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Cocaine withdrawal reduces $GABA_BR$ transmission at entopeduncular nucleus – lateral habenula synapses

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Abstract

Lateral habenula (LHb) hyperactivity plays a pivotal role in the emergence of negative emotional states, including those occurring during withdrawal from addictive drugs. We have previously implicated cocaine-driven adaptations at synapses from the entopeduncular nucleus (EPN) to the LHb in this process. Specifically, ionotropic $GABA_A$ receptor (R)-mediated neurotransmission at EPN-to-LHb synapses is reduced during cocaine withdrawal, due to impaired vesicle filling. Recent studies have shown that metabotropic $GABA_BR$ signaling also controls LHb activity, although its role at EPN-to-LHb synapses during drug withdrawal is unknown. Here, we predicted that cocaine treatment would reduce $GABA_BR$-mediated neurotransmission at EPN-to-LHb synapses. We chronically treated mice with saline or cocaine, prepared brain slices after two days of withdrawal and performed voltage-clamp recordings from LHb neurons whilst optogenetically stimulating EPN terminals. Compared with controls, mice in cocaine withdrawal exhibited reduced $GABA_AR$-mediated input to LHb neurons, and a reduced occurrence of $GABA_BR$-signaling at EPN-to-LHb synapses. We then assessed the underlying mechanism of this decrease. Application of $GABA_BR$ agonist baclofen evoked similar postsynaptic responses in EPN-innervated LHb neurons in saline- and cocaine-treated mice. Release probability at EPN-to-LHb GABAergic synapses was also comparable between groups. However, incubating brain slices in glutamine to facilitate GABA vesicle filling, normalized $GABA_BR$-currents at EPN-to-LHb synapses in cocaine-treated mice. Overall, we show that during cocaine withdrawal, together with reduced $GABA_AR$ transmission, also $GABA_BR$-mediated inhibitory signaling is diminished at EPN-to-LHb synapses, likely via the same presynaptic deficit. In concert, these alterations are predicted to contribute to the emergence of drug withdrawal symptoms, facilitating drug relapse.

Introduction

Drug addiction is a psychiatric disorder defined as compulsive substance use despite negative consequences (Everitt, 2014). Cessation of continuous drug use typically triggers a negative withdrawal syndrome, which can in turn reinitiate drug intake to help alleviate this aversive state (Koob & Le Moal, 2001; Sinha, 2008). This is also the case for the prototypical psychostimulant cocaine for which the withdrawal state is associated with depressive-like negative mood and anxiety, symptoms which emerge within hours after the last dose and which can persist for weeks (Barr *et al.*, 2002; Kosten & O'Connor, 2003). The manifestation of this cocaine withdrawal syndrome requires complex neural circuit adaptations, and unraveling their precise identity remains a pressing issue (Koob & Le Moal, 2001; Volkow & Morales, 2015; Koob & Volkow, 2016).

Recent findings have identified that the lateral habenula (LHb) plays a key role in encoding aversive experiences (Lecca *et al.*, 2014; Proulx *et al.*, 2014). Specifically, LHb hyperactivity contributes to the expression of depressive-like symptoms (Sartorius *et al.*, 2010; Li *et al.*, 2011, 2013; Proulx *et al.*, 2014; Lecca *et al.*, 2016; Tchenio *et al.*, 2017), including those occurring during cocaine withdrawal (Jhou *et al.*, 2013; Meye *et al.*, 2015, 2016; Neumann *et al.*, 2015). In particular, cocaine-driven hyperactivity occurs in neurons within the lateral segment of the LHb that project to GABAergic cells in the midbrain, which in turn inhibit mesolimbic dopamine neurons of the reward system (Ji & Shepard, 2007; Jhou *et al.*, 2009; Matsumoto & Hikosaka, 2009; Lecca *et al.*, 2011; Maroteaux & Mameli, 2012; Meye *et al.*, 2015, 2016; Gao *et al.*, 2017).

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This lateral segment of the LHb is prominently innervated by the entopeduncular nucleus (EPN), the rodent equivalent of the primate globus pallidus interna (Herkenham & Nauta, 1979; Hong & Hikosaka, 2008; Shabel *et al.*, 2014; Stephenson-Jones *et al.*, 2016). A significant population of EPN neurons that project to the LHb corelease GABA and glutamate (Shabel *et al.*, 2014; Meye *et al.*, 2016; Root *et al.*, 2018). Recent findings describe that reductions in vesicular GABA content occur at EPN-to-LHb synapses during cocaine withdrawal, thereby diminishing ionotropic $GABA_A$ receptor ($GABA_AR$)-mediated transmission (Meye *et al.*, 2016). However, GABA release from EPN terminals onto the LHb also activates metabotropic $GABA_B$ receptors ($GABA_BRs$), which have a pivotal role in controlling LHb neuronal activity (Lecca *et al.*, 2016; Tchenio *et al.*, 2017). Yet the effect of cocaine withdrawal on $GABA_BR$-mediated inhibition of LHb neurons, particularly at EPN-to-LHb synapses, remains unassessed. Here, we predicted that $GABA_BR$-dependent signaling would diminish at EPN-to-LHb synapses during cocaine withdrawal, due to a presynaptic deficit in GABA vesicle filling.

Materials and methods

Animals

Male experimentally naive mice (C57Bl6/J) of 4 weeks of age were obtained from Janvier Labs (France). These mice were group-housed (3–5 within same cage) and maintained on a 12 : 12 light cycle (lights on at 7:00 am). Mice were kept on *ad libitum* chow (Safe Diets, France #A03) and water at all times. Mice were used in accordance with the guidelines of the French Agriculture and Forestry Ministry for handling animals (Darwin5 Ethical Committee of the University Pierre et Marie Curie, Paris, France) and according to the regulations of the Cantonal Veterinary Offices of Vaud and Zurich (Switzerland; License VD3310). A total of 118 mice were used for this study.

