LHb, dorsal striatum and lateral hypothalamus (Supplementary Fig. 9c), which was consistent with previous reports [27, 35, 36]. The relative number of dsRed<sup>+</sup> input neurons in the CeA was not different between Sal-Ens and Mor-Enss groups (Supplementary Fig. 9c). Fluorescent probes targeting CRh and dsRed mRNA were used to identify the CRH neurons in the CeA connecting to Sal- or Mor-Enss. The proportion of CRh<sup>+</sup> population in Mor-Enss connective CeA neurons was higher than that in Sal-Enss (Fig. 4f, g), suggesting that CRH<sup>CeA-VTA</sup> inputs preferentially form synaptic connections with the Mor-Enss. To achieve specific manipulation of CRH neurons and VTA ensembles in the same mouse, we used a doxycycline (Dox)-dependent AAV-Ram-tTA-TRE-EGFP system to label the ensembles (Fig. 4h). CRH<sup>ires-Cre</sup> mice were infected with AAV-DIO-hCrhr2-mCherry in the CeA, and RAM-tTA-TRE-EGFP in the VTA (Fig. 4i). The RAM-captured ensembles (EGFP<sup>+</sup> neurons) were largely overlapped with E-SARE-captured ensembles (mCherry<sup>+</sup> neurons) recruited by saline or morphine, and showed similar spatial distribution and comparable cell number to those labeled by E-SARE system (Supplementary Fig. 10a–d). Instead, there were less than 30% overlapping distributions of the E-SARE-captured Mor-Enss with RAM-captured Sal-Enss in the same mouse (Supplementary Fig. 10e–g), indicating they were separated subpopulations. Synaptic responses of EGFP<sup>+</sup> Sal-Enss and Mor-Enss, as well as the adjacent EGFP<sup>+</sup> neurons that were not recruited by initial morphine exposure (Non-Mor-Enss) with optical stimulation were recorded (Fig. 4i). Activation of CRH<sup>CeA-VTA</sup> terminals by 20-Hz optical stimulation increased the frequency of IPSCs in Mor-Enss (Fig. 4j, k), while it had no effect on that in Sal-Enss (Fig. 4l, m). Additionally, the amplitude of IPSC in Non-Mor-Enss was slightly decreased by activation of CRH<sup>CeA-VTA</sup> terminals (Supplementary Fig. 10h, i). To investigate the effect of chronic morphine administration on the inhibitory CRH<sup>CeA-VTA</sup> transmission in Mor-Enss, we recorded the O-IPSCs in Mor-Enss in response to the 473-nm laser, and identified the dopaminergic Mor-Enss via backfill staining (Fig. 4n, o). We found that majority of the responsive
neurons were dopaminergic neurons, and Mor-Ens exhibited decreased paired-pulse ratio (PPR) value and increased O-IPSC amplitude after chronic morphine administration (Fig. 4p–s), revealing that the enhanced inhibitory tone from CRH CeA → VTA projections to dopaminergic Mor-Ens following chronic morphine administration involves both presynaptic and postsynaptic mechanisms.

Specific modulation of CRH<sup>Cre</sup>→VTA and Mor-Ens pathway is required for the development of negative effect

