

# Opioid Withdrawal Abruptly Disrupts Amygdala Circuit Function by Reducing Peptide Actions

Gabrielle C. Gregoriou, Sahil D. Patel, Sebastian Pyne,  Bryony L. Winters, and Elena E. Bagley

Sydney Pharmacy School, Faculty of Medicine and Health and Charles Perkins Centre, University of Sydney, Sydney, New South Wales, Australia, 2111

While the physical signs of opioid withdrawal are most readily observable, withdrawal insidiously drives relapse and contributes to compulsive drug use, by disrupting emotional learning circuits. How these circuits become disrupted during withdrawal is poorly understood. Because amygdala neurons mediate relapse, and are highly opioid sensitive, we hypothesized that opioid withdrawal would induce adaptations in these neurons, opening a window of disrupted emotional learning circuit function. Under normal physiological conditions, synaptic transmission between the basolateral amygdala (BLA) and the neighboring main island (Im) of GABAergic intercalated cells (ITCs) is strongly inhibited by endogenous opioids. Using patch-clamp electrophysiology in brain slices prepared from male rats, we reveal that opioid withdrawal abruptly reduces the ability of these peptides to inhibit neurotransmission, a direct consequence of a protein kinase A (PKA)-driven increase in the synaptic activity of peptidases. Reduced peptide control of neurotransmission in the amygdala shifts the excitatory/inhibitory balance of inputs onto accumbens-projecting amygdala cells involved in relapse. These findings provide novel insights into how peptidases control synaptic activity within the amygdala and presents restoration of endogenous peptide activity during withdrawal as a viable option to mitigate withdrawal-induced disruptions in emotional learning circuits and rescue the relapse behaviors exhibited during opioid withdrawal and beyond into abstinence.

**Key words:** addiction; amygdala; opioids; peptidase; withdrawal

## Significance Statement

We find that opioid withdrawal dials down inhibitory neuropeptide activity in the amygdala. This disrupts both GABAergic and glutamatergic transmission through amygdala circuits, including reward-related outputs to the nucleus accumbens. This likely disrupts peptide-dependent emotional learning processes in the amygdala during withdrawal and may direct behavior toward compulsive drug use.

## Introduction

Opioid addiction is characterized by cycles of compulsive drug use, withdrawal and relapse (Koob and Volkow, 2016). During withdrawal, relapse is strongly motivated by disrupted emotional learning processes. Maladaptive reinforcement mechanisms drive relapse triggered by the desire to alleviate the aversive withdrawal state (Wikler and Pescor, 1967; Kenny et al., 2006) and by exposure to cues/contexts previously associated with drug use or withdrawal (Wikler and Pescor, 1967; Childress et al., 1993; Hellems et al., 2006; Crombag et al., 2008). Then, if opioid reuse during withdrawal does occur, disrupted reward learning processes elevate the incentive value of opiates, which persistently escalates

reward-seeking and taking behaviors (Hutcheson et al., 2001; Wassum et al., 2016). The cellular adaptations that underlie these disruptions likely develop in the basolateral amygdala (BLA), as neural circuits within this area are associated with reinforcement-driven relapse (Stinus et al., 1990; Fuchs and See, 2002; Frenois et al., 2005) and disrupted reward learning during withdrawal (Wassum et al., 2016).

Endogenous opioids acting at  $\mu$ -opioid receptors (MORs) in the BLA mediate the aversive opioid withdrawal state (Frenois et al., 2002) and learning changes in incentive value during withdrawal (Wassum et al., 2016). While their involvement in cue/context-induced reinstatement of opiates is unresolved, endogenous opioids are also implicated in cue/context-driven relapse to other drugs such as nicotine (Liu et al., 2009), alcohol (Ciccocioppo et al., 2002), and cocaine (Burattini et al., 2008). This suggests that changes to endogenous opioid activity in the BLA during withdrawal may underlie the disruptions to emotional learning processes that support and escalate drug use. Whether BLA endogenous opioid actions are changed by opioid withdrawal is unknown.

The endogenous opioids that disrupt BLA processes during opioid withdrawal are likely sourced from neighboring GABAergic

Received July 3, 2022; revised Oct. 18, 2022; accepted Nov. 21, 2022.

Author contributions: E.E.B. designed research; G.C.G., S.D.P., S.P., B.L.W., and E.E.B. performed research; G.C.G., S.D.P., S.P., and B.L.W. analyzed data; G.C.G. wrote the first draft of the paper; G.C.G. and E.E.B. edited the paper; E.E.B. wrote the paper.

This work was supported by the National Health and Medical Research Council Grant APP1047372.

The authors declare no competing financial interests.

Correspondence should be addressed to Elena E. Bagley at elena.bagley@sydney.edu.au.

<https://doi.org/10.1523/JNEUROSCI.1317-22.2022>

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intercalated cells (ITCs). We have recently shown that neurons in the main island (Im) of ITCs, which sits at the base of the BLA, makes direct synaptic connections with BLA principal neurons (Gregoriou et al., 2020), and readily release endogenous opioids (Winters et al., 2017). Once released, these endogenous opioids inhibit neurotransmitter release from both BLA principal neurons, and from Im neurons themselves (Winters et al., 2017). A major controller of endogenous opioid function in the amygdala are the peptidases that break down opioid peptides into inactive fragments, with inhibition of these enzymes doubling endogenous opioid actions (Winters et al., 2017). Withdrawal increases biochemical measures of enkephalin-degrading peptidase activity in several brain regions important for drug dependence, including the periaqueductal gray and striatum (Malfroy et al., 1978; Zhou et al., 2001). It is possible that increased peptidase activity represents an adaptation to chronic opioids, common throughout opioid-sensitive circuits, that could alter endogenous opioid function during withdrawal. Im ITCs are likely to develop adaptations to chronic opioid treatment, such as increases in peptidase activity, because they express very high levels of both the MOR and also enkephalin-degrading peptidases (Pollard et al., 1989; Krizanová et al., 2001; Banegas et al., 2005; Poulin et al., 2006). Whether increased peptidase activity is unmasked in the amygdala during opioid withdrawal is unknown.

Here, we show that opioid withdrawal alters peptide regulation of amygdala synapses. Using whole-cell patch recordings of BLA principal neurons and Im ITCs from brain slices spontaneously withdrawn from morphine, we demonstrate that opioid withdrawal reduces peptide control of synaptic transmission at reciprocal BLA-Im synapses, including synapses onto BLA neurons that project to the NAc. This reduced peptidergic control is dependent on protein kinase A (PKA)-driven upregulation of peptidase activity. Thus, we determine that amygdala-dependent emotional learning processes that rely on endogenous opioid actions are vulnerable during withdrawal and suggest that withdrawal opens a window where long-term disruptions to these processes can be established, contributing to the development of compulsive drug use.

## Materials and Methods

### General methods

All experimental procedures were conducted under protocols approved by the University of Sydney Animal Ethics Committee. Experimental procedures were performed on male Sprague Dawley rats (4–13 weeks old) obtained from the Animal Resources Centre. Rats were housed in groups of up to six in a low background noise room. Rats were maintained on a 12/12 h light/dark cycle with food and water available *ad libitum*. Room temperature was maintained at 21 ( $\pm$ 1)°C.

### Acute brain slice preparation

Coronal slices were prepared according to previously described methods (Gregoriou et al., 2020). Rats were anesthetized with isoflurane and killed by decapitation. The brain was rapidly removed from the skull and immersed in ice-cold external solution (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2.5 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 11 glucose, and 25 NaHCO<sub>3</sub>; pH 7.3, 295 mOsm/l) bubbled with carbogen (95% O<sub>2</sub>; 5% CO<sub>2</sub>).

Coronal brain slices (280  $\mu$ m) containing the rostral amygdala were cut using a VT1200S vibratome (Leica Biosystems) and then transferred to a holding chamber containing carbogenated cutting solution. In some instances (when animals were over six weeks old or when cells were being recorded from the BLA), slices were incubated in a recovery solution (in mM: 93 NMDG, 2.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 30 NaHCO<sub>3</sub>, 20 HEPES, 25 glucose, 2 thiourea, 3 sodium pyruvate, 5 sodium ascorbate, 10 MgCl<sub>2</sub>, and 0.5 CaCl<sub>2</sub>; pH 7.3, 300–310 mOsm/l) for 10 min before being transferred to the holding chamber. Occasionally slices had to be pretreated with drugs before recording. In these cases, drugs were included in the external

solution (noted in results where applicable). For all experiments, once submerged in cutting solution, slices were incubated at 34°C for at least 1 h and then stored at room temperature until used for recording.

### Electrophysiology

For recording, brain slices were transferred to a recording chamber and continuously superfused at 2 ml/min with artificial CSF (aCSF) of composition (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 11 glucose and 25 NaHCO<sub>3</sub>, saturated with carbogen and heated to 32–34°C. Brain slices were visualized with a BX51WI upright microscope (Olympus, Japan) fitted with Dodt gradient contrast optics (Thorlabs). Neurons of interest were readily identified by their location and morphology. Fluorescent neurons were visualized using an X-cite series 120Q excitation light source (Excelitas Technologies Corp) and appropriate filters. To verify correct targeting of neurons, either the electrophysiological properties of cells were examined, or slices were fixed following recording and kept for *post hoc* staining (see below). Whole-cell voltage-clamp recordings were made using patch electrodes (2–5 M $\Omega$ ) pulled on a P-1000 Micropipette puller (Sutter Instruments).

Unless otherwise stated, whole-cell voltage-clamp recordings were made from neurons voltage clamped at  $-70$  mV using patch pipettes filled with internal solution containing (in mM): 140 CsCl, 5 HEPES, 10 EGTA, 2 CaCl<sub>2</sub>, 2 MgATP, 0.3 NaGTP, and 3 QX-314-Cl (pH 7.3, 280–285 mOsm/l, liquid junction potential of  $-4$  mV was not corrected). For experiments measuring the inhibition/excitation ratio onto BLA neurons, the internal solution contained (in mM): 135 K-gluconate, 10 HEPES, 11 EGTA, 8 NaCl, 2 Mg<sub>2</sub>ATP, 0.3 NaGTP, and 3 QX-314-Cl (pH 7.3, osmolarity 280–285 mOsm, liquid junction potential of  $-13.6$  mV was not corrected). 0.1% Biocytin was routinely added to the internal solution. In all experiments, series resistance ( $<20$  M $\Omega$ ) was compensated by 60% and continuously monitored. Experiments in which series resistance changed by  $>20\%$  were excluded from analysis.