Stereotactic injections

In the case of stereotactic injections, mice (4–7 weeks) were first anaesthetized with a mixture of ketamine (150 mg/kg) and xylazine (100 mg/kg) (Sigma-Aldrich/Merck). Subsequently, they were bilaterally injected in the EPN using a glass pipette in a stereotactic frame (Kopf) with rAAV2/1-CAG-hChR2(H134R)-mCherry with a titer of 1×10^{12} gc/mL (University of Pennsylvania viral vector core). For the experiments assessing the effects of glutamine on ionotropic $GABA_AR$-mediated transmission, we injected mice with rAAV2/1-hSyn-CoChR-eGFP with a titer of 3.8×10^{12} gc/mL (University of North Carolina viral vector core). Injections were placed at 1.25 mm posterior to bregma, 1.80 mm lateral to bregma, and −4.65 mm from the skull. The injection volume ranged between 200 and 400 nL per side, and was delivered at a rate of approximately 100 nL/min. After the total injection volume was delivered, the injection pipette remained in the brain for another 5 min before it was slowly retracted. Injected mice were allowed to recover for a minimum of 3 weeks before recordings were made.

Pharmacological treatment

Prior to recordings, mice were i.p. injected for five consecutive days with either saline or cocaine-hydrochloride (20 mg/kg; Sigma-Aldrich/Merck), after which they were placed in a separate cage for 30–40 min prior to being put back in the home cage. In case of a single injection per day, the injection was performed at 5 p.m. In case of two injections per day, the first injection was performed at 10 a.m. and the second injection at 5 p.m. Slice electrophysiology experiments after pharmacological treatment were always performed two days after the last saline/cocaine injection.

Electrophysiology

Slicing procedure

On the day of recordings animals were first anaesthetized (ketamine/xylazine; 150 mg/100 mg/kg) and then killed by decapitation, after which their brains were rapidly isolated in an ice-cold carbogenated (95% O_2/5% CO_2) solution containing (in mM): Choline Cl 110; glucose 25; $NaHCO_3$ 25; $MgCl_2$ 7; ascorbic acid 11.6; Na^+-pyruvate 3.1; KCl 2.5; NaH_2PO_4 1.25; $CaCl_2$ 0.5. Subsequently, coronal brain slices of 250 μm thickness were prepared and transferred to a warmed solution (34 °C) of identical composition for 10 min. Slices were then transferred to room temperature carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124; $NaHCO_3$ 26.2; glucose 11; KCl 2.5; $CaCl_2$ 2.5; $MgCl_2$ 1.3; NaH_2PO_4 1.

Patch-clamp recordings

After the minimal recovery period of 1 h after slice preparation, slices were used for patch-clamp recordings. A slice was transferred to the recording set-up, where it was immersed in and continuously superfused with ACSF, at a flow rate of 2.5 mL/min at 30 °C. Neurons were patch-clamped using borosilicate glass pipettes (3.0–4.5 MΩ; Phymep, France) under an Olympus-BX41 microscope (Olympus, France). For voltage/current clamp recordings, the signal was amplified, filtered at 5 kHz and digitized at 10 kHz (Multiclamp 200B; Molecular Devices, USA). Data were acquired using Igor Pro with NIDAQ tools (Wavemetrics, USA). Access resistance was continuously monitored with a −4 mV step delivered at 0.1 Hz. Experiments were discarded if the access resistance increased by more than 20% during the recording.

Recording miniature and spontaneous inhibitory postsynaptic currents

Spontaneous inhibitory currents (sIPSCs)

Voltage-clamp recordings from lateral LHb neurons (from the posterior and middle segment, rather than from the rostral pole) were made at −50 mV with an internal medium consisting of (in mM): KCl 100, K-gluconate 30, $MgCl_2$ 4, EGTA 0.2, HEPES 5, Na_2ATP 3.4, Na_3GTP 0.1, Na^+ creatine phosphate 10; pH 7.3, 300 mOsm. As a result of the high chloride concentration in this medium, $GABA_AR$ responses are inward at −50 mV and easier to detect. After patching, LHb neurons were allowed to recover for at least 10 min before another epoch of 10 min of sIPSCs was recorded. Recordings were done in the presence of the AMPA/kainate receptor antagonist NBQX (20 μM; Tocris) and the NMDA receptor antagonist D-APV (100 μM; Tocris) to pharmacologically isolate $GABA_AR$ responses. These responses were mediated by $GABA_ARs$ as they were blocked by the $GABA_AR$ antagonist (−)-Bicuculline-methiodide (20 μM; Tocris), as assessed in a subset of recordings.

Miniature inhibitory currents (mIPSCs)

mIPSCs were recorded in the presence of tetrodotoxin (TTX; 1 μM; Tocris) to abolish action potential-dependent transmission.

Furthermore, to allow for better detection of mIPSCs, we voltage clamped cells at −50 mV with a cesium-based internal solution, consisting of (in mM): CsCl 130, NaCl 4, MgCl2 2, EGTA 1.1, HEPES 5, Na2ATP 2, sodium creatinephosphate 5, Na3GTP 0.6, spermine 0.1; pH 7.3, 300 mOsm.