To verify the interplay of the stress and reward systems in vivo, CRH-ires-Cre mice were infected with AAV-RAM-tTA-TRE-hM3Dq-HA in the VTA to express hM3Dq in Mor- or Sal-Ens (Fig. 5a–c and Supplementary Fig. 11a–c). Chemogenetic activation of Mor-Ens, but not Sal-Ens during place conditioning, resulted in the
Fig. 2 The inhibitory transmission in dopaminergic Mor-Ens is enhanced and this is required for the development of negative effect during opiate withdrawal. a Experimental scheme of the VTA ensembles recording before (day 1), or after the escalating dose of morphine treatment (day 7). b Representative images of neurons for the recordings in the VTA and the neuron filled with dye. Scale bar, 20 μm. c Mor-Ens or Sal-Ens were injected with biocytin, and co-stained with anti-Th antibody after recording. Red: mCherry; Green: biocytin; Blue: Th. Scale bar, 10 μm. d Representative traces of mIPSCs. e, f Cumulative probability distribution and average amplitude and frequency of mIPSCs recorded from Th^+^ VTA ensembles after escalating-dose of morphine administration. g Representative traces of mEPSCs. h i Cumulative probability distribution and average amplitude and frequency of mEPSCs recorded from Th^+^ VTA ensembles after escalating-dose of morphine administration. Sal-Ens: 9 mice, Mor-Ens: 11 mice. Unpaired t test, frequency: t = 3.237, df = 49, P = 0.0022 in (f); Mann–Whitney U test, frequency: U = 291, P = 0.5429 in (h), amplitude: U = 311, P = 0.8151 in (i); two-sample Kolmogorov–Smirnov (KS) test for cumulative probability distribution, interval: P < 0.0001 in (e), amplitude: P < 0.0001 in (f). j - k Schematic representation of the virus injection and behavioral tests in mice without withdrawal. The effect of inhibition of Mor-Ens in the VTA on CPA and anxiety (j). Quantiﬁcation of CPA score in hM4Di groups (k). Two-way RM ANOVA, F groups × session (1, 26) = 5.342, P = 0.0290. Sal-Ens vs Mor-Ens within test, P = 0.0397. (j) Quantification of the time in open arm in hM4Di groups without withdrawal. Mann–Whitney U test, U = 58, P = 0.0411. m Experimental process of the virus injection and behavioral tests after the ensemble labeling in withdrawal mice. n-p The effect of inhibition of Mor-Ens on morphine-withdrawal-induced CPA and anxiety. Representative images of expression of hM4Di-mCherry in VTA Sal-Ens and Mor-Ens (n). Scale bar, 100 μm. Quantification of morphine-withdrawal-induced CPA score in hM4Di groups (o). Two-way RM ANOVA, F groups × session (1, 23) = 7.248, P = 0.013, Sal-Ens vs Mor-Ens within test, P = 0.0429. Quantification of the time in open arm in hM4Di groups (p). Mann–Whitney U test, U = 52, P = 0.0350. q-s The effect of activation of Mor-Ens on morphine-withdrawal-induced CPA and anxiety. Representative images of expression of hM3Dq-mCherry in VTA Sal-Ens and Mor-Ens (q). Scale bar, 100 μm. Quantification of morphine-withdrawal-induced CPA score in hM3Dq groups (r). Two-way RM ANOVA, F groups × session (1, 33) = 5.125, P = 0.0303, Sal-Ens vs Mor-Ens within test, P = 0.0170. Quantification of the time in open arm in hM3Dq groups (s). Mann–Whitney U test, U = 73, P = 0.0387. t Experimental process of virus injection and 20-min behavioral recording in the home cages. u Analysis of the eight behaviors in mice that activation of Sal-Ens and Mor-Ens during the spontaneous withdrawal period. Each column represents individual behavior. Each row represents one mouse. v t Distributed stochastic neighbor embedding (t-SNE) representation of 40 mice behavioral patterns showing three clusters: negative (black), neutral (green), and chemo-activated (magenta). w Top: Proportion of mice displaying neutral or negative pattern. Bottom: Proportion of CNO-treated mice displaying neutral pattern. Top, χ^2^ test, P = 0.0066; Bottom, χ^2^ test, P = 0.0384. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are presented as mean ± SEM.
decreased O-IPSC amplitude, while it had no effect on PPR (Fig. 6q–s), indicating that postsynaptic CRHR1 modulates the inhibitory inputs from CRH-CeA → VTA neurons to Mor-Ens. These results demonstrate that CRH–CRHR1 signaling contributes to the synaptic and functional remolding of the circuit of inhibitory CeA CRH inputs and Mor-Ens following chronic morphine administration, which mediates the negative effect during opiate withdrawal.