Electrically evoked synaptic currents were elicited via a bipolar stimulating electrode placed in various positions depending on the synaptic input studied. To record evoked EPSCs (eEPSCs) at BLA-Im and external capsule-BLA synapses, the stimulating electrode was placed close to the basomedial or basolateral edge of the BLA, respectively, and the GABA<sub>A</sub> receptor antagonists picrotoxin (100  $\mu$ M) and gabazine (10  $\mu$ M) were included in the aCSF (unless otherwise stated) to block fast inhibitory synaptic transmission. To record evoked IPSCs (eIPSCs) at Im-BLA synapses, the stimulating electrode was placed within the Im and the AMPA receptor antagonist CNQX (10  $\mu$ M) was used (unless otherwise stated) to block fast excitatory synaptic transmission.

### Electrophysiology data acquisition and analysis

All recorded electrophysiological signals were amplified, low pass filtered (5 kHz), digitized and acquired (sampled at 10 kHz) using Multiclamp 700B amplifier (Molecular Devices) and online/offline analysis was performed with Axograph Acquisition software (Molecular Devices).

For all experiments examining synaptic transmission, synaptic currents were evoked every 15 s by delivery of paired stimulating pulses (1–100 V, 100  $\mu$ s, 50 ms interpulse interval), except for endogenous opioid release experiments where a moderate stimulus protocol was used (1–100 V, five stimuli at 150 Hz, followed by one test pulse 200 ms later). Stimulus intensity was defined for each experiment as the voltage at which a synaptic current could be easily and consistently distinguished yet yielded submaximal current amplitudes. Peak amplitude was quantified as the mean peak amplitude of four to eight consecutive currents, after responses reached a stable plateau. The peak amplitude of the currents elicited by pulse 1 of the paired stimuli, or the test pulse of the moderate stimuli, were used for analysis of drug effects. Drug effects were quantified as the percentage change in mean peak amplitude between drug superfusion and the average baseline. The average baseline was an average of the peak amplitude of currents at the beginning of each experiment and the peak amplitude of currents at the end of each experiment (on reversal of drug effects by antagonist or drug washout). Reversal of drug effects was calculated as the percentage of the baseline amplitude recovered by drug washout or antagonist application. In experiments where antagonists increased peak amplitude of currents, drug effects

were calculated from the baseline. To determine whether synaptic connections were monosynaptic, the synaptic jitter, or the variability of latency to peak onsets, was measured. Latency to peak onset was defined as the time from the end of the stimulation artifact to 1% of the ePSC peak. Synaptic jitter of <0.7 ms indicates that a connection is monosynaptic (Doyle and Andresen, 2001), thus, only neurons with a jitter <0.7 ms were kept for further analysis. The synaptic jitter of a response was calculated as the standard deviation of the peak onsets.

#### Drugs and reagents

Stock solutions of all drugs were diluted to working concentrations in aCSF immediately before use and applied to brain slices by superfusion. The stock solutions for all drugs, except thiorphan, were made in Milli-Q water. Thiorphan was dissolved in DMSO to achieve a working DMSO concentration of 0.01%. Drugs and reagents were obtained from the following sources: CNQX, DAMGO, SR-95531 (gabazine), thiorphan and bestatin were from Abcam. Picrotoxin, methionine-enkephalin (met-enk) and captopril were from Sigma. CTAP, [D-Ala<sup>2</sup>]-Deltorphin II (Delt II), ICI-174864, naloxone and nociceptin were from Tocris. H-89 and forskolin were from Cayman Chemicals and morphine base was from Sun Pharma.

#### Chronic morphine treatment

Morphine dependence was induced using the chronic morphine treatment regimen described previously (Bagley et al., 2011). Rats received a series of subcutaneous injections of a sustained release emulsion containing 50 mg of morphine base (100 mg/kg, Sun Pharma) suspended in a vehicle solution comprised of 0.1 ml mannide monooleate (Arlacel A), 0.4 ml light liquid paraffin and 0.5 ml 0.9% w/v NaCl. Injections of warmed emulsion were made while animals were under light isoflurane anesthesia on days 1, 3, and 5. A group of rats were injected with a morphine-free emulsion on the same schedule and used as controls. Vehicle-treated and chronic morphine-treated rats were used on days 6 and 7 for experiments. The animal used on day 6 alternated between chronic morphine treated and vehicle throughout experiments.

#### Withdrawal behaviors

To test for dependence, a group of rats were injected with the opioid receptor antagonist naloxone hydrochloride (5 mg/kg, i.p.). Withdrawal signs including jumping, wet-dog shakes, tremor and teeth chattering were counted before and after withdrawal was precipitated.

#### Stereotaxic surgery

Rats were placed in induction chamber and administered 5% isoflurane. When deeply anesthetized, rats' heads were shaved and placed in stereotaxic apparatus (Model 942, Kopf Instruments) while maintaining incisor bar to achieve a flat skull position. Subcutaneous injection of caprofen (5 mg/kg, Cenvet) and bupivaine (5%, Cenvet) were made under the skin of the injection site. A single incision was made down the center of the rats' heads to expose the skull. Holes were drilled above the NAc and bilateral injections of fluorescent beads (1:1 dilution in saline, red-orange fluorescent microspheres 565/580, 0.04 μm, ThermoFisher Scientific) were made by hydraulic pressure at 10 nl/min for 1 min at stereotaxic coordinates: AP: +1.8 mm, ML: 1.4 mm, DV: -7.2 mm (horizontal skull, reference from bregma). Injections were made with glass micropipettes (30–50 μm tip, Drummond Scientific) pulled with a PC-10 micropipette puller (Narishige). On completion of the injection, the micropipette was left in place for a further 15 min before retraction. Following pipette withdrawal, skull openings were sealed with bone wax (Coherent Scientific) and the incision was closed with silk sutures. Rats were given cephalosporin (100 mg/ml, Hospira, Cenvet) and saline postsurgery and allowed to recover for 7 d before being used for experiments.

#### Immunohistochemistry

Slices were prepared for immunohistochemistry from perfused animals or following electrophysiological experiments for *post hoc* staining. For perfusion, animals were deeply anesthetized with isoflurane and/or a lethal dose of pentobarbital sodium (120–150 mg/kg, i.p.). Once reflexes were abolished, animals were perfused through the ascending aorta with 3000 units/ml heparin in a 0.5% NaNO<sub>2</sub>/0.9% saline solution followed by 4% paraformaldehyde (PFA) solution in 0.1 M PBS (pH 7.4). Brains were removed and fixed overnight in 4% PFA (4°C). Brains were washed

three times with PBS and then stored for no more than one week in PBS (4°C) before sectioning. Coronal sections (40 μm) containing the amygdala were cut using a vibratome (Leica Biosystems). Sections were stored in a cryoprotectant solution comprised of 40% PBS, 30% glycerol, 30% ethylene glycol cryoprotectant at -30°C until required for immunohistochemistry. Following electrophysiological experiments, coronal slices (280 μm) containing the amygdala were fixed overnight in 4% PFA (4°C). Slices were washed three times with PBS and then stored in cryoprotectant solution at -30°C until required for immunohistochemistry.

Before staining, cryoprotectant was removed from slices by washing three times with PBS, then incubated for 30 min at room temperature in 10% donkey serum and 0.5% BSA in PBS to block nonspecific binding. Primary antibodies for met-enk (Millipore; rabbit anti-Met-enkephalin; ab5026, RRID:AB\_91644; 1:200), MOPR (Aves Labs; chicken anti-MOPR; 1:5000), and NEP (R&D Systems; donkey anti-goat; AF1126; lot: JGIO116081, RRID:AB\_2144426; 1:80) were diluted in 0.1% BSA/0.25% Triton X-100 in PBS and sections were incubated overnight (4°C). Secondary antibodies for rabbit (Abcam; donkey anti-rabbit Alexa Fluor 568; ab175692; lot: GR322655-2, RRID:AB\_2884939; 1:500), chicken (Jackson ImmunoResearch; donkey anti-chicken Alexa Fluor 647, RRID:AB\_2340379; 1:500) and goat (Abcam; donkey anti-goat Alexa Fluor 488; ab150133; lot: GR312722-2, RRID:AB\_2832252; 1:500) were diluted in 0.1% BSA in PBS, and incubated 2 h at room temperature (light protected). The nuclear stain DAPI (1:500, ThermoFisher Scientific) was added for the final 30 min of this incubation. Sections were then washed three times in PBS and mounted onto slides using ProLong Gold Antifade (Life Technologies).

For *post hoc* staining of brain slices (280 μm) used in electrophysiological experiments and containing cells filled with 0.1% biocytin (Sigma) and fluorescent beads (ThermoFisher Scientific), slices were briefly washed before being incubated for 1 h at room temperature in 10% goat serum, 0.5% BSA and 0.3% Triton X-100 in PBS. Streptavidin conjugated antibody (Alexa Fluor 647 streptavidin, 1:2000, ThermoFisher Scientific S32357) was diluted in 1% BSA/0.1% Triton X-100 in PB and incubated for 2 h at room temperature (light protected). DAPI (1:500, ThermoFisher Scientific) was added for the final 30 min of this incubation period. Slices were then washed three to four times (10 min) with PBS and mounted onto slides using Fluoromount-G (SouthernBiotech).

Sections were visualized using a Zeiss LSM800 Meta confocal microscope (lasers 405, 488, 561 nm; Carl Zeiss) and a Leica SP8 STED confocal microscope (lasers 405, 561, 647 nm; Leica). Images were taken sequentially with different lasers using low power dry 10× [numerical aperture (NA) 0.45] and 20× objectives (NA 0.8), as well as high power oil immersion 63× (NA 1.40) objective. Single images were collected using the 10× objective and Z-stacks were collected for the 20× and 63× objectives, respectively.