Recording EPN-to-LHb GABAergic responses

To record $GABA_AR$ transmission at EPN-to-LHb synapses, voltage-clamp recordings were made at –50 mV using an internal medium consisting of (in mM): K-Gluconate 140; KCl 5; HEPES 10; EGTA 0.2; MgCl2 2; Na2ATP 4; Na3GTP 0.3; creatine phosphate 10. Light pulses (470 nm, 1–10 ms) for opto-stimulation of EPN nerve terminals were delivered with a LED (CoolLed, UK) illumination system. To isolate $GABA_AR$ responses we added the AMPA/kainate receptor antagonist NBQX (20 μM; Tocris) and the NMDA receptor antagonist D-APV (100 μM; Tocris) during the recording. To evoke $GABA_BR$ responses we delivered a train of 10 pulses at 5, 10, or 20 Hz each sweep (0.1 Hz inter-sweep interval). We utilized the same stimulation frequency for at least 10 consecutive sweeps and then averaged these sweeps. In this average trace, slow outward IPSCs were quantified as the delta current between the 50 ms baseline prior to opto-stimulation and the current remaining between 40 and 50 ms after the tenth optogenetic pulse was delivered (when fast ionotropic $GABA_AR$-dependent responses had ended). A cell was classified as having a $GABA_BR$ outward IPSC in the event that there was at least a 5 pA delta current quantified in this way that could be blocked by the $GABA_BR$ antagonist CGP54626 (10 μM; Tocris UK). To detect the presence or absence of $GABA_BR$ currents specifically in LHb neurons innervated by EPN (i.e. EPN-responsive LHb neurons), we only used cells in which single pulse optogenetic stimulation of EPN nerve terminals gave rise to at least 50 pA of fast ionotropic synaptic current at −50 mV (AMPAR and/or $GABA_AR$-mediated). In the case of glutamine incubation experiments assessing effects on $GABA_BR$ transmission, we preincubated the slice in ACSF with L-Glutamine (2 mM; Sigma-Aldrich/Merck) for at least 20 min prior to the recording and then maintained this presence of glutamine throughout the recording.

Recording pharmacological $GABA_BR$ responses in LHb neurons

For experiments in which $GABA_BR$-responses were evoked pharmacologically we first ensured, by means of optogenetic stimulation of EPN terminals, that the recorded lateral LHb neuron was innervated by the EPN. Subsequently, in the absence of opto-stimulation, we recorded the holding current required to maintain the cell at −50 mV during a baseline period (5 min). Then we applied the $GABA_BR$ agonist (+)-Baclofen (100 μM; Sigma-Aldrich/Merck) for 5 min, and subsequently the $GABA_BR$ antagonist CGP54626 (10 μM). The pharmacological $GABA_BR$ response was quantified as the delta holding current between the baseline period and the peak current upon baclofen application.

Histology

In each animal, we evaluated the location of the stereotactic injection site in the EPN after slice preparation. To this end, brain slices containing the EPN from injected mice were directly examined under an epifluorescent microscope. Only animals in which fluorescent injection sites were confined to the EPN were included for the analysis.

Data analysis

Compiled data are expressed either in bar graphs as the mean ± SEM (with individual data points shown in the form of circles alongside the bars) or as pie charts. Data collection and analysis were not done blind to the conditions of the experiments, but were performed by multiple experimenters. Online/offline analyses were performed using IGOR-PRO 6.37 (Wavemetrics) and SPSS 17 (IBM Corp). When applicable (indicated in the results section for each test), statistical tests employed were Two-Way ANOVAS, or Repeated Measures ANOVAS. In case of nonparametric data, the Mann–Whitney *U* tests and Pearson's Chi-Square Test were used. Significance was always tested two-tailed, with α set at 0.05.

Results

Reduced $GABA_AR$-transmission in EPN-innervated LHb segment in cocaine withdrawal

First we evaluated the effect of cocaine withdrawal on fast ionotropic $GABA_AR$-mediated inhibitory input to those LHb neurons likely innervated by the EPN. To this end we first mapped the area of the LHb receiving EPN input. We injected an anterograde tracer (rAAV2/1-CAG-ChR2-mCherry) in the EPN of C57B6 mice and prepared brain slices after at least three weeks of viral incubation (Fig. 1A). In accordance with previous findings we observed that EPN projections specifically target the lateral part of the LHb (Fig. 1B) (Herkenham & Nauta, 1979; Hong & Hikosaka, 2008; Shabel *et al.*, 2014; Meye *et al.*, 2016; Stephenson-Jones *et al.*, 2016).

We took advantage of this topographical innervation pattern of the LHb by the EPN to determine if those lateral LHb neurons would exhibit reductions in $GABA_AR$ neurotransmission during cocaine withdrawal. We injected another batch of experimentally naïve C57B6/J mice with either saline or cocaine (20 mg/kg/day, i.p. injection) for five consecutive days, prepared brain slices two days afterward and obtained patch-clamp recordings from lateral LHb neurons. We recorded pharmacologically isolated $GABA_AR$-dependent spontaneous inhibitory postsynaptic currents (sIPSCs; Fig. 1C). Compared with saline-treated mice, there was a decrease in the frequency [Saline: $n = 24$ cells/5 mice, Cocaine: $n = 22$ cells/5 mice, Mann–Whitney *U* test; $U = 165.5$, $Z = -2.166$, $P = 0.03$] and the amplitude [Mann–Whitney *U* test; $U = 174.5$, $Z = -1.968$, $P = 0.049$] of $GABA_AR$-mediated sIPSCs in the cocaine-treated group (Fig. 1C and D), indicating a reduction in fast inhibitory transmission onto lateral LHb neurons during cocaine withdrawal. We next assessed whether action potential-independent miniature (m)IPSCs were also altered after cocaine treatment. We did not observe cocaine-driven alterations in mIPSC frequency [Saline: $n = 13$ cells/2 mice, Cocaine: $n = 13$ cells/2 mice, Mann–Whitney *U* test; $U = 62.00$, $Z = -1.154$, $P = 0.249$] or amplitude [Mann-Whitney *U* test; $U = 68.00$, $Z = -0.846$, $P = 0.397$] (Fig. 1E). Overall these findings suggest that $GABA_AR$-mediated neurotransmission in the lateral LHb (i.e., the EPN-innervated portion) diminishes during cocaine withdrawal, and that this may especially occur during conditions of action potential-dependent synaptic transmission.