**DISCUSSION**

An increase in the mesolimbic dopamine is the commonality for positive reinforcement of addictive drugs. Previous studies have shown that optically activated VTA dopaminergic neurons are sufficient to trigger compulsive taking in mice, and evoke synaptic plasticity in the NAc associated with drug addiction [38]. Morphine acts via binding to opiate receptors to inhibit the VTA GABAergic
The inhibitory innervation of TH+
inputs to TH inhibitory inputs to TH might inhibit VTA GABAergic neurons, and then decrease mechanism underlies the negative effect during opiate withdrawal (D7). Mann–Whitney U test, frequency: U = 136, P = 0.0086 in (g), amplitude: U = 145, P = 0.0119 in (h); two-sample KS test for interval: P < 0.0001, amplitude: P < 0.0001. i, j Representative AP traces (i) and quantification of the induced spike number (j) of the CRHR1 pathway facilitates GABAergic tone to the stressful situations [61]. Our results showed that CRHR1 deletion of CRH neurons is interrupted by the pulses of 594-nm laser. m Experimental process of the optical inhibition during the behavioral assays. n–p The effect of optical inhibition on CRH−/− VTA terminals during morphine-withdrawal-induced CPA (o) and anxiety (p). Representative traces illustrate the CPA test sections (n). Two-way RM ANOVA, Fvirus × session(1, 21) = 4.553, P = 0.0430, EYPF vs enPH3.0 within test, P < 0.01 in (o); unpaired t test, t = −3.413, df = 19, P = 0.0029 in (p). q Schematic representation of the viral injection and optical-fiber implantation in CRH−/− VTA mice. r The action potentials of a CRH neuron in the CeA induced by 473-nm laser (20 Hz, 5 ms). s Experimental process of the optical activation during the behavioral assays. t–v Optical stimulation of the CRH−/− VTA terminals drives CPA (u) and anxiety-like behavior (v). Representative tracks (t) illustrate the CPA test section. Two-way RM ANOVA, Fvirus × session(1, 21) = 7.677, P = 0.0115, mCherry vs hChR2 within test, P < 0.01 in (u); unpaired t test, t = 2.195, df = 22, P = 0.0390 in (v). **P < 0.05, ***P < 0.01, ****P < 0.0001. Data are presented as mean ± SEM.
In this study, we investigated into the underlying CRH-mediated inhibitory neurotransmission and plasticity changes of the neuronal ensembles encoding drug-reward experience. The inhibitory inputs of CRH\(^{\text{Cre}}\) neurons onto Mor-Ens were enhanced following chronic morphine administration, and thus promoted the negative effect by dysregulating the function of those ensembles. We proposed that activation of ensembles encoding a drug-reward experience offers a potential node for alleviating the negative effect driven by enhanced inhibitory inputs from CeA, and thus preventing opiate dependence. Expression of immediate early genes has been used to identify diverse experience-defined ensembles, and drive distinct behavior.
reinforcement [62, 63]. In our studies, the activation of dopamine-
recruited ensembles labeled by either the enhanced Arc or c-fos
promoter is sufficient to elicit appetitive responses and positive
reinforcement. Considering the heterogeneous composition of the
ensemble, phasic firing pattern in DA neurons, delayed DA release,
and GABA activation [64, 65] should be considered for the
specific neurotransmission and plasticity modulation in the
ensembles. Therefore, specifically labeling opiate-recruited dopa-
minergic and GABAergic ensembles will facilitate the identifica-
tion of the functional neuronal subtype in the heterogeneous
VTA neurons involved in the diverse information processing of the
opiate reward in future. Nevertheless, our data suggest that
modulating CRH GABAergic circuit between CeA and VTA may be
a potential strategy for treating the negative reinforcement
produced by drug withdrawal.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in accordance with the animal care
guidelines approved by the Animal Care and Use Committee of the School
of Basic Medical Sciences of Fudan University and the guidelines of the
National Institutes of Health. CRH-ires-Cre B6(Cg)-Chtmtm1(cre)Zjh/J mice
(012704) were obtained from Jackson Lab (CA, USA), and were bred on a
C57 BL/6 (012704) were obtained from Jackson Lab (CA, USA), and were bred on a
standard 12-h light/dark cycle with food and
China). The mice used for experiments were housed in plastic cages with
were purchased from Shanghai Laboratory Animal Center (CAS, Shanghai,
the CeA colocalized with c-Fos. Green: EYFP; Red: tdTomato; Magenta: c-Fos. Scale bar: 100 μm. c Quantification of the percentage of c-Fos" cells in CRH
TRE-EGFP) vector. i Shematic representation of the IPSC recordings from the Mor-Ens or Sal-Ens. Right: the expression of CRH
C57 BL/6 mouse strains. All genotypes were confirmed by polymerase chain reaction
of input cells in the CeA colocalized with Crh (green) mRNA probe, Red: dsRed; Green: EYFP; Blue: DAPI. Scale bar, 100 μm. f Representative imaging
by Tamoxifen (Sigma-Aldrich) was prepared in a mixed oil solution (of nine
80 mg/ml) for surgery in the stereotactic
system, 10 mg/ml DMSO solvent. d Phasic firing pattern: in the baseline condition, VTA neurons exhibited a phasic firing pattern in DA neurons, delayed DA release,
and GABA activation [64, 65] should be considered for the specific neurotransmission and plasticity modulation in the ensembles. Therefore, specifically labeling opiate-recruited dopaminergic and GABAergic ensembles will facilitate the identification of the functional neuronal subtype in the heterogeneous VTA neurons involved in the diverse information processing of the opiate reward in future. Nevertheless, our data suggest that modulating CRH GABAergic circuit between CeA and VTA may be a potential strategy for treating the negative reinforcement produced by drug withdrawal.

Viral vectors

To obtain the neuronal ensembles that respond to specific stimuli, we used a
synthetic promoter, the enhanced synaptic activity-responsive element (E-
SARE), which was constructed by multiplexing the 104-bp SARE enhancer

distinct from the templates of pAAV-5hsyn-HM3Dq-mCherry (Addgene: 50474). All the AAV vectors were titered by AAV- and packaged by Gene
Technology Co., Ltd (Shanghai, China). AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-EYFP, AAV-Retro-Hsyn-tdTomato, AAV-CAG-DIO-saCas9, AAV-CAG-DIO-saCas9, AAV-
EYFP were purchased from Taitool Bioscience Co., Ltd. (Shanghai, China). AAV-EF1a-DIO-HM3Dq-HA, and AAV-EF1a-DIO-eNhR130-EYFP were purchased from the University of North Carolina (Vector Core, NC, USA).