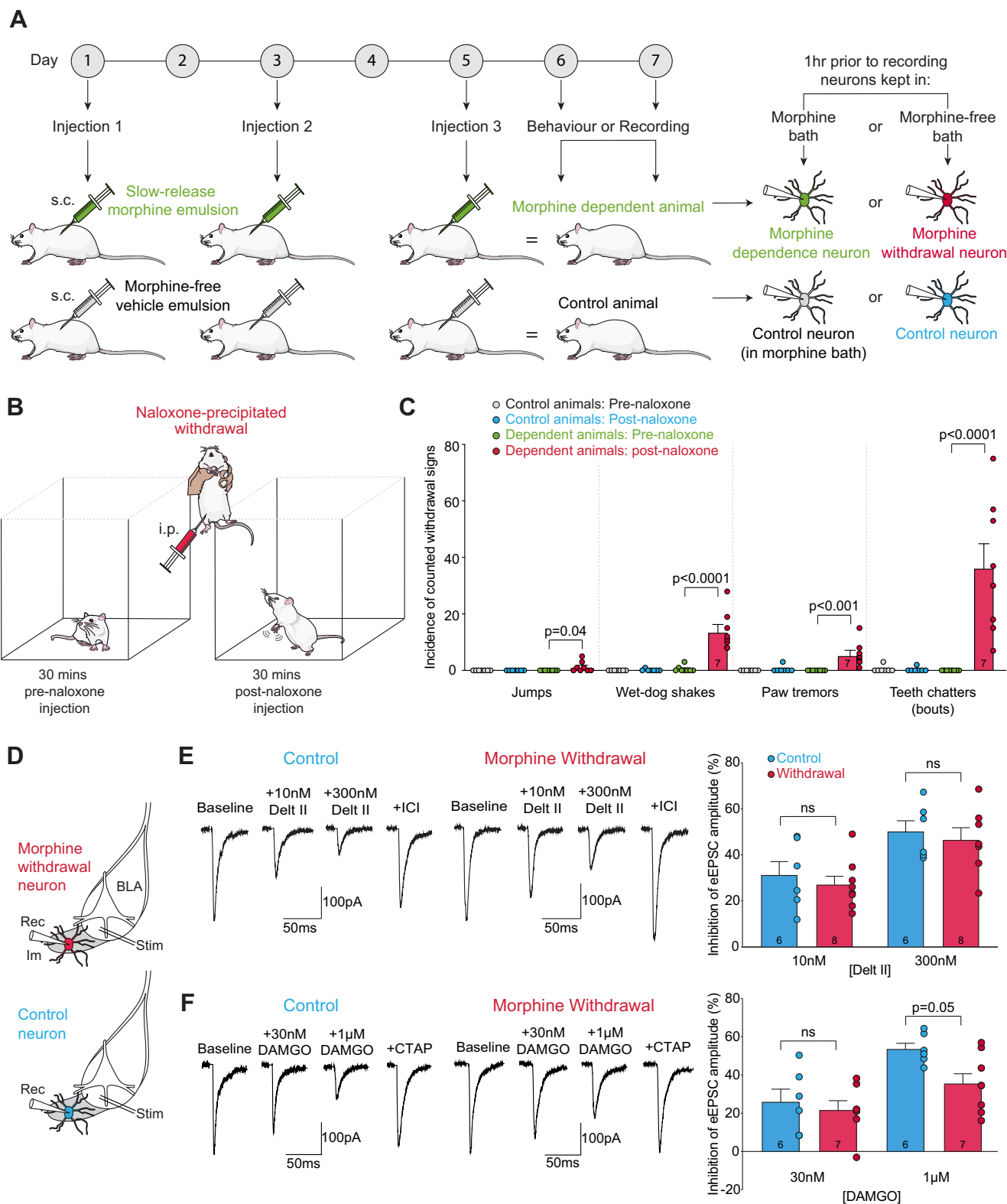
#### Data collection and analysis

All data are presented as the mean ± SEM. No statistical methods were used to predetermine sample size; however, the numbers obtained are similar to those generally used within the field. Numbers vary because of effect size, variability, and difficulty of experiment. Data distribution was assumed normal in all cases. Statistical analysis was determined before any results were obtained. All statistical analysis was performed using GraphPad Prism 6. Data were considered significant if  $p < 0.05$ .

## Results

### Opioid withdrawal produces tolerance to MOR, but not DOR, mediated inhibition of glutamate release at BLA-Im ITC synapses

To induce morphine dependence, Sprague Dawley rats were chronically treated with a slow-release morphine emulsion (Bagley et al., 2011) and control rats were treated with a morphine-free vehicle emulsion on the same schedule (Fig. 1A). Brain slices taken from these animals were incubated in either a bath of artificial ACSF containing morphine (5 μM) to study the effects of chronic morphine treatment or a morphine-free bath to study the effects of spontaneous *in vitro* morphine withdrawal (Bagley et al., 2005; Fig. 1A). To test whether this chronic morphine treatment induced



**Figure 1.** Opioid withdrawal produces homologous tolerance to MOR-mediated inhibition of glutamate release at BLA-Im synapses. **A**, Schematic of the dependence paradigm showing that a slow-release emulsion containing 50 mg/kg morphine was injected sub cutaneously (s.c.) into rats every second day to induce dependence. Control rats were injected with a morphine-free vehicle emulsion on the same schedule. Morphine-dependent and control animals were used for experiments on day 6 or 7. Brain slices taken from these rats were incubated in either a bath containing 5  $\mu$ M morphine or a morphine-free bath to study morphine dependence or spontaneous *in vitro* withdrawal, respectively. **B**, Schematic showing that rats were observed for 30 min preinjection and postinjection of 5 mg/kg naloxone hydrochloride intraperitoneal (i.p.) and withdrawal signs were counted during each observation period. **C**, Bar chart of withdrawal signs. Treatment group did not affect the incidence of each withdrawal sign prenaloxone injection. Postnaloxone injection, there was a significant increase in the incidence of each withdrawal sign in dependent animals compared with control animals (control vs dependent jumps,  $F_{(1,13)} = 3.23$ ,  $p = 0.035$ ; control vs dependent wet-dog shakes,  $F_{(1,13)} = 24.05$ ,  $p < 0.0001$ ; control vs dependent paw tremor,  $F_{(1,13)} = 9.39$ ,  $p = 0.0004$ ; and control vs dependent teeth chattering,  $F_{(1,13)} = 15.88$ ,  $p < 0.0001$ ). In morphine-dependent animals ( $n = 8$ ), naloxone injection significantly increased all withdrawal signs (prenaloxone vs postnaloxone jumps,  $F_{(1,13)} = 3.23$ ,  $p = 0.04$ ; prenaloxone vs postnaloxone wet-dog shakes,  $F_{(1,13)} = 34.82$ ,  $p < 0.0001$ ; prenaloxone vs

opioid dependence we examined whether injection of the opioid receptor antagonist naloxone (5 mg/kg) increased opioid withdrawal signs (Fig. 1B). Neither vehicle-treated control animals nor morphine-dependent animals displayed any signs of withdrawal before the naloxone challenge (Fig. 1C) and no withdrawal signs were observed in vehicle-treated rats after the naloxone challenge (Fig. 1C). In contrast, after chronic morphine-treatment rats exhibited all the counted withdrawal signs postnaloxone injection (Fig. 1C). This indicates that, as seen previously (Bagley et al., 2005, 2011), the current chronic morphine treatment protocol successfully induced opioid dependence.

To study whether opioid withdrawal alters peptidase control of neuropeptide activity in the amygdala our initial experiments focused on regulation of the BLA principal neuron synapse onto Im neurons (BLA-Im synapse). We chose to study this synapse as peptidases strongly limit inhibition of this synapse by both exogenous and endogenous enkephalins in untreated rats (Winters et al., 2017; Gregoriou et al., 2020). Our measurement of peptidase activity during withdrawal relied on unchanged opioid receptor signaling, however, in other brain regions, withdrawal can either increase or decrease opioid receptor coupling to their effectors (Chieng and Christie, 1996; Ingram et al., 1998, 2008; Hack and Christie, 2003; Bagley et al., 2005). To control for the possibility of withdrawal-induced changes in opioid receptor signaling, we initially tested whether spontaneous withdrawal from chronic morphine treatment induced changes to opioid receptor coupling to inhibition of glutamate release at BLA-Im synapses. To do this we electrically stimulated the BLA (Fig. 1D) and recorded the resulting evoked EPSCs (eEPSCs) in Im neurons during whole cell patch clamp recordings. As activation of  $\mu$ -opioid and  $\delta$ -opioid receptors (MORs and DORs), but not  $\kappa$ -opioid receptors (KORs), inhibits glutamate release at BLA-Im synapses (Winters et al., 2017), selective agonists for MORs and DORs were used to test opioid receptor coupling at this synapse. Nonhydrolyzable MOR and DOR agonists were chosen so that drug effects were independent of peptidase activity. Deltorphin II (Delt II), a selective and nonhydrolyzable agonist at the DOR, was tested at a submaximal (10 nM) and maximal concentration (300 nM; Hack et al., 2005). Delt II inhibited eEPSCs at the BLA-Im synapse in a concentration-dependent manner in both control and morphine-withdrawn neurons (Fig. 1E). Morphine-withdrawal did not alter the efficacy of Delt II to inhibit glutamate release at either concentration (Fig.

1E). This indicates that withdrawal does not modify DOR coupling at this synapse. The DOR-selective antagonist, ICI-174864 (1  $\mu$ M), reversed the Delt II-induced inhibition in all neurons [control neurons reversal =  $101.91 \pm 5.43\%$  ( $n = 6$ ), withdrawn neurons reversal =  $89.30 \pm 4.31\%$  ( $n = 8$ )]. DAMGO, a selective and nonhydrolyzable agonist at the MOR, was also tested at submaximal (30 nM) and maximal concentrations (1  $\mu$ M; Ingram et al., 1998). While submaximal DAMGO inhibited glutamate release to the same extent in both groups, at the maximal concentration, there was a reduction in the effect of DAMGO in morphine-withdrawn neurons (Fig. 1F). The MOR antagonist CTAP (1  $\mu$ M) reversed the DAMGO inhibition of glutamate release in all cells [control neurons reversal =  $97.25 \pm 3.96\%$  ( $n = 6$ ), withdrawn neurons reversal =  $100.01 \pm 3.28\%$  ( $n = 8$ )]. These data indicate that spontaneous withdrawal from chronic morphine treatment reduces the ability of MORs to regulate glutamate transmission from BLA principal neurons to Im ITCs while DOR-dependent regulation of glutamate release remains unaffected. This suggests MOR activity on BLA terminals is reduced after chronic treatment and withdrawal from the MOR-preferring agonist, morphine. To prevent this change from distorting our interpretation of peptidase function, all subsequent experiments (unless otherwise stated) were conducted in the presence of the MOR antagonist CTAP (1  $\mu$ M) and thus only opioid actions at DOR on BLA synaptic terminals were measured.