Decrease in $GABA_BR$-signaling at EPN-to-LHb synapses during cocaine withdrawal

Aside from fast $GABA_AR$ neurotransmission, slower inhibition via $GABA_BR$-signaling is an important determinant of LHb activity

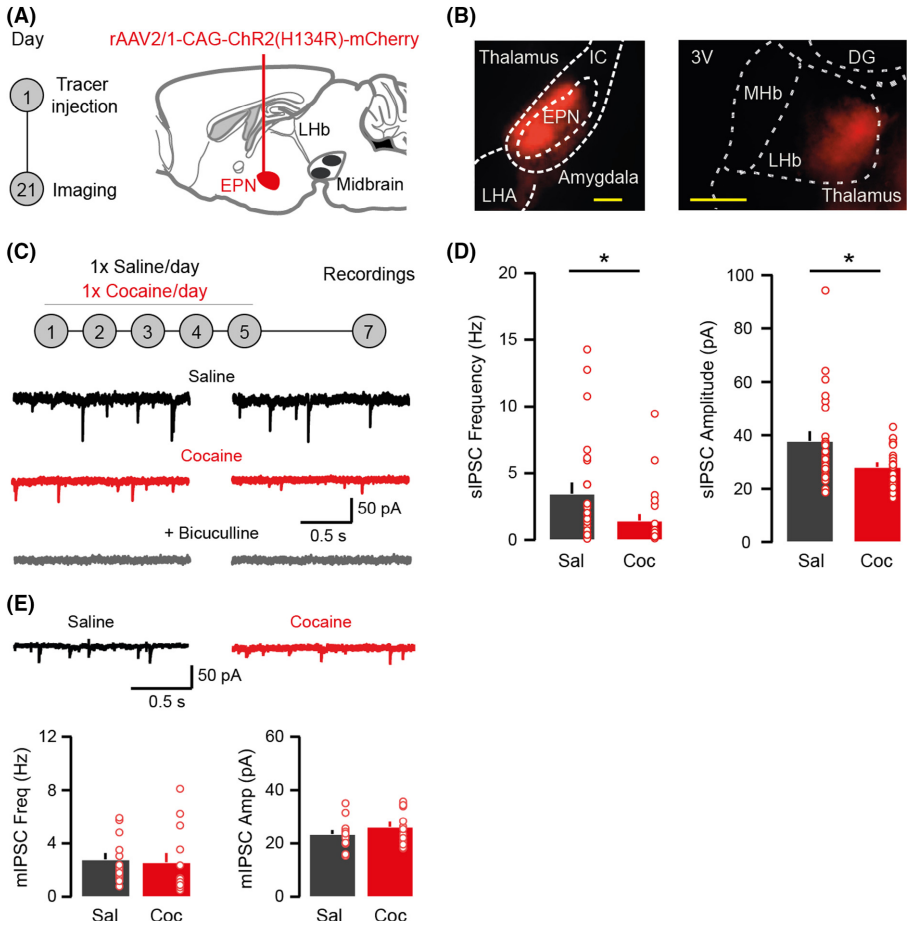

FIG. 1. Reduced spontaneous GABA$_A$R neurotransmission in the lateral LHb during cocaine withdrawal. (A) Experimental timeline for viral injections in the EPN and recordings in the LHb. (B) Representative injection sites for AAV-CAG-hChR2(H134R)-mCherry in the EPN and terminal expression in the LHb. Yellow horizontal scale bars represent 250 μm. (C) Timeline of saline and cocaine treatment (1 injection/day, 5 days) and representative traces of sIPSCs recorded in lateral LHb neurons from saline- and cocaine-treated mice. sIPSCs were blocked by GABA$_A$R antagonist bicuculline. (D) Bar graphs for the frequency (left) and amplitude (right) of recorded sIPSCs in the lateral LHb in saline- and cocaine-treated mice. (E) Representative traces and bar graphs for the frequency (left) and amplitude (right) of recorded mIPSCs in the lateral portion of the LHb in saline- and cocaine-treated mice. *$P < 0.05$.

levels (Lecca *et al.*, 2016) and such signaling can be evoked at EPN-to-LHb synapses (Tchenio *et al.*, 2017). However, it is currently unknown if GABA$_B$R signaling is diminished at EPN-to-LHb synapses during cocaine withdrawal and, if so, through which mechanism. To assess this we first determined whether we could functionally assess EPN-to-LHb synapse function. We injected channelrhodopsin-2 (ChR2) in the EPN of C57B6 mice and prepared brain slices after at least three weeks of viral incubation (Fig. 2A). We confirmed that EPN neurons responded to optogenetic stimulation by firing action potentials (Fig. 2B). When we recorded from lateral LHb neurons in the presence of a pharmacological blocker for AMPA receptors, single-pulse opto-stimulation of EPN nerve terminals resulted in inhibitory postsynaptic currents (oIPSCs) at EPN-to-LHb synapses, dependent on GABA$_A$Rs (Fig. 2B). These oIPSCs were indeed functionally inhibitory as they were capable of interrupting LHb neuron firing (Fig. 2C).

Whereas GABA$_A$R-mediated postsynaptic currents arise after single pulses of stimulation, GABA$_B$R activation typically requires synchronized trains of stimulation at intermediate-to-high frequencies (Ulrich & Bettler, 2007; Padgett *et al.*, 2012; Tchenio *et al.*, 2017). Therefore, we investigated whether trains of 10 pulses delivered at 20 Hz would reveal GABA$_B$R-mediated currents at EPN-to-LHb synapses in mice that had not received pharmacological treatment. Across 11 mice, in 18 of 38 EPN-innervated LHb neurons such stimulation trains indeed yielded slow outward IPSCs (Fig. 2D and E). These slow currents exhibited a frequency-dependent increase in maximal amplitude when we compared 10-pulse trains delivered at 5, 10, and 20 Hz [Repeated Measures ANOVA: $n = 17$ cells/11 mice, $F_{2,32} = 19.641$, $P < 0.0001$] (Fig. 2D and F) and were largely blocked by GABA$_B$R antagonist CGP54626 (10 μM) [Repeated Measures ANOVA: $n = 19$ cells/11 mice, $F_{1,18} = 26.604$, $P < 0.0001$] (Fig. 2D and G).