Plaque assay

To generate the pAAV-RAM-d2TTA-TRE-EGFP-WPRE-pA plasmid we replaced the

EGFP in pAAV-RAM-d2TTA-TRE-EGFP-WPRE-pA (Addgene: 88469) with the

Cre sequence obtained by PCR from pAAV-Cre-GFP (Addgene: 68544). To generate the

pAAV-RAM-d2TTA-pA-TRE-Fp-lpRE-EGFP-WPRE-pA plasmids, we replaced

EGFP in pAAV-RAM-d2TTA-TRE-EGFP-WPRE-pA with the Fp sequence obtained by PCR from pAAV-TRE-Fp-lpRE (Addgene: 119019). To generate the

plasmids pAAV-RAM-d2TTA-pA-TRE-HM3Dq-Cre-lpRE-WPRE-pA, we replaced EGFP

in pAAV-RAM-d2TTA-TRE-EGFP-WPRE-pA with the HM3Dq cre sequence

obtained from the additive vector pAAV-5hsyn-HM3Dq-mCherry (Addgene: 50474). All the AAV vectors were titered by AAV- and packaged by Gene
Technology Co., Ltd (Shanghai, China). AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-EYFP, AAV-Retro-Hsyn-tdTomato, AAV-CAG-DIO-saCas9, AAV-CAG-DIO-saCas9, AAV-
EYFP were purchased from Taitool Bioscience Co., Ltd. (Shanghai, China). AAV-EF1a-DIO-HM3Dq-HA, and AAV-EF1a-DIO-eNhR130-EYFP were purchased from the University of North Carolina (Vector Core, NC, USA).

Retroviral labeling

To label the ensembles with the E-SARE-CreERT2 system, 10 mg/ml tamoxifen (Sigma-Aldrich) was prepared in a mixed oil solution (of nine
corns oil and 1-part ethanol), and was injected intraperitoneally (i.p.) at the dose of 125 mg/kg. Mice were given morphine (10 mg/kg, i.p.) or equivalent volume of saline injection 24 h after TAM administration to label the ensembles. To label ensembles with the RAM-1TA-TRE system, mice were taken off doxycycline (Dox) diet (40 mg/kg, 48 hrs before the injection of morphine or saline, and then kept back on Dox 12 h later. Mice were given at least five days to allow protein expression before the electrophysiological and behavior experiments.

Pavlovian conditioning

Conditioned-place preference (CPP) was performed by unbiased proce-
dures in the two-chamber apparatus with distinct tactile environments to
count and determine the percentage of c-Fos cells in the CeA
distinct from the templates of pAAV-5hsyn-HM3Dq-mCherry (Addgene: 50474). All the AAV vectors were titered by AAV- and packaged by Gene
Technology Co., Ltd (Shanghai, China). AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-EYFP, AAV-Retro-Hsyn-tdTomato, AAV-CAG-DIO-saCas9, AAV-CAG-DIO-saCas9, AAV-
EYFP were purchased from Taitool Bioscience Co., Ltd. (Shanghai, China). AAV-EF1a-DIO-HM3Dq-HA, and AAV-EF1a-DIO-eNhR130-EYFP were purchased from the University of North Carolina (Vector Core, NC, USA). RN/EN/∆deltaG∆dsRed, AAV-HSyn-DAA4, AAV-HDIO-H2B-EGFP-TVA, AAV-DIO-RVG, AAV-CMV-sgRNA (Scramble)-mCherry, and AAV-CMV-sgRNA (Chfr)-mCherry were purchased from BrainVTA Co., Ltd. (Wuhan, Hubei, China).

Stereotaxic surgery and laser stimulation

Mice were anesthetized with 2% isoflurane for surgery in the stereotactic
instrument (Stoelting, Kiel, WI, USA). Microinjections were performed using 33-gauge needles connected to a 10-μl microsyringe (Hamilton, Nevada, USA). The intended stereotaxic coordinates for the ventral tegmental area (VTA) were −3.2-mm AP; ML ±0.9-mm ML (with an angle of 10° from the middle to the lateral); −4.4-mm DV; for CeA were −1.3-mm AP; ±2.70-
mm ML; −4.40-mm DV. Each site was injected with 0.5 μL of purified and mixed AAV (10^12 IU/ml) with a slow-injection rate of 0.1 μl/min. About 200-
μm-diameter glass optical fiber cannula (3077 N) was used to target VTA
with 120-mm DV coordinates. Laser stimulation was delivered through a 473-nm or 594-nm laser diode (Brain-King, China). The light intensity at the fiber tip was measured using a light sensor (Thorlabs, Newton, NJ, USA). An 8–10-mW laser pulse (20-Hz frequency, 5-ms duration) generated by a Master-8 pulse stimulator (AMPI, Jerusalem, Israel) was delivered through the embedded optical fiber in the VTA. The mice with off-target mCherry or EGFP location were excluded from analysis.