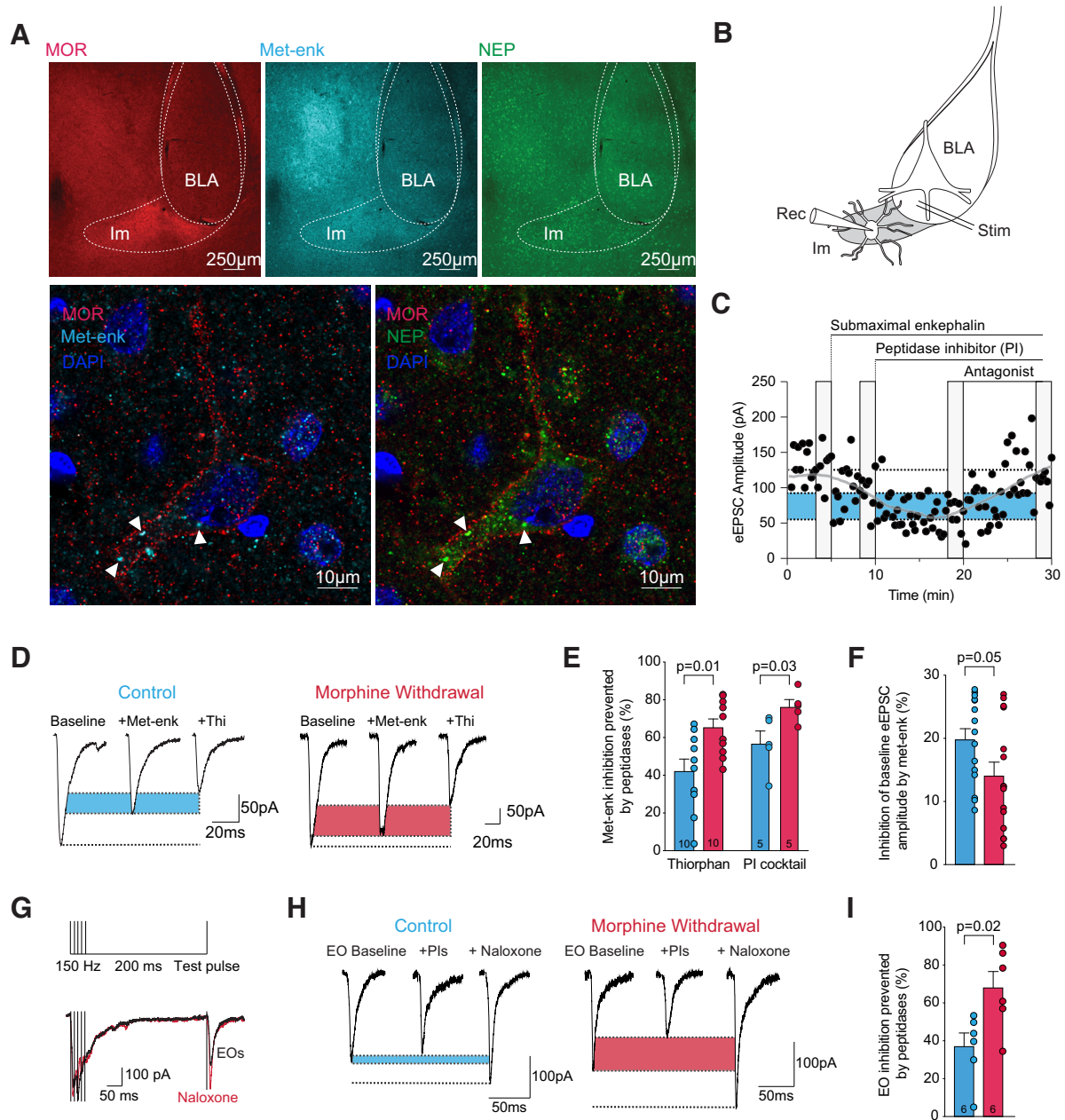
#### Nepriylsin activity at the BLA-Im synapse is upregulated during opioid withdrawal

The ability of endogenously released enkephalins to inhibit Im neurons and their associated synapses (Winters et al., 2017) is limited by the activity of neprilysin (Gregoriou et al., 2020), an enkephalin-degrading peptidase that cleaves the glycine-phenylalanine bond of enkephalin molecules (Hiranuma and Oka, 1986; Erdős and Skidgel, 1988; Hiranuma et al., 1997). In areas with high expression of opioid receptors, such as the striatum and periaqueductal gray, neprilysin is abundantly coexpressed (Waksman et al., 1986). Given that withdrawal from chronic morphine treatment increases biochemical activity of a neprilysin-like peptidase in these same brain regions (Malfroy et al., 1978; Zhou et al., 2001), we hypothesized that elevated neprilysin activity during opioid withdrawal may be a widespread phenomenon, common in cells with overlapping neprilysin and opioid receptor expression. Considering this, we examined whether neprilysin expression overlapped with opioid receptor expression in the Im. We found high immunoreactivity for neprilysin throughout all amygdala nuclei, including within the Im (Fig. 2A). We also found high immunoreactivity for MOR in the Im (Poulin et al., 2008; Winters et al., 2017) and moderate immunoreactivity for enkephalins in both the Im and CeA, as previously observed (Poulin et al., 2008; Winters et al., 2017; Fig. 2A). Within the Im, neprilysin co-localized with enkephalins in MOR-expressing neurons (Fig. 2A), suggesting that when Im neurons release enkephalins, neprilysin is well positioned to cleave this enkephalin near its release site, thus critically regulating the extent, timing, and spread of endogenous enkephalin activity within the Im.

Opioid withdrawal increases biochemical measures of neprilysin-like activity (Malfroy et al., 1978; Zhou et al., 2001), but whether this alters peptide actions is unknown. If opioid withdrawal increases neprilysin activity in the Im it would likely reduce peptidergic control of synaptic transmission and cellular excitability. To study this, we examined whether inhibition of the BLA-Im synapse by a submaximal concentration of met-enkephalin (met-enk; 100 nM) is limited to a greater degree by neprilysin activity

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postnaloxone paw tremor,  $F_{(1,13)} = 12.74$ ,  $p = 0.0006$ ; and prenaloxone vs postnaloxone teeth chattering,  $F_{(1,13)} = 16.48$ ,  $p < 0.0001$ ). Naloxone injection did not affect the incidence of any withdrawal sign in control animals ( $n = 7$ ). D, Representative BLA stimulation (stim) and Im recording (rec) locations. E, Example eEPSCs from individual cells showing the effect of the selective DOR agonist Delt II in both control and withdrawal conditions. Bar chart showing percentage inhibition from baseline. Maximal Delt II inhibited eEPSCs more than submaximal Delt II in both groups (control, 10 nM vs 300 nM,  $F_{(1,23)} = 14.42$ ,  $p = 0.04$ ; withdrawal, 10 nM vs 300 nM,  $F_{(1,23)} = 14.42$ ,  $p = 0.02$ ). Delt II inhibited eEPSC amplitude in controls to a similar extent to withdrawn neurons at both submaximal and maximal concentrations. F, Example eEPSCs from individual cells showing the effect of the selective MOR agonist DAMGO in both control and withdrawal conditions. Bar chart showing percentage inhibition from baseline. Maximal DAMGO inhibited eEPSCs more than submaximal DAMGO in control neurons but not in withdrawn neurons (control, 30 nM vs 1  $\mu$ M,  $F_{(1,23)} = 14.85$ ,  $p = 0.004$ ; withdrawal, 30 nM vs 1  $\mu$ M,  $F_{(1,23)} = 14.85$ ,  $p = 0.13$ ). There was a significant effect of treatment group on percent inhibition by DAMGO at the maximal, but not submaximal, concentration (30 nM, control vs withdrawal,  $F_{(1,23)} = 4.32$ ,  $p > 0.9999$ ; 1  $\mu$ M, control vs withdrawal,  $F_{(1,23)} = 4.32$ ,  $p = 0.05$ ), with the effect of DAMGO at maximal concentration reduced in morphine withdrawn neurons compared with control. Bar charts show mean  $\pm$  SEM. Circles represent individual neurons. Neuron number is shown in bars. Data were analyzed using two-way ANOVAs with comparisons on graphs showing results from *post hoc* Bonferroni's multiple comparisons tests.



**Figure 2.** Neprilysin activity at the BLA-Im synapse is upregulated during opioid withdrawal. **A**, Neprilysin (NEP) is co-localized with met-enk and MOR in the Im. From left, Low power confocal images showing immunoreactivity for MOR (red), met-enk (blue), and neprilysin (green) in the amygdala. Magnification of an Im neuron reveals that MOR, met-enk, and neprilysin immunoreactivity co-localizes within a single Im neuron. All images were taken at bregma  $-2.00$ . **B–E**, Peptidase regulation of submaximal met-enk ( $100$  nM) is enhanced during morphine withdrawal. **B**, Representative BLA stimulation (stim) and Im recording (rec) locations. **C**, Representative timeplot of experimental protocol. Gray bars show where drug effect measurements were taken; gray line is the moving average amplitude of eEPSC amplitude; dotted line is average baseline amplitude; blue bar represents the amount of peptide inhibition prevented by peptidases. **D**, Example eEPSCs from representative experiments with colored bars representing the amount of peptide inhibition prevented by the thiorphan-sensitive peptidase, neprilysin. **E**, Bar chart showing the proportion of met-enk inhibition prevented by the activity of neprilysin alone (thiorphan bars), or by neprilysin plus two other major met-enk degrading peptidases, which were blocked by the peptidase inhibitor cocktail [PI cocktail bars, PI cocktail = thiorphan ( $10$   $\mu$ M), captopril ( $1$   $\mu$ M), bestatin ( $10$   $\mu$ M)]. The activity of neprilysin and cocktail sensitive peptidases was significantly greater during opioid withdrawal (thiorphan alone, control vs withdrawal,  $t_{(18)} = 2.87$ ,  $p = 0.01$ ; PI cocktail, control vs withdrawal,  $t_{(8)} = 2.61$ ,  $p = 0.03$ ). **F**, Bar chart showing inhibition of baseline eEPSC amplitude by submaximal met-enk (control vs withdrawal,  $t_{(28)} = 2.03$ ,  $p = 0.05$ ). **G**, Stimulus paradigm for endogenous opioid (EO) release using a protocol of a five-stimuli train followed by a single test pulse. Example traces show eEPSCs evoked by the stimulus paradigm with and without naloxone ( $10$   $\mu$ M). **H**, Example test pulse eEPSCs from single representative experiments. Dotted line from naloxone trace is the “true” baseline eEPSC amplitude when EO actions blocked by naloxone; colored bars represent the amount of endogenous opioid inhibition prevented by peptidases. **I**, Bar chart showing the proportion of endogenous opioid inhibition prevented by cocktail-sensitive peptidases. Withdrawal significantly enhanced peptidase control of the actions of endogenously released enkephalin (control vs withdrawal,  $t_{(10)} = 2.75$ ,  $p = 0.02$ ). Bar charts show mean  $\pm$  SEM. Circles represent individual neurons. Neuron number is shown in bars. Data were analyzed using two-way ANOVAs with Bonferroni’s multiple comparisons and unpaired Student’s  $t$  tests. Comparisons on graphs show results from  $t$  tests.

during opioid withdrawal (Fig. 2B–E). In both control and morphine-withdrawn neurons, submaximal met-enk inhibited eEPSC amplitude and this inhibition was increased by inhibiting neprilysin with thiorphan (Table 1). However, in morphine-withdrawn neurons, a significantly larger proportion of the met-enk response was prevented by neprilysin activity (Fig. 2D,E, see thiorphan bars in E). This suggests that the ability of neprilysin to protect met-enk from degradation is enhanced during opioid withdrawal.