We next assessed whether cocaine-treatment would affect GABA$_B$R-dependent signaling at EPN-to-LHb synapses. We again treated mice with five injections of either saline or cocaine and prepared brain slices 2 days afterward. We patch-clamped lateral LHb neurons and first confirmed that they responded to EPN nerve terminal opto-stimulation with ionotropic (AMPAR and/or GABAaR responses > 50 pA). In such EPN-innervated LHb neurons from saline-treated mice, 20 Hz opto-stimulation resulted in GABA$_B$R-mediated slow outward currents in 18 of 33 cells from 10 mice (Fig. 2H), a similar occurrence compared with naïve controls [Chi-Square Test; $\chi(1) = 0.364$, $P = 0.55$]. Instead, in cocaine-treated mice 20 Hz opto-stimulation only resulted in GABA$_B$R-mediated slow outward currents in 10 of 37 cells from 12 mice (Fig. 2H), a reduced occurrence compared with saline-treatment [Chi-

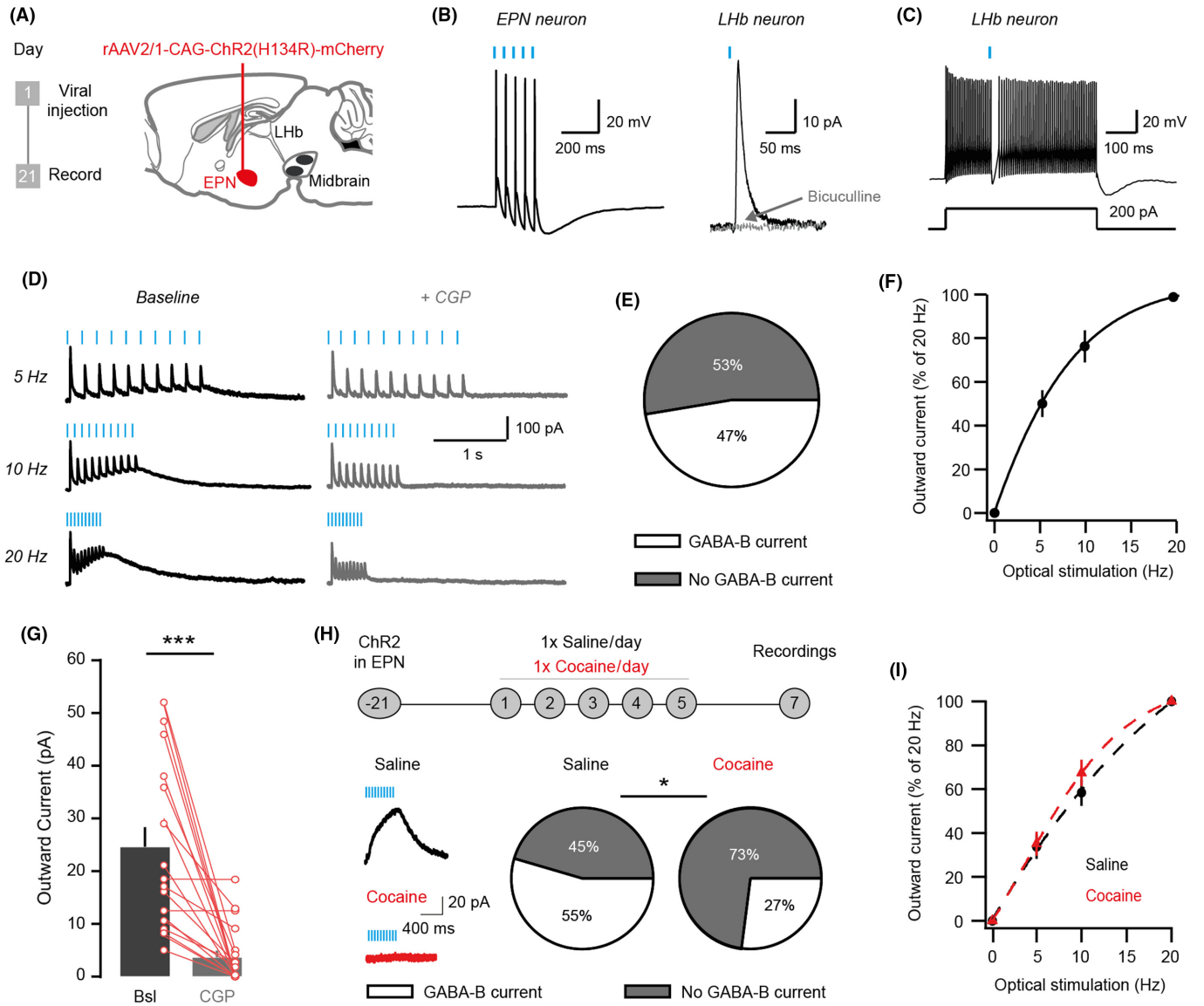

FIG. 2. Reduced $GABA_BR$ signaling at EPN-to-LHb synapses during cocaine withdrawal. (A) Experimental timeline for viral injections of channelrhodopsin-2 (ChR2) in the EPN and recordings in the LHb. (B) Example traces of (left) an EPN neuron optogenetically stimulated to fire action potentials; (right) an LHb neuron, in AMPA receptor-transmission blocked, responding with an oIPSC to EPN terminal stimulation, which is blocked by $GABA_AR$ antagonist bicuculline. Blue rectangles indicate onset of optogenetic stimulation. (C) Example traces of how a single oIPSC evoked in an EPN-responsive LHb neuron causes a direct interruption of LHb neuron firing of action potentials in response to a 200 pA current injection. (D) Representative traces of 10 pulse trains of stimulation driving optogenetically evoked slow outward oIPSCs at EPN-to-LHb synapses, which were blocked by the $GABA_BR$ antagonist CGP54626. (E) Pie chart indicating the probability of $GABA_BR$-signaling at EPN-responsive LHb neurons of drug-naïve mice (F) Frequency-dependent magnitude of synaptically evoked $GABA_BR$-dependent EPN-to-LHb signaling. (G) Bar graph with quantification of the sensitivity of the slow outward IPSC to $GABA_BR$ antagonist CGP54626. (H) Timeline of saline and cocaine treatment (1 injection/day, 5 days) and pie charts indicating the occurrence of LHb neurons exhibiting $GABA_BR$-dependent responses to EPN opto-stimulation. Example traces indicate the amount of CGP54626-dependent (i.e. $GABA_BR$-mediated) current upon trains of 10 pulses at 20 Hz in saline (black) and cocaine (red) conditions. (I) Frequency-dependent magnitude of synaptically evoked $GABA_BR$-dependent EPN-to-LHb signaling in saline and cocaine conditions. $^*P < 0.05$, $^{***}P < 0.001$.