Labeling-activated ensembles

To label the ensembles with the E-SARE-CreERT2 system, 10 mg/ml tamoxifen (Sigma-Aldrich) was prepared in a mixed oil solution (of nine
corns oil and 1-part ethanol), and was injected intraperitoneally (i.p.) at the dose of 125 mg/kg. Mice were given morphine (10 mg/kg, i.p.) or equivalent volume of saline injection 24 h after TAM administration to label the ensembles. To label ensembles with the RAM-1TA-TRE system, mice were taken off doxycycline (Dox) diet (40 mg/kg, 48 hrs before the injection of morphine or saline, and then kept back on Dox 12 h later. Mice were given at least five days to allow protein expression before the electrophysiological and behavior experiments.

Pavlovian conditioning

Conditioned-place preference (CPP) was performed by unbiased proce-
dures in the two-chamber apparatus with distinct tactile environments to
count and determine the percentage of c-Fos cells in the CeA
distinct from the templates of pAAV-5hsyn-HM3Dq-mCherry (Addgene: 50474). All the AAV vectors were titered by AAV- and packaged by Gene
Technology Co., Ltd (Shanghai, China). AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-HM3Dq-mCherry, AAV-EF1a-DIO-EYFP, AAV-Retro-Hsyn-tdTomato, AAV-CAG-DIO-saCas9, AAV-CAG-DIO-saCas9, AAV-
EYFP were purchased from Taitool Bioscience Co., Ltd. (Shanghai, China). AAV-EF1a-DIO-HM3Dq-HA, and AAV-EF1a-DIO-eNhR130-EYFP were purchased from the University of North Carolina (Vector Core, NC, USA). RN/EN/∆deltaG∆dsRed, AAV-HSyn-DAA4, AAV-HDIO-H2B-EGFP-TVA, AAV-DIO-RVG, AAV-CMV-sgRNA (Scramble)-mCherry, and AAV-CMV-sgRNA (Chfr)-mCherry were purchased from BrainVTA Co., Ltd. (Wuhan, Hubei, China).
for next. The procedures consisted of pretest, and conditioning test. On
day 1, mice were placed in the middle of the conditioning chambers and
allowed to freely explore the entire apparatus for 20 min (pretest). The
sessions were recorded by infrared-tracking instrument and the time spent
in each chamber was determined. Mice that stayed in one chamber for
more than 13 min were excluded from the experiment. On days 2, 3, and 4,
mice received the intraperitoneal injection of CNO (2 mg/kg) or laser
stimulation while confined to one of the chambers for 30 min (conditioning)
and then received an equivalent volume of saline while confined to the
other chamber for 30 min 6 h later. On day 5, mice were allowed to
freely explore the entire apparatus for 20 min (test). The time spent in each
chamber was recorded during the pretest and test sessions. The CPP score
was defined as the time (in seconds) spent in the CNO-paired chamber
minus the time spent in the saline-paired chamber.
Fig. 5  Chemogenetic activation of Mor-Ens in VTA alleviates the negative effect induced by activating CRH CeA–VTA terminals. a Schematic representation of AAV-ARM-VTA-TRE-HM3Dq vector. b Experimental process of the CNO-evoked CPP experiment. c Representative images of expression of hm3Dq-HA in VTA Sal-Ens and Mor-Ens. Scale bar, 100 μm. d, e Chemoactivation of Mor-Ens drives CPP and promotes anxiolytic behavior. Two-way RM ANOVA, Fgroups × session (1, 38) = 7.365, P = 0.011, Sal-Ens + hm3Dq vs Mor-Ens + hm3Dq within test, P = 0.006; Fgroups × session (1, 30) = 4.917, P = 0.034, Mor-Ens + hm3Dq vs Mor-Ens + EGFP within test, P = 0.029; Fgroups × session (1, 24) = 0.0006, P = 0.9799, Sal-Ens + hm3Dq vs Sal-Ens + EGFP within test, P > 0.999 in (d); Mann–Whitney test, Sal-Ens + hm3Dq vs Mor-Ens + hm3Dq, U = 29, P = 0.0065; Mor-Ens + hm3Dq vs Mor-Ens + EGFP, U = 26, P = 0.0233; Sal-Ens + hm3Dq vs Sal-Ens + EGFP, U = 60, P = 0.9851 in (e). f Schematic representation of virus injection and optic-fiber implantation in the VTA. g Experimental process of the opto- and chemogenetic manipulated behavioral tests. h Representative images of CRH neurons in the CeA expressing hChR2-EYFP (top), and Sal-Ens or Mor-Ens in the VTA labeled with hm3Dq-HA (bottom). Green: EYFP; Red: HA; Blue: DAPI. Scale bars, top: 100 μm; bottom: 20 μm. i, j The effect of chemogenetic activation of Mor-Ens and optogenetic activation of CRH CeA–VTA terminals on the CPA and EPM performance. Two-way RM ANOVA, Fgroups × session (1, 24) = 2.257, P = 0.146, hChR2-EYFP × session (1, 24) = 3.583, P = 0.0614 in (i); k Left: Schematic representation of virus injection and optic-fiber implantation in the VTA for negative reinforcement task. Right: Experimental process of the opto and chemogenetic manipulated operant-response training. l Representative images of Chr2-EYFP in CeA, and hm3Dq-HA in VTA. The dashed white lines indicated the optic fiber trace. Green: EYFP; Scale bars, 100 μm. m Average number of nose pokes in 60-min session on day 4. Unpaired t test, t = 3.026, df = 17, P = 0.0076. In total, 8–11 mice/group. n Left: Schematic representation of virus injection and optic-fiber implantation in the VTA for negative-reinforcement task. Right: Experimental process of the optical and chemogenetic manipulated operant-response training. o Representative images of Chr2-EYFP in CeA, and hm3Dq-HA in VTA. The dashed white lines indicated the optic-fiber trace. Green: EYFP; Red: HA; Blue: DAPI. Scale bars, 100 μm. p Average number of nose pokes in 60-min session on day 4. Unpaired t test, t = 2.215, df = 17, P = 0.0407. In total, 9–10 mice/group. *P < 0.05, **P < 0.001. Data are presented as mean ± SEM.