Under normal physiological conditions, neprilysin selectively degrades enkephalins in the Im (Gregoriou et al., 2020). However, several other enkephalin-degrading peptidases, such as angiotensin-converting enzyme (ACE) and aminopeptidase N (APN), are expressed in the amygdala (Pollard et al., 1989; Krizanová et al., 2001; Banegas et al., 2005). To examine whether opioid withdrawal alters ACE and/or APN hydrolysis of met-enk we applied a peptidase inhibitor (PI) cocktail to amygdala slices in the presence of submaximal met-enk (100 nM). The PI cocktail consisted of thiorphan (10  $\mu$ M) to inhibit neprilysin, captopril (1  $\mu$ M) to inhibit ACE, and bestatin (10  $\mu$ M) to inhibit APN (Winters et al., 2017; Gregoriou et al., 2020). Similar to the results from experiments using thiorphan alone, in both control and morphine-withdrawn neurons, submaximal met-enk (100 nM) inhibited the eEPSC amplitude and this inhibition was increased by the cocktail of PIs (Table 1). A greater proportion of the met-enk inhibition was prevented by peptidase activity during opioid withdrawal (Fig. 2E, see PI cocktail data). Importantly, in both groups the total met-enk inhibition produced in the presence of the PI cocktail was no different from the total met-enk inhibition produced in the presence of thiorphan alone, as can be appreciated by comparing the percent inhibition caused by met-enk + thiorphan and met-enk + PI cocktail in both control and morphine withdrawn cells in Table 1 (two-way ANOVA with Bonferroni's multiple comparisons). In other words, the addition of captopril and bestatin did not increase the effect of thiorphan alone. This indicates that increased peptidase control of opioid activity during opioid withdrawal is likely because of changes in neprilysin activity alone, not changes to the activity of any other enkephalin-degrading peptidases, such as ACE or APN.

As met-enk inhibition of synaptic transmission gives us a readout of how much met-enk is able to activate opioid receptors, we could use this readout to both, (1) determine how much of the enkephalin response was being prevented by peptidases in control versus withdrawn cells (by applying peptidase inhibitors, as presented above), and (2) quantify the extent to which peptidase activity dials down opioid peptide actions in each group (by measuring the enkephalin-induced inhibition of baseline eEPSC amplitude before peptidase inhibitor application). Thus, to determine whether increased neprilysin activity during withdrawal had a functional consequence on opioid actions, we consolidated all previous submaximal met-enk data and compared inhibition of the BLA-Im synapse in control and withdrawn cells. During withdrawal, the inhibitory actions of met-enk at the BLA-Im were reduced (Fig. 2F), confirming that increased neprilysin activity during withdrawal increases metabolism of opioid peptides and dials down their inhibitory actions at the BLA-Im synapse.

To understand how the increased neprilysin activity during opioid withdrawal might limit the actions of endogenously released peptides we stimulated BLA principal neurons at moderate frequency (Fig. 2G). This releases enkephalins from the Im at levels limited by peptidase activity, but still sufficient to inhibit

neurotransmission from BLA principal neurons (Winters et al., 2017). To determine the level of peptidase control, we applied the PI cocktail, and to determine the level of endogenous opioid inhibition, we applied naloxone (10  $\mu$ M) at a concentration able to antagonise the DORs mediating the endogenous opioid inhibition (Winters et al., 2017), with CTAP 1  $\mu$ M present throughout the experiment. Naloxone increased eEPSC amplitudes above baseline levels in all neurons indicating that the BLA-Im synapse is under tonic endogenous opioid control [control neurons: naloxone increase =  $16.93 \pm 2.13\%$  ( $n=6$ ), withdrawn neurons: naloxone increase =  $17.15 \pm 8.65\%$  ( $n=6$ )]. Therefore, the eEPSC amplitude following naloxone superfusion reflected the “true baseline” of glutamatergic signaling at the BLA-Im synapse once endogenous opioid inhibition was blocked (Fig. 2G,H). This value was used to determine the proportion of endogenous opioid signaling that was under peptidase control. We found that endogenously released opioids yielded a small inhibition of eEPSCs, which was enhanced by addition of the PI cocktail in morphine withdrawn neurons (Fig. 2H,I; Table 1). The ability of peptidases to prevent endogenous opioid activity was enhanced in withdrawn neurons compared with control (Fig. 2I). These data indicate that, as we saw with exogenous enkephalin, morphine withdrawal increases peptidase control over endogenously released enkephalin activity in the Im.

### Neprilysin upregulation occurs through a PKA-dependent mechanism

Having demonstrated that neprilysin control of endogenous peptide actions at the BLA-Im synapse is upregulated during withdrawal, the following experiments addressed how this occurred. As opioid withdrawal results from cellular adaptations induced by chronic opioid exposure (Williams et al., 2001) two possibilities emerged: neprilysin activity is (1) upregulated during morphine dependence to counteract prolonged occupation of opioid receptors, or (2) is upregulated in response to spontaneous withdrawal from chronic morphine *in vitro*. To address this question, we made recordings from Im neurons in slices that had either been spontaneously withdrawn or maintained in morphine (5  $\mu$ M; Fig. 3A). In experiments described above, that established neprilysin upregulation during the withdrawal state, the MOR antagonist CTAP was present so that homologous tolerance of MORs would not distort our measurement of peptidase activity. However, this was not possible in the present experiment because CTAP would precipitate withdrawal in the slices maintained in morphine. Therefore, we studied whether peptidase control over the activity of another peptide, nociceptin, which is also controlled by neprilysin at this synapse (Gregoriou et al., 2020), is altered by chronic morphine treatment and/or opioid withdrawal. In withdrawn and control neurons, submaximal nociceptin (100 nM) caused a small inhibition of eEPSC amplitude, which was potentiated by thiorphan (10  $\mu$ M) in withdrawn cells (Fig. 3B; Table 1). Similar to met-enk, a larger proportion of the nociceptin inhibition was prevented by neprilysin in cells withdrawn from chronic morphine (Fig. 3B). In contrast, when slices from morphine-dependent rats or control rats were stored in a morphine-containing bath, the extent to which thiorphan was able to boost synaptic inhibition was no different between groups (Fig. 3B). Together, these data indicate that increased neprilysin activity is revealed during the withdrawal state rather than during chronic morphine treatment. These data also suggest that withdrawal-induced changes in neprilysin activity can limit the activity of non-opioid peptides.

**Table 1. Percent inhibition of baseline synaptic transmission at the BLA-Im synapse by peptides, alone and in the presence of peptidase inhibitors**

Group	Slices incubated in H89									
	Met-enk	Met-enk + thiorphan	Met-enk	Met-enk + PI cocktail	EOs	EOs + PI cocktail	Nociceptin	Nociceptin + thiorphan	Met-enk + thiorphan	
Control (morphine-free bath)	19.42 ± 2.39 (10)	34.40 ± 4.01* (10)	20.48 ± 2.58 (5)	48.53 ± 3.33* (5)	14.43 ± 1.56 (6)	23.21 ± 2.30* (6)	21.65 ± 2.92 (6)	31.41 ± 2.50 (6)	23.29 ± 3.40 (6)	39.10 ± 4.38* (6)
Morphine withdrawal	15.08 ± 3.27 (10)	38.98 ± 5.57* (10)	11.85 ± 1.55 (5)	54.43 ± 7.72* (5)	12.69 ± 5.31 (6)	34.09 ± 5.89* (6)	13.33 ± 1.09 (5)	39.54 ± 6.78* (5)	19.46 ± 3.56 (6)	32.58 ± 4.50 (6)
Control (morphine bath)							15.11 ± 4.43 (5)	24.91 ± 4.48* (5)		
Morphine dependence							13.94 ± 1.24 (5)	27.36 ± 2.37* (5)		
Naive (+ forskolin)	8.26 ± 2.66 (5)	25.78 ± 7.87* (5)								
Naive (– forskolin)	18.60 ± 3.82 (5)	25.14 ± 5.84 (5)								

Values are mean inhibition of eEPSC amplitude from baseline (%) ± SEM. Neuron number for each group is shown in brackets. Data were analyzed using two-way repeated measures ANOVAs with *post hoc* Bonferroni's multiple comparisons tests to assess the effects of peptidase inhibition on the ability of peptides (met-enk, EOs, and nociceptin) to inhibit the BLA-Im synapse in various treatment groups. \*, significant results from *post hoc* Bonferroni's tests ( $p < 0.05$ ) comparing percent inhibition produced by peptides alone (met-enk, EO, and nociceptin columns) with percent inhibition produced by peptides + peptidase inhibitors (met-enk + thiorphan, met-enk + PI cocktail, EO + PI cocktail, nociceptin + thiorphan columns, respectively) for each treatment group.

Hypertrophy in the adenylyl cyclase/PKA signaling pathway is a neuroadaptation revealed in many opioid-sensitive cells during opioid withdrawal (Maldonado et al., 1995; PUNCH et al., 1997; Williams et al., 2001; Nestler, 2004). Thus, we hypothesized that the withdrawal-induced increase in neprilysin control of peptide activity may result from elevated adenylyl cyclase/PKA signaling. Therefore, we inhibited PKA by incubating morphine-withdrawn slices in H89 (10  $\mu$ M) for 40 min before recording and found that, while neprilysin inhibition still potentiated met-enk inhibition of eEPSCs (Table 1), the amount of the met-enk response prevented by neprilysin activity was no different between groups (Fig. 3C,D). This suggests that the upregulation of neprilysin activity revealed during opioid withdrawal is produced as a result of increased PKA activity. Thus, increasing adenylyl cyclase activity in neurons from untreated rats should mimic the effect of opioid withdrawal. To test this further, we activated adenylyl cyclase with forskolin (10  $\mu$ M) in slices from naive animals and found that forskolin potentiated baseline eEPSC amplitudes (68 ± 11.61% increase from baseline,  $p = 0.02$ , paired Student's *t* test,  $n = 5$ ). In the presence of forskolin, submaximal met-enk (100 nM) inhibited eEPSCs and the met-enk inhibition was further increased by neprilysin inhibition in the presence of forskolin (Table 1). Further, a significantly larger proportion of the met-enk response was prevented by neprilysin activity in neurons with forskolin present (Fig. 3D). Forskolin rapidly increases neprilysin breakdown of peptides of within 5–10 min of application. The rapid actions of forskolin are consistent with the rapidly developing and then stable withdrawal-induced increase in neprilysin activity that we observed. In control ( $n = 19$ ) and withdrawn cells ( $n = 17$ ), the size of the neprilysin upregulation remained consistent from 2–11 h in the morphine-free bath (Control cells:  $r_{17} = -0.3945$ ,  $p = 0.09$ ; withdrawn cells:  $r_{15} = 0.3305$ ,  $p = 0.18$ , data analyzed by two-tailed Pearson's correlation coefficient). Together, these data indicate that inhibition of PKA prevents the withdrawal induced increase in neprilysin activity and that stimulation of the adenylyl cyclase pathway induces an upregulation of neprilysin activity analogous to the increase observed in withdrawn slices. Therefore, it is likely that enhanced adenylyl cyclase and PKA activity during withdrawal boosts neprilysin activity and, through this, limits peptide regulation of neural circuits in the amygdala.