Square Test; $\chi(1) = 5.50$, $P = 0.019$]. These effects were not due to differences in viral infection efficiency between the saline and cocaine groups, as the absolute magnitude of optogenetically evoked fast inward currents in these cells was comparable [Saline: $n = 32$ cells/10 mice, Cocaine: $n = 37$ cells/12 mice, Mann–Whitney U test; $U = 527.5$, $Z = -0.776$, $P = 0.438$, data not shown]. As opposed to the difference in the occurrence of evoked $GABA_BR$-currents at EPN-to-LHb synapses after cocaine or saline treatment, the frequency-dependent activation kinetics of such $GABA_BR$-signaling remained unaltered after cocaine treatment (Fig. 2I) [Repeated Measures ANOVA: Frequency × Treatment interaction: Saline $n = 17$ cells/10 mice, Cocaine $n = 10$ cells/12 mice, $F_{2,50} = 0.7$, $P = 0.50$].

Underlying mechanism of reduced EPN-to-LHb $GABA_BR$-signaling during cocaine withdrawal

The behavioral effects of drugs of abuse are dependent on the number of exposures (Steketee & Kalivas, 2011). We therefore asked whether the occurrence of $GABA_BR$ currents at EPN-to-LHb

synapses would be differentially altered by a more extensive cocaine exposure protocol, involving a total of 10 cocaine injections (2x/day, 5 days). Again, 2 days after this treatment we prepared brain slices and patch-clamped lateral LHb neurons while opto-stimulating EPN-terminals. We observed that also this protocol caused a reduction in the occurrence of GABA$_B$R currents at EPN-to-LHb synapses in cocaine-treated animals compared with the saline control group [Chi-Square Test; $\chi(1) = 4.187$, $P = 0.041$]. Indeed in 2x/day saline-treated mice, 20 Hz opto-stimulation of EPN nerve terminals resulted in GABA$_B$R-mediated slow outward currents in 10 out of 23 EPN-responsive cells from 9 mice. In 2x/day cocaine-treated mice, 20 Hz opto-stimulation resulted in such GABA$_B$R currents in 8 out of 41 EPN-responsive cells from 13 mice (Fig. 3A). The occurrence of GABA$_B$R currents was not significantly further reduced in the 2x/day cocaine protocol compared with the 1x/day cocaine protocol [Chi-Square Test; $\chi(1) = 0.619$, $P = 0.432$].

Using the 2x/day cocaine protocol we further investigated the underlying mechanism for the diminished GABA$_B$R signaling at EPN-to-LHb synapses during cocaine withdrawal. We tested three different scenarios. First we determined whether postsynaptic GABA$_B$R-function of LHb neurons was diminished by cocaine treatment, since recent studies have demonstrated that aversive stimuli (other than drug withdrawal) cause postsynaptic internalization of GABA$_B$Rs in the LHb (Lecca *et al.*, 2016; Tchenio *et al.*, 2017). We recorded from lateral LHb neurons that were innervated by the EPN, as confirmed by optogenetic stimulation. By bath-applying the GABA$_B$R agonist baclofen we determined the maximal GABA$_B$R current that could be postsynaptically evoked in these EPN-innervated LHb neurons. All cells recorded in these experimental conditions responded to baclofen with outward currents, and the size of these baclofen-induced responses was comparable between mice treated with saline or cocaine [Saline: $n = 10$ cells/6 mice, Cocaine: $n = 10$ cells/5 mice, Mann–Whitney U test; $U = 39.00$, $Z = -0.832$, $P = 0.41$] (Fig. 3B). Overall this suggests that diminished EPN-to-LHb GABA$_B$R signaling after cocaine treatment likely did not occur due to reduced number or function of GABA$_B$Rs on the postsynaptic membranes of lateral LHb neurons.

Next we determined if altered presynaptic release probability for GABA vesicles at EPN-to-LHb synapses could account for diminished GABA$_B$R signaling after cocaine treatment. To address this we calculated the paired-pulse ratio (PPR) for GABA$_A$R-mediated transmission at EPN-to-LHb synapses upon trains of 10 pulses delivered at 20 Hz in mice treated with saline or cocaine. Neither with the two injections/day, nor with the one injection/day protocol was there a difference between the saline and cocaine conditions [Two-Way ANOVA: Saline 1x/day: $n = 15$ cells/8 mice, Cocaine 1x: $n = 8$ cells/7 mice, Saline 2x/day: $n = 11$ cells/10 mice, Cocaine 2x: $n = 8$ cells/6 mice, Pharmacological Treatment Main effect: $F_{1,39} = 0.03$, $P = 0.864$, Pharmacological Treatment × Injections per day Interaction: $F_{1,39} = 0.332$, $P = 0.568$] (Fig. 3C). This suggests, in accordance with our previous findings (Meye *et al.*, 2016), that there is no reduced release probability of GABA vesicles at EPN-to-LHb synapses after cocaine treatment that could explain the diminished GABA$_B$R signaling at these synapses.