Morphine-withdrawal-induced conditioned-place aversion (CPA) procedure: mice were allowed to freely explore both sides of the CPA apparatus for 20 min to assess their baseline place preference (pretest). Then, these mice received an injection of morphine (i.p.; saline, 10, 20, 40, 60, and 60 mg/kg for five consecutive days) in their home cages. About 9 h after each of the last 3 morphine injections, when somatic withdrawal symptoms were induced, mice were confined in one chamber (withdrawal-paired) of the apparatus for 30 min. Mice were reexposed to the apparatus for 20 min six days after the last conditioning trial. The CPA score was defined as the time (in seconds) spent in the withdrawal-paired chamber minus the time spent in the other side of the chamber. For chemogenetic manipulations, mice were given an injection of CNO (2 mg/kg, i.p.) 30 min prior to each of the CPA conditioning.

Optical intracranial self-stimulation (ICSS) Three weeks following virus infection, mice labeled with Mor-Ens or Sal-Ens were given six daily intracranial self-stimulation (ICSS) training sessions. Behavioral training and testing were performed in a mouse-operant apparatus (Med Associates) interfaced with optogenetic stimulation equipment (Newdoon Inc and Inper Tech, Hangzhou, Zhejiang, China). Each operant behavior chamber was equipped with a dim-light source and a nose-poke hole equipped with infrared photobeams connected to a computer. A 1-h fixed-ratio one (FR1) schedule was performed every training day. A nose poke in the target hole produced 2 s of 473-nm light (5 ms, 20 Hz, 10 mW). For drug-infusion studies, mice received injections of either fluorescent probes (0.5 mg/kg), Tocris, Bristol, UK) or saline vehicle intraperitoneally 30 min prior to ICSS sessions. For negative-reinforcement procedures [68], mice were placed into the chamber and delivered continuous 473-nm (5 ms, 20 Hz, 10 mW) optical stimulation with an interstimulus interval of 1 s. The mice were trained on a FR1 training schedule, in which each active nose poke produced a 20-s laser shut-off and the terminals were not optogenetically activated.

Open-field test (OFT) and elevated-plus-maze (EPM) test An activity-monitor system (43.2-cm length × 43.2-cm width × 30.5 cm height, Med-Associates, USA) was used to detect the locomotor activity. Each mouse was placed in the center of the open field and allowed to explore freely for 30 min. The center was defined as a square of 50% of the total OFT area. The entries in the center zone, time in the center zone, and total distance traveled were recorded and analyzed. For chemogenetic manipulations, mice were given an intraperitoneal injection of CNO (2 mg/kg) 30 min prior to behavior test.

The elevated plus maze consisted of four arms (34.5-cm length × 8.3-cm width × 19.5-cm height) and a center platform placed 75 cm above the floor. Two of the arms had 20 cm high dark walls (closed arms), and two had 0.8-cm high ledges (open arms). The arms were angled at 90° to each other. The apparatus was placed in a quiet and dimmed room. Mice were placed in the center and allowed to freely explore the maze for 6 min. Their behaviors were recorded with a camera located above the maze and analyzed by Etho Vision XT 8.5 video-tracking program (Leesburg, VA, USA). The arms were cleaned with water and dried between each test to ensure the absence of olfactory cues.