### Balance is shifted toward inhibition at synapses between Im ITCs and NAc-projecting BLA principal neurons during withdrawal

Having established that peptide regulation of the BLA-Im synapse is turned down during opioid withdrawal because of

increased neprilysin activity, we wondered whether peptide signaling is also disrupted at GABAergic projections from Im ITCs onto BLA principal neurons. Given the participation of endogenous opioid signaling in the BLA in the relapse-triggering processes that occur during opioid withdrawal, this could influence several distinct emotional learning mechanisms, such as learning about the aversiveness of withdrawal, reward re-evaluation, and cue-driven relapse. We were particularly interested in a subpopulation of BLA principal neurons involved in encoding reward values and mediating reward-seeking behaviors (Stuber et al., 2011; Britt et al., 2012; Namburi et al., 2015; Ramirez et al., 2015; Beyeler et al., 2016). These neurons are characterized by their projections to the nucleus accumbens (NAc). Indeed, studies that optically stimulate, inhibit (Stuber et al., 2011), or lesion (Shiflett and Balleine, 2010) the neural pathway from the BLA to the NAc have confirmed that the activity of NAc-projecting BLA principal neurons is required for animals to correctly process the subjective values of rewards and use these values to guide reward-seeking behaviors. While the Im strongly inhibits randomly sampled BLA principal neurons (Gregoriou et al., 2019), it is not known whether Im neurons specifically target NAc-projecting BLA principal neurons. To identify NAc-projecting BLA principal neurons, fluorescent retrograde beads were injected into the NAc (Fig. 4A,B; Extended Data Fig. 4-1). Then, the Im was electrically stimulated and whole cell recordings were made from BLA principal neurons containing fluorescent beads (Fig. 4C–F). BLA principal neurons were characterized by their marked pyramidal morphology with clear differentiation of a thicker apical dendrite from thinner basal dendrites. Synaptic jitter, or the variability of eIPSC latency (the time interval between the end of the stimulation artifact and the onset of the IPSC), indicates whether a synaptic connection is monosynaptic or polysynaptic, with jitters < 0.7 ms signifying monosynaptic connections, and jitters > 0.7 ms representing polysynaptic connections (Doyle and Andresen, 2001). The synaptic jitter of each Im-BLA (NAc-projector) synapse was calculated and only neurons with direct connections were kept for further analysis (Fig. 4F). Fifty-four NAc-projecting BLA principal neurons were recorded from and of these 49 neurons (91%) had direct inhibitory synaptic connections from the Im (synaptic jitter 0.35 ± 0.02 ms). These data suggest that Im neurons do indeed target the NAc-projecting subpopulation of BLA principal neurons and that withdrawal-induced adaptations could directly affect BLA-NAc reward processes. To address whether peptide control of the Im-BLA (NAc-projector) synapse is also disrupted during opioid withdrawal we tested whether neprilysin more strongly controls nociceptin inhibition of this synapse in morphine withdrawn versus control neurons (Fig. 4G,H). Nociceptin was used as we

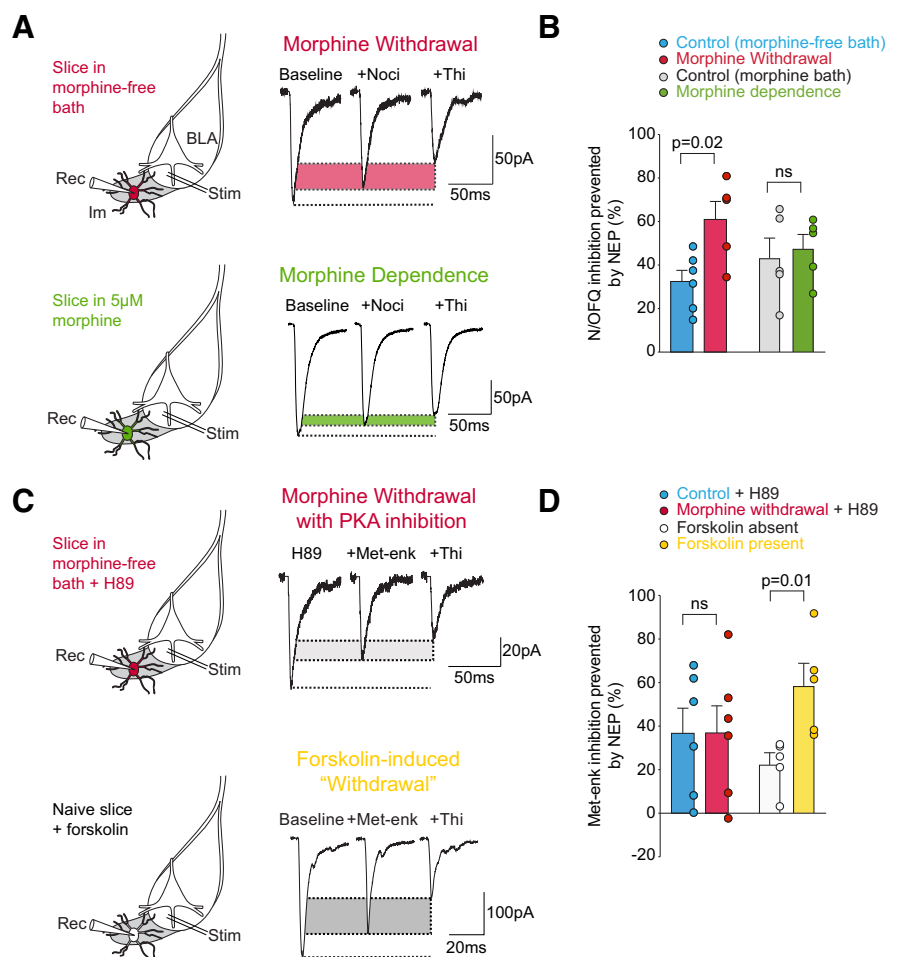


know its actions are limited by neprilysin (Gregoriou et al., 2020) and using opioids was inappropriate as Im terminals are not inhibited by DOR agonism (Winters et al., 2017). Also, MOR activity is likely disrupted at Im-BLA synapses during opioid withdrawal, as we saw at the BLA-Im synapse (Fig. 1). In both morphine-withdrawn and control neurons, submaximal nociceptin (100 nM) inhibited eIPSC amplitude at the Im-BLA (NAC-projector) synapse and this inhibition was potentiated by thiorphan (10  $\mu$ M; Table 2). However, a larger proportion of the nociceptin response was prevented by neprilysin in spontaneously withdrawn cells than control (Fig. 4H). This indicates that, as we saw at BLA-Im synapses, neprilysin degradation of peptides is enhanced in opioid withdrawn slices at Im-BLA synapses. This disruption of peptide signaling in the BLA, and thus, disruption of GABAergic inhibition of NAC-projecting BLA principal neurons, may disrupt the reward learning processes and resulting behaviors that are mediated by this pathway.

To determine whether the withdrawal-induced neprilysin effect indeed disrupted GABAergic inputs onto these cells, we examined the ratio of inhibitory-to-excitatory inputs onto BLA projection neurons in cells pre-CTAP and post-CTAP-precipitated withdrawal. To measure inhibition and excitation in the same cell, we placed stimulating electrodes in the Im and external capsule and measured the resulting eIPSCs and eEPSCs, respectively. By using a K-gluconate based internal, we could isolate eIPSCs versus eEPSCs without synaptic transmission blockers present by clamping BLA neurons close to their excitatory (10 mV) and inhibitory (−70 mV) reversal potentials. We then measured the I/E ratio (eIPSC amplitude/eEPSC amplitude) of inputs onto BLA neurons pre-CTAP and post-CTAP-precipitated withdrawal in slices from morphine-dependent and control animals. Superfusion of CTAP (1  $\mu$ M) potentiated eIPSCs, but not eEPSCs, in neurons from dependent animals compared with controls (Fig. 4J,K), suggesting that the balance of synaptic transmission onto BLA principal neurons is shifted toward inhibition during opioid withdrawal.