Our previous study suggested that a reduction in the GABA content in vesicles at EPN-to-LHb synapses caused diminished fast ionotropic GABA$_A$R neurotransmission at EPN-to-LHb synapses (Meye *et al.*, 2016). Here, we assessed if this also contributed to the reduced GABA$_B$R signaling at these synapses. To this end we aimed to enhance vesicular GABA content at EPN-to-LHb synapses by applying L-glutamine to the bath, a precursor to GABA previously described to induce a facilitation of GABA synthesis and vesicular packaging (Battaglioli & Martin, 1991; Wang *et al.*, 2013). We opto-stimulated EPN-to-LHb synapses and recorded GABA$_A$R transmission. Administration of L-glutamine (2 mM) to the bath caused an increase in the amplitude of oIPSCs compared with baseline [Repeated Measures ANOVA; Main effect Glutamine: Saline: $n = 9$ cells/3 mice, Cocaine: $n = 9$ cells/4 mice, $F_{1,16} = 7.955$, $P = 0.012$], which did not differ significantly between the groups [Repeated Measures ANOVA; Glutamine × Group interaction, $F_{1,16} = 0.247$, $P = 0.626$] (Fig. 3D). This effect on amplitude was in the absence of changes in the PPR, suggesting that this manipulation enhanced GABA release without affecting the release probability [Repeated Measures ANOVA; Main effect Glutamine: Saline: $n = 9$ cells/3 mice, Cocaine: $n = 9$ cells/4 mice, $F_{1,16} = 0.699$, $P = 0.416$, data not shown]. This suggests that L-glutamine application is an effective strategy to enhance vesicular GABA content at EPN-to-LHb synapses.

To determine whether facilitation of GABA release could rescue EPN-to-LHb GABA$_B$R signaling, we incubated slices from saline- and cocaine-treated mice in 2 mM L-glutamine prior to recording from LHb neurons whilst opto-stimulating EPN nerve terminals (20 Hz, 10 pulses/train). Under these conditions, EPN-innervated LHb neurons from saline-treated mice showed GABA$_B$R currents in 4/8 neurons from three mice, whereas in cocaine-treated mice GABA$_B$R currents were observed in 6/10 neurons from four mice (Fig. 3E). The rate of occurrence of GABA$_B$R currents in saline- and cocaine-treated mice when recording in glutamine was not significantly different [Chi-Square Test; $\chi(1) = 0.014$, $P = 0.906$] (Fig. 3E). Moreover, L-glutamine preincubation, compared with recording in the absence of L-glutamine (Fig. 3A), caused a significant increase in the occurrence of GABA$_B$R currents at EPN-to-LHb synapses in cocaine-treated mice [Chi-Square Test; $\chi(1) = 6.617$; $P = 0.01$] (Fig. 3E). Overall these data suggest that cocaine drives reductions in GABA$_B$R-signaling at EPN-to-LHb synapses (Fig. 3F) due to reductions in presynaptic GABA release.

Discussion

The dysphoric cocaine withdrawal state exerts a motivational drive for drug intake in order to alleviate depressive-like symptoms (Koob & Le Moal, 2001; Barr *et al.*, 2002). Hyperactivity of the epithalamic LHb is a prominent neurobiological substrate for depressive-like symptoms, including those encountered during drug withdrawal (Meye *et al.*, 2017). One of the critical modifications contributing to LHb hyperactivity in depressive-like states is a reduction in GABAergic inhibition of the LHb (Shabel *et al.*, 2014; Lecca *et al.*, 2016; Meye *et al.*, 2016; Tchenio *et al.*, 2017). However, the mechanisms underlying this diminished LHb inhibition remain incompletely understood. In the current study, we provide evidence that: (i) Cocaine treatment results in diminished fast inhibitory GABAergic input onto lateral LHb neurons; (ii) Cocaine treatment also decreases slow inhibitory GABA$_B$R signaling at EPN synapses to the lateral LHb; and (iii) Facilitation of presynaptic GABA vesicle filling reinstates EPN-to-LHb GABA$_B$R neurotransmission in cocaine-treated mice. We now further discuss the implications of these findings.

Presynaptic deficit of GABA release at EPN-to-LHb synapses during cocaine withdrawal

Presynaptic alterations in the strength of GABA synapses have often been linked to changes in the release probability of neurotransmitter release, as demonstrated by changes in the paired-pulse ratio (Nugent *et al.*, 2007; Mameli *et al.*, 2008; Creed *et al.*,