Saccharin-preference test (SPT) and tail-suspension test (TST) The morphine-withdrawal-induced decrease in the preference for saccharin is thought to reflect anhedonia-like states. Animals were habituated with two bottles of water for 36 h, followed by one bottle of 0.1% (w/v) saccharin solution and one bottle of water. Bottle positions were switched every 12 h. After training, mice were restricted from water for 12 h and then exposed to a choice of two bottles for 1.5 h test. The saccharin preference was calculated by dividing the total consumption of saccharin by the total consumption of both water and saccharin. For chemogenetic manipulations, mice were given an intraperitoneal injection of CNO (2 mg/kg, i.p.) 30 min prior to the test session. For tail-suspension test, the tail of the mouse was fixed with a tape at a distance of 1 cm from the end, and then suspended upside down on a crossbar 15–20 cm from the floor without any accessible surface for 6 min. After a period of agitation, there was intermittent immobility, indicating a state of depression. Videos were taken for every session and the time of exploration (approach, sniffing, and rearing within 2 cm from the cage) of the stranger mouse and the empty cage by the test mouse was recorded. The social-preference score = the time interact with the stranger mouse/the total exploration time.

Social-preference test As previously described [69], the mouse was put in a three-chambered arena (60 × 40 × 22 cm). The luminosity was around 7 lux. Each mouse in the experiment group and the control group was placed in the center chamber for 10 min for habituation period. Then in the test period, two rodent models, which were isolated during one night, were placed in these cages. A binocular microscope was used to record the time of struggling and immobility within 6 min. The box was cleaned with 75% ethanol and dried between trials.

Behavioral-spectrum analysis Mice infected with AAADIO-hM3Dq-mCherry combined with AAV-E-SARE-CrFP were labeled with morphine-positive or saline-positive ensembles. These mice received intraperitoneal injections of morphine (i.p.) twice daily in their home cages for five consecutive days (with escalating dose as described above). Nine hours after the last morphine injection, mice received an injection of CNO (2 mg/kg, i.p.) and withdrawal symptoms were recorded for 20 min by a camera at 30 FPS. Somatic symptoms (walking, stilling, grooming, sniffing, digging, hanging, rearing, and tremors) were manually quantified by an individual blinded to the group allocation. t-Distributed stochastic neighbor embedding (t-SNE) was used to evaluate the changes of...
the whole spectrum of behaviors. We performed t-SNE on the ethogram matrix composed of eight behaviors (walk, dig, still, hang, groom, rear, sniff, and tremor) during spontaneous withdrawal. Each column represents one behavior. Each row represents one mouse. The variable values are the total duration or times of each behavior during 20 min of spontaneous withdrawal.

The main setting parameters for t-SNE: Output dimensionality = 2, Perplexity parameter = 5, Number of iterations = 500.

In vivo optic-fiber recording

An optical fiber with an outer diameter of 200 or 400 μm and 0.50 NA (Inper Tech, Hangzhou, Zhejiang, China) was implanted into the VTA or NAc of mice as described above. Fluorescence signals were recorded using a Fiber Photometry system equipped with a 470- and 410-nm excitation lasers (Inper Tech). Optical stimulation (594 nm, 10 mW, 5-ms pulse, 20 Hz, 2-s duration) was delivered every 20 s for 10–15 trials through the fiber implanted in the
For light-evoked inhibitory postsynaptic current recordings, neurons were clamped at -10 mV. D-APV (100 mM) and CNQX (20 mM) were added to the intracellular solution to block excitatory currents. CRHR2-expression fibers were excited with a train of 473-nm light pulses (20 Hz, duration 5 ms), and the postsynaptic responses were recorded for 1 min. For evoked IPSC recordings, the duration of the pulse was 1–2 ms. Once a stable evoked response was achieved, a baseline was recorded (5 min) and then the perfusion was switched to ACSF containing 600 μM antalarmin (Sigma-Aldrich, A8727, St Louis, WA, USA) or 300 μM antisavagine-30 (Tocris, #2071) for 20-min recording. Recordings with Rs > 30 MΩ were excluded from statistical analysis. Electrophysiological data were analyzed off-line with Clampfit 10.3 (Molecular Devices, Union City, CA, USA) or MiniAnalysis Program (Synaptosoft Inc, Fort Lee, NJ, USA).

Rabies tracing
Trans-synaptic tracing studies were carried out as previously described [75] with minor modifications. For rabies tracing, mice were infected with AAV-E-SARE-CreNT2, AAV-DIO-H2B-EFP-TVA, and AAV-DIO-RVG in the VTA. Three weeks later, mice were injected with TAM (125 mg/kg, i.p.) 24 h before ensemble labeling. A single dose of morphine (10 mg/kg) or saline was injected intraperitoneally for labeling. RV-ENW-delta-gsRed was injected into the VTA at the same coordinates on the next day. Mice were housed in the ABSL-3 facility for 7 days before the histological processing. For rabies-tracing analysis, consecutive brain slices (50-μm thickness) selected from every fifth slice were collected throughout the brain, and dsRed− input neurons and starter cells were manually counted by an experimenter, blind to the experimental condition.