## Discussion

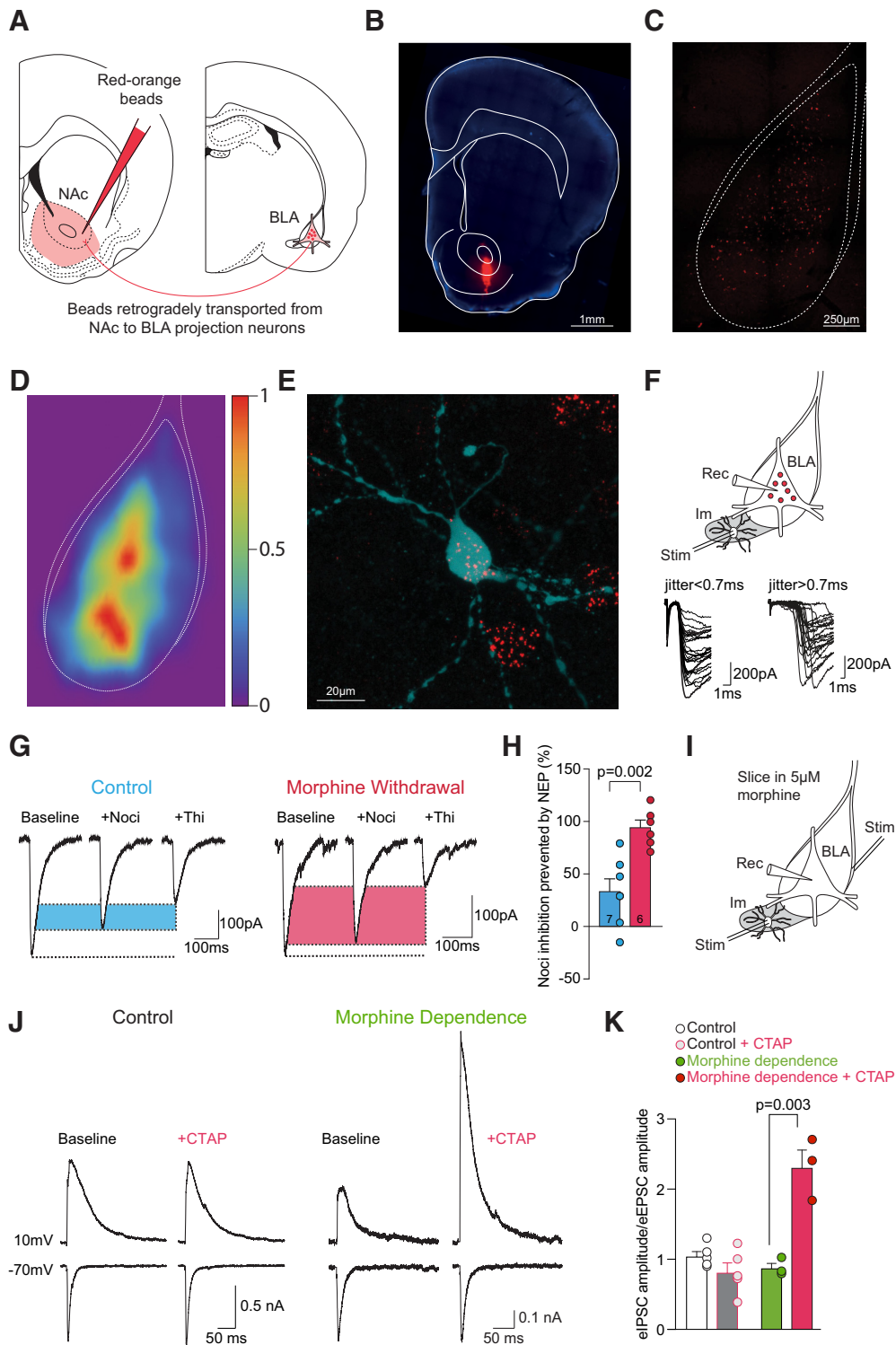
Here, we reveal a loss of control of synaptic modulation by endogenous peptides because of abnormally high peptidase activity in the Im during withdrawal from chronic morphine treatment. As a consequence, inhibition of glutamate release from BLA terminals in the Im is reduced and GABA release from Im terminals onto BLA principal neurons is enhanced during opioid withdrawal (Fig. 5). These data establish that there is a functional increase in neprilysin activity in the amygdala as an adaptive



**Figure 3.** Adenyl cyclase/PKA signaling is necessary and sufficient for neprilysin upregulation to occur. **A, B**, Morphine withdrawal, not dependence, enhances neprilysin control of nociceptin inhibition. **A**, Example eEPSCs from single representative experiments. **B**, Bar chart showing the proportion of nociceptin inhibition prevented by neprilysin activity. Neprilysin activity was significantly increased during opioid withdrawal, but not during morphine dependence (morphine-free bath, control vs withdrawal,  $t_{(9)} = 2.96$ ,  $p = 0.02$ ; morphine-bath, control vs dependence,  $t_{(8)} = 0.39$ ,  $p = 0.71$ ). **C, D**, Increased neprilysin activity during morphine withdrawal was prevented by inhibiting adenyl cyclase/PKA signaling with 10  $\mu$ M H89 and was produced in naive slices by activating adenyl cyclase with 10  $\mu$ M forskolin. **C**, Example eEPSCs from single representative experiments. **D**, Bar chart showing that the proportion of met-enk inhibition prevented by neprilysin activity was no different between treatment groups with H89 present (control vs withdrawal,  $t_{(10)} = 0.01$ ,  $p = 0.99$ ), and also that neprilysin activity was increased when forskolin was present compared with absent (forskolin present vs forskolin absent,  $t_{(8)} = 3.15$ ,  $p = 0.01$ ). Bar charts are mean  $\pm$  SEM. Circles represent individual neurons, with neuron number shown in bars. Data were analyzed using two-way ANOVAs with Bonferroni's multiple comparisons and unpaired Student's  $t$  tests. Comparisons on graphs show results from  $t$  tests.

response to chronic drug administration, and further, that increases in neprilysin activity rely on withdrawal-driven increases in PKA activity. The upregulated neprilysin activity altered peptide regulation of synapses at multiple sites within the amygdala and for multiple peptides.

The capacity for breakdown of opioid peptides is increased in multiple brain regions during opioid withdrawal (Malfroy et al., 1978; Zhou et al., 2001). Here, we used a functional measure of peptidase activity to demonstrate that peptide breakdown is also disrupted in the amygdala and crucially, we showed that this has functional consequences for amygdala neural circuits. To allow us to distinguish withdrawal-induced changes in peptidase activity from changes in receptor function and/or peptide release we used two approaches. First, we studied peptide activation of DOR or nociceptin receptors, whose activity was not altered by chronic treatment or opioid withdrawal. Second, we studied



**Figure 4.** Withdrawal increases inhibitory synaptic transmission from Im ITCs to NAC-projecting BLA principal neurons during withdrawal. **A**, Approach for recording NAC-projecting BLA principal neurons. Schematic shows injection site for fluorescent beads in the NAc and retrogradely transported beads in NAC-projecting BLA principal neurons. **B**, Representative fluorescence image showing a fluorescent bead injection site in the NAc (red) with DAPI labeling (blue). All other injection sites are shown in Extended Data Figure 4-1. **C**, Representative fluorescence image of the BLA showing retrogradely transported beads (red) visible in NAC-projecting BLA principal neurons. **D**, Heatmap of bead density in intermediate BLA slices. Bregma  $-1.80$ ,  $n = 6$  slices. In each slice, the position of every NAC-projecting principal neuron was quantified by their  $x$  and  $y$  coordinates and then normalized to the top of the BLA. The normalized bead plots were superimposed to generate a heatmap of bead density, with density represented by color, from purple (low density) to red (high density). **E**, Biocytin labeled BLA pyramid (blue) positive for retrogradely transported beads (red). **F**, Typical stimulating (stim) site in the Im and recording (rec) site in the BLA. Example traces from BLA neurons demonstrating a monosynaptic connection with jitter  $< 0.7$  ms and polysynaptic connection with jitter  $> 0.7$  ms. **G**, **H**, Morphine withdrawal enhances neprilysin control of nociception inhibition of the Im-BLA(NAC-projecting) principal neuron synapse. **G**, Example eEPSCs from single representative experiments. **H**, Bar chart showing the proportion of nociception inhibition prevented by neprilysin activity. Neprilysin activity was increased by opioid withdrawal (control vs withdrawal,  $t_{(11)} = 4.10$ ,  $p = 0.002$ ). **I**, Typical stimulating site in the external capsule and recording site in the BLA. **J**, **K**, Morphine withdrawal enhances GABA inputs onto BLA principal neurons. **J**, Example eIPSCs and eEPSCs recorded at 10 and  $-70$  mV, respectively, from single representative experiments. **K**, Bar chart showing the inhibition/excitation ratio (I/E ratio; eIPSC amplitude/eEPSC amplitude). The I/E ratio was significantly increased during CTAP-precipitated withdrawal in dependent, but not control, slices (control,

**Table 2. Percent inhibition of baseline synaptic transmission at the Im-BLA (NAc-projecting) synapse by peptides, alone and in the presence of peptidase inhibitors**

Group	Nociceptin	Nociceptin + thiorphan
Control (morphine-free bath)	19.61 ± 11.65 (7)	34.34 ± 18.21* (7)
Morphine withdrawal	2.89 ± 5.88 (6)	35.53 ± 12.81* (6)

Values are mean inhibition of eEPSC amplitude from baseline (%) ± SEM. Neuron number for each group is shown in brackets. Data were analyzed using two-way repeated measures ANOVAs with *post hoc* Bonferroni's multiple comparisons tests to assess the effects of peptidase inhibition on the ability of nociceptin to inhibit the Im-BLA(NAc-projecting) synapse in control and withdrawal. \*, significant results from *post hoc* Bonferroni's tests ( $p < 0.05$ ) comparing percent inhibition produced by nociceptin alone with percent inhibition produced by nociceptin + thiorphan for each group.

peptidase regulation of exogenous peptide which was not influenced by any changes to endogenous peptide release. This approach allowed us to firmly conclude that opioid withdrawal increased neprilysin activity and thus peptide breakdown, although there may also be other changes, such as reduced responses at the MOR and the possibility of changes in peptide release may occur.

While only male rats are included in the current study, similar changes are likely in females as withdrawal induced elevations of PKA activity, which is the key driver of the increased neprilysin activity during withdrawal, also occurs in females (Contet et al., 2008). Additionally, at least under control conditions, brain neprilysin activity does not differ between the sexes (Carter et al., 2006). Nevertheless, there is some evidence that females are more vulnerable to opioid withdrawal, craving and relapse than males (Yu et al., 2007; Bobzean et al., 2019; Bakhti-Suroosh et al., 2021). Therefore, future studies will determine whether similar, or possibly more pronounced, changes in neprilysin activity occur during opioid withdrawal in females.