RNAseq ISH
As previously described [74], the frozen brain tissue was sliced into 10-μm coronal sections and mounted onto Colorfrost Plus slides (Thermo Scientific). Slices were incubated with hydrogen peroxide for 10 min at RT, and then performed target retrieval and proteolysis using RNAscope® 2.5 Universal Pretreatment Reagents (ACD: 322380). SfMISH for all genes examined, Cre (#402551), Chr (#316901), dsRed (#481361-C2), Chr1 (#418011-C3), and mCherry (#431201-C2) performed hybridizedization for 2 h. After hybridization, we used RNAscope® Multiplex Fluorescent Detection Kit v2 (ACD: 323110) to amplify the signal. Images were acquired with 20× objective (Nikon A1, Tokyo, Japan). IOD in mCherry- and Cre-positive cells or Chr-positive neurons was analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). The observer analyzing the expression of Chr1 and Chrin neuronal ensembles was blinded to the group allocation.

Immunohistochemistry
Mice were anesthetized and perfused as described previously. The brain was isolated, fixed in 4% PBS-buffered PFA, and dehydrated in sucrose solutions (30%) at 4°C. The 50-μm frozen coronal slices were prepared by Leica CM3050 S Cryostat (Buffalo Grove, IL, USA). Slices were incubated in primary antibody in block buffer (10% goat serum and 0.2% Triton X-100) over night. After washing by PBS, the slices were incubated in secondary antibody for 1 h and DAPI for 5 min at room temperature. The slices were mounted in mounting medium (Sigma). Primary antibodies used were anti-
HA (H6908-0.5 ml, Sigma, 1:500), anti-c-Fos (sc-52, Santa Cruz, Dallas, TX, USA), anti-CRH (ab8901, Abcam, Cambridge, MA, USA), anti-TH antibodies (MAB318, Merck, 1:1000), and DAPI (D9534, Sigma). Secondary antibodies were anti-mouse 488 (711-545-150, Jackson Immunoresearch, 1:1000) and anti-rabbit Cy3 (115-165-116, Jackson Immunoresearch, 1:500). Images were acquired on a Nikon-AI confocal microscope (Tokyo, Japan) using a 20× objective lens. Bioinert-backfill slices were incubated with Cy3- or Alexa Fluor-488-conjugated streptavidin (Jackson Immunoresearch, 016-160-084, 016-540-084, 1:1000) to secondary antibody solution.

**Ensemble axonal projection collateralization**

Sample fixation and slides were processed as described above in the “Immunohistochemistry” section. Coronal sections (30-μm thickness) were analyzed with Image J by applying equal thresholds to all images and measuring the integrated density (IntDen) contained in a 250 × 250 pixel zone around target brain area. The intensity of mCherry-expressing fibers could be assessed for major VTA Sal-Ens or Mor-Ens anatomical target. Data were presented as a relative-intensity ratio normalized with Sal-Ens in the NAc, mPFC, amygdala, and LHB.

**Analysis of off-target effects of CRISPR-mediated genome editing**

sgRNAs were designed using online CRISPR tools (http://crispr.mit.edu/ and http://chopchop.cbu.ubc.ca/). sgRNA (5′-ACTCCACCCGCTGCTGCAGGGTAATC-3′) targeting the exon of Chr1, and scramble sgRNA (5′-GACTACCA AGCTACTAATCTTGATGAGGA-3′) were analyzed by two-way RM ANOVA. The Bonferroni following primers:

- **Crhr1**

Two-way ANOVA. Two-sample analysis was performed after one-way ANOVA or two-way ANOVA. The normality test of the data was performed by Shapiro-Wilk test and the homoscedasticity was performed by F test. The nonnormalized data were analyzed with nonparametric test. Comparison between groups were made by student’s t test (unpaired, two tailed), paired t test, Mann-Whitney U test, χ2 test, and one-way ANOVA or two-way ANOVA.

Two-sample Kolmogorov-Smirnov test was used for cumulative frequency and amplitude plot analysis. Behavior results of CPA and CPP were analyzed by two-way RM ANOVA. The Bonferroni’s post hoc analysis was performed after one-way ANOVA or two-way ANOVA.

**Statistical signicances were represented as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. All data are presented as mean ± SEM.**

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AUTHOR CONTRIBUTIONS
FW, LM and CJ designed the experiments and analyzed the data. CJ carried out the behavioral tests. CJ, XY and GH did immunostaining and RNAscope ISH and the behavioral tests. CJ, XY and GH did immunostaining and RNAscope ISH experiments. ZW constructed the viral vectors. CJ drafted the paper. YM, WX, FW and LM revised the paper. FW and LM supervised the study.
COMPETING INTERESTS
The authors declare no competing financial interests.

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