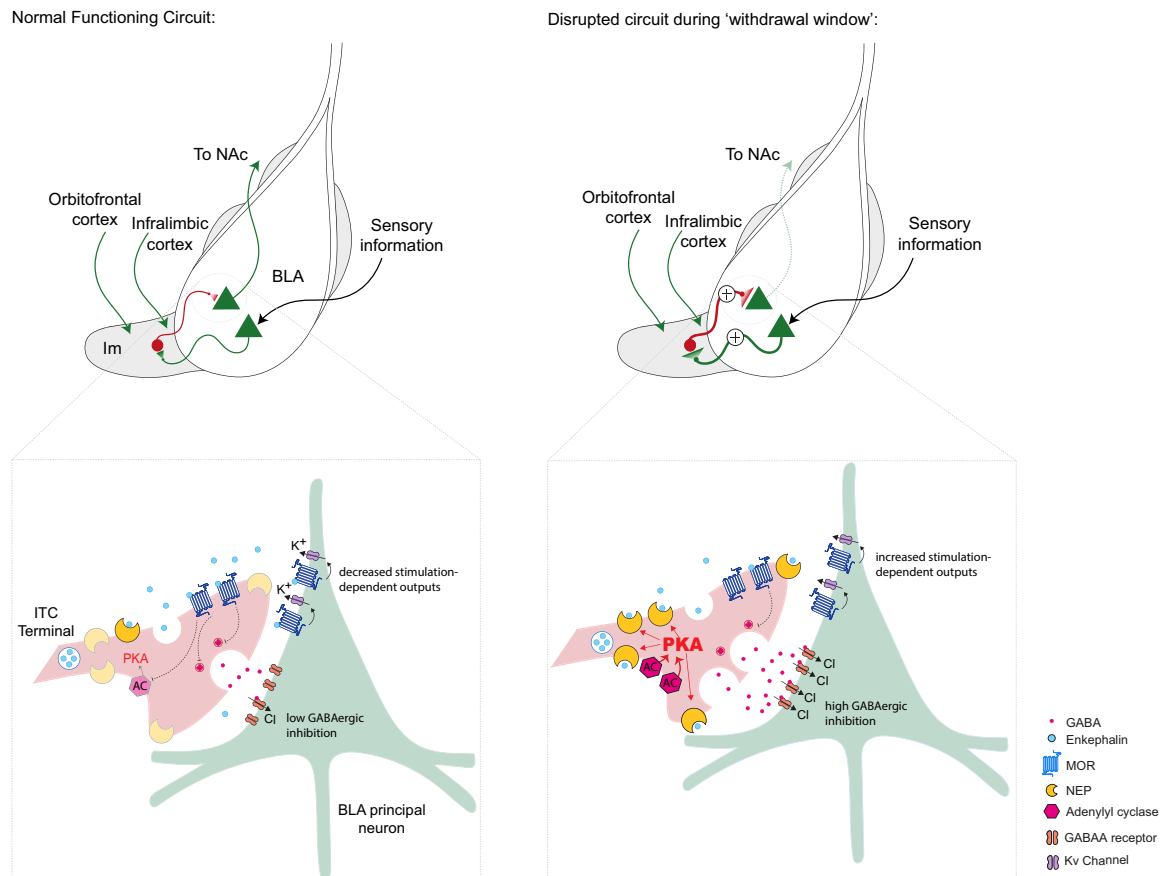
Withdrawal from chronic opioids increases adenylyl cyclase and resultant PKA activity in MOR-sensitive neurons in brain regions such as the periaqueductal gray, striatum, and ventral tegmental area (Bonci and Williams, 1997; Williams et al., 2001; Nestler, 2004). This elevation of adenylyl cyclase and PKA is responsible for a range of changes in neuronal activity, including intrinsic excitability, generation of extracellular adenosine and neurotransmitter release (Williams et al., 2001; Bagley et al., 2011). In these experiments, we have discovered that adenylyl cyclase signaling in the amygdala increases the activity of the peptidase neprilysin. The mechanism by which adenylyl cyclase/PKA activity alters neprilysin activity in neurons has not been defined, however, in endothelial cells and fibroblasts, activation of adenylyl cyclase increases both neprilysin activity and total neprilysin protein levels (Kondepudi and Johnson, 1993; Graf et al., 1995), effects that are mimicked by activation of cAMP and PKA (Graf et al., 1995). As we found that opioid withdrawal and activation of adenylyl cyclase with forskolin rapidly increases neprilysin activity, this suggests that PKA phosphorylation of neprilysin, or another regulatory protein, increases the activity of existing neprilysin rather than relying on expression of neprilysin molecules, which takes at least 90 min (Stewart and Kenny, 1984; Lemay et al., 1990). As a Type II membrane protein, neprilysin is comprised of a large extracellular C-terminal

domain that contains its catalytic center, a single transmembrane domain, and a small N-terminal cytoplasmic tail, where several amino acid residues that could possibly be phosphorylated reside. Indeed, several serine/threonine-specific protein kinases, including casein kinase II (Siepmann et al., 2010) and MAPK/ERK (Kakiya et al., 2012), transiently interact with the Ser-6 residue on the N-terminal of neprilysin to mediate its phosphorylation, thus, it is plausible that adenylyl cyclase/PKA acts in an analogous manner to directly regulate neprilysin activity. Although, additional boosts to neprilysin activity during later phases of withdrawal via increased expression cannot be discounted. The reliance of neprilysin activity on adenylyl cyclase/PKA has several important implications. First, that neprilysin activity could be increased in MOR sensitive neurons in other brain regions where adenylyl cyclase/PKA activity is increased during withdrawal (Nestler, 2004). Consistent with this, elevations of adenylyl cyclase/PKA activity occur in the periaqueductal gray, striatum and ventral tegmental area (Zhou et al., 2001), where there are also elevations in biochemical measures of peptidase activity (Malfroy et al., 1978). The consequence of elevating neprilysin activity will depend on enkephalin function in each brain region. For example, in the striatum it could alter reward learning and behaviors, including state dependent reward consumption, which has recently been shown to rely on enkephalins (Castro et al., 2021). Contrastingly, in the periaqueductal gray, inhibition of neprilysin significantly attenuates the naloxone-precipitated withdrawal syndrome in rats (Haffmans and Dzoljic, 1987; Maldonado et al., 1992), suggesting that peptidase inhibitors are able to mitigate the reduced peptide function and rescue the impaired peptide signaling that contributes to the withdrawal syndrome. Second, the PKA dependence suggests that the changes in neprilysin activity and consequent limitation of peptide levels and actions could disrupt synaptic transmission for at least as long as the adenylyl cyclase/PKA activity is elevated. Whether this elevation is transient or long-term, any learning and underlying synaptic plasticity that relies on preserved functioning of amygdala circuits would be disrupted during this time, which could have long-term behavioral consequences. Finally, that it is possible that other interventions or behavioral states that increase adenylyl cyclase/PKA signaling, such as such as fear learning in the BLA (Goosens et al., 2000; Schafe and LeDoux, 2000; Weeber et al., 2000), or chronic alcohol or cocaine (Nestler, 2004), could enhance neprilysin activity and thus disrupt peptide signaling.

The dialling down of peptide levels during opioid withdrawal will limit the extent and also the temporal and spatial characteristics of peptide regulation at multiple targets. These targets include neurotransmitter release (Winters et al., 2017; Gregoriou et al., 2020) and potassium channel activation (Faber and Sah, 2004; Winters et al., 2017; Fig. 5). Of particular interest is our finding that peptide control of GABAergic inhibition onto BLA neurons projecting to the NAc is reduced. This is likely to impact behavioral responses that require activity of BLA-NAc pathway, such as cue-guided and reward seeking behaviors (Everitt et al., 1999; Ambroggi et al., 2008; Stuber et al., 2011). Therefore, when Im neurons are stimulated to release GABA by their excitatory inputs from the BLA (Winters et al., 2017), infralimbic cortex (Amir et al., 2011), and orbitofrontal cortex (Ghashghaei and Barbas, 2002), there will be greater GABAergic inhibition of BLA-NAc pathway during withdrawal (Fig. 5) which could disrupt BLA principal neurons encoding, updating and/or

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pre-CTAP vs post-CTAP,  $F_{(1,6)} = 14.09$ ,  $p = 0.58$ ; dependent, pre-CTAP vs post-CTAP,  $F_{(1,6)} = 14.09$ ,  $p = 0.003$ ). Bar charts are mean ± SEM. Circles represent individual neurons, with neuron number shown in bars. Data were analyzed using two-way ANOVAs with Bonferroni's multiple comparisons and unpaired Student's *t* tests. Comparisons on graphs show results from *t* tests in **H** and *post hoc* Bonferroni's comparisons in **K**.



**Figure 5.** Proposed opioid withdrawal-induced changes to synaptic transmission within the amygdala. Schematic shows withdrawal-induced changes at the circuit level. Under normal conditions, the activation of BLA principal neurons by sensory and also direct cortical inputs generates Im neuronal activity, which drives feedback inhibition onto Im targets, including NAc-projecting BLA reward neurons. During the withdrawal window, reduced opioid inhibition of BLA principal neuron inputs to the Im will increase Im activation. This increased activation and reduced opioid inhibition of GABA release from Im neurons will result in greater feedback inhibition of BLA principal neurons and thus will likely reduce amygdala outputs (i.e., inhibition of BLA outputs to the NAc) and any associated behaviors. Magnification of an Im ITC-BLA(NAc-projecting) principal neuron synapse shows synaptic level changes. Under normal physiological conditions, appropriate stimulation will induce the release of endogenous enkephalins from ITC terminals at concentrations sufficient to overcome peptidase degradation and signal at their presynaptic receptors, inhibiting GABA release from Im terminals, and at their postsynaptic receptors, reducing input-driven activation of BLA principal neurons. During chronic treatment with opioids there is upregulation of adenylyl cyclase/PKA signaling. Withdrawal removes opioid inhibition of the adenylyl cyclase/PKA system and the resulting superactivation rapidly boosts neprilysin peptidase activity. The increased neprilysin activity reduces synaptic concentrations of endogenous enkephalins and impairs the ability of these peptides to signal at their receptors. This increases GABA release from Im terminals and reduces opioid activation of Kv channels on BLA principal neurons.

retrieving reward values (Wassum and Izquierdo, 2015; Malvaez et al., 2019). This is likely because these processes rely on the ability of BLA principal neurons to form stimulus-outcome associations, which are thought to be mediated, in part, by synaptic plasticity at cortical and thalamic inputs to these cells (Maren, 2005; Bocchio et al., 2017). This process is altered by other neuromodulators that change GABAergic inhibition (Huang and Kandel, 1998; Clem and Haganir, 2010), such as dopamine (Bissière et al., 2003). Therefore, the disruption of peptide control of the Im during withdrawal may contribute to long-term impairments in the ability to correctly evaluate and respond to rewards both directly, by reducing the actions of BLA endogenous opioids, on which these processes rely (Wassum et al., 2016), and indirectly, by altering plasticity via changed GABAergic inhibition. The disruption of peptide function could extend to other peptides that neprilysin degrades, such as dynorphin (Hiranuma et al., 1998), corticotropin releasing factor (Ritchie et al., 2003) and substance P (Matsas et al., 1984), which each play critical and specific roles in opioid addiction (Heinrichs et al., 1995; Gadd et al., 2003; Koob, 2015).

The reduction in peptide function that we observed and thus disruption of amygdala neural pathways could be mitigated using two approaches that we have previously shown enhance endogenous opioid function in the amygdala (Winters et al., 2017). First, inhibition of neprilysin would rescue levels of all neprilysin-sensitive peptides, including enkephalins (Matsas et al., 1984) and nociception (Sakurada et al., 2002), and to a lesser degree other peptides associated with drug use, such as dynorphin (Matsas et al., 1984). This approach is successful in reducing the physical signs of withdrawal when neprilysin inhibitors are injected into the periaqueductal gray (Haffmans and Dzoljic, 1987; Maldonado et al., 1992). The second approach would more selectively mitigate the lower levels of peptides during withdrawal by enhancing receptor responsiveness to their cognate peptides using positive allosteric modulators (PAMs), such as MOR and DOR PAMs (Winters et al., 2017). Both of these approaches may act to close the aberrant learning window that opioid withdrawal opens in the amygdala and should be pursued as viable targets for the development of novel pharmacotherapies for relapse during withdrawal and beyond.

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