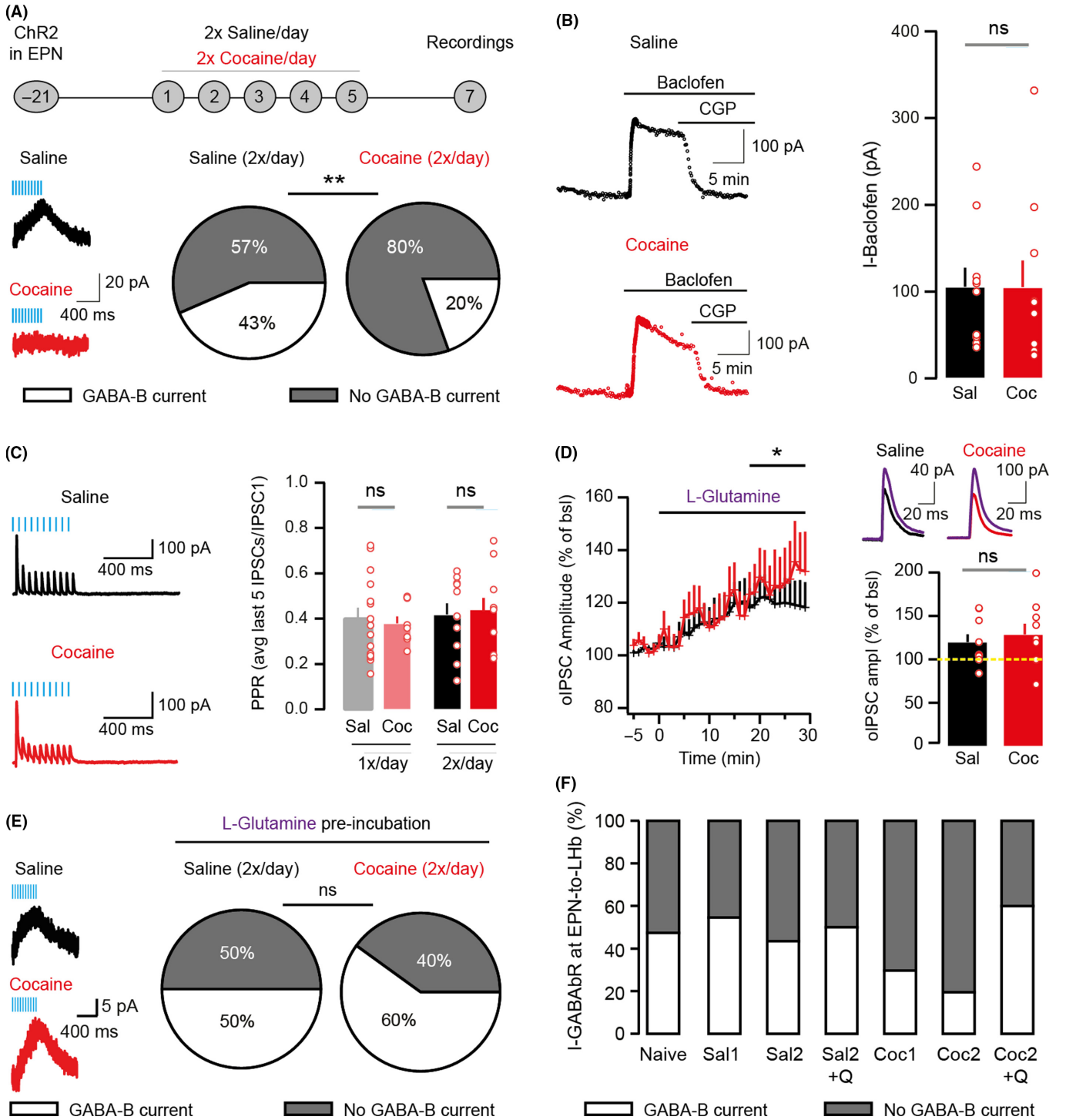

FIG. 3. Mechanisms underlying reduced $GABA_BR$ signaling at EPN-to-LHb synapses during cocaine withdrawal. (A) Timeline of saline and cocaine treatment (2 injections/day, 5 days) and pie charts indicating the occurrence of $GABA_BR$-mediated transmission at EPN-to-LHb synapses in mice that underwent the 2 injections/day protocol. Example traces indicate the amount of CGP54626-dependent (i.e. $GABA_BR$-mediated) current upon trains of 10 pulses at 20 Hz in saline (black) and cocaine (red) conditions. (B) Example traces (left) and bar graph (right) of baclofen-induced $GABA_BR$-mediated currents in EPN-responsive LHb neurons from saline and cocaine-treated mice (5 days, 2 injections/day). (C) Paired-pulse ratios (PPR) for $GABA_AR$-oIPSCs at EPN-to-LHb synapses in mice treated with saline or cocaine with either 1 or 2 injections/day. Example traces (left) of PPR for $GABA_AR$-oIPSCs for animals treated with saline or cocaine for 2x/day over 5 days. Recordings in $GABA_BR$ antagonist CGP54626. (D) Effect of L-Glutamine (2 mM) on $GABA_AR$-dependent oIPSCs at EPN-to-LHb synapses. Timeline of the average effect (left) and bar graphs quantifying the average oIPSC during the last 10 min of glutamine application compared to baseline (right). Example oIPSC traces (top right) for the baseline period for a saline- (black) and cocaine-treated mouse (red) with an overlaid average of $GABA_AR$-oIPSCs after glutamine application (purple). (E) Pie charts indicating the occurrence of $GABA_BR$-mediated transmission at EPN-to-LHb synapses in mice that underwent the two injections/day protocol, when recording in 2 mM L-glutamine. Example traces indicate the amount of CGP54626-dependent (i.e. $GABA_BR$-mediated) current upon trains of 10 pulses at 20 Hz in saline (black) and cocaine (red) conditions (F) Overview of EPN-to-LHb $GABA_BR$ occurrence across all assessed experimental conditions. Sal1 and Sal2 refer to 1 or 2 injections/day respectively. Similar terminology for cocaine injections. Q refers to glutamine (Q) incubation conditions during patching. $*P < 0.05$, $**P < 0.01$.

2016). In this study we find that, at EPN-to-LHb synapses, cocaine withdrawal did not result in alterations in paired-pulse ratio, in accordance with our previous findings (Meye *et al.*, 2016). Instead, several lines of converging evidence suggest that an alternative presynaptic deficit occurs at EPN-to-LHb synapses during cocaine withdrawal.

First, we have previously shown a diminished presence of the vesicular GABA transporter (VGAT or VIAAT) at EPN nerve terminals in the LHb during withdrawal, and rescuing this resulted in normalized GABA$_A$R currents (Meye *et al.*, 2016). Second, an electron microscopy study showed that cocaine treatment for 5 days followed by either 1 or 14 days of withdrawal caused a reduction in immunolabeled GABA (but not glutamate) in synapses onto LHb neurons (Meshul *et al.*, 1998). Third, in a rat model for depression, which shares symptomatology with cocaine withdrawal (Lecca *et al.*, 2014; Meye *et al.*, 2017), mRNA levels of the GABA synthetizing enzyme glutamic acid decarboxylase (GAD) were reduced in EPN-to-LHb boutons. Interestingly, this also coincided with diminished GABA release, despite unaltered paired-pulse ratios, and antidepressant treatment rescued both GAD mRNA levels and GABA transmission (Shabel *et al.*, 2014). Finally, in the current study we show that incubating slices in glutamine (a precursor to GABA) resulted in the rescue of GABA$_B$R signaling at EPN-to-LHb synapses, while not affecting the paired-pulse ratio of GABAergic neurotransmission. This, together with the experiment assessing baclofen-induced GABA$_B$R currents, indicates that functional GABA$_B$Rs are in place on EPN-innervated LHb neurons, but are less engaged by GABA during cocaine withdrawal.

Altogether, this indicates that cocaine treatment induces presynaptic deficits at EPN-to-LHb synapses, likely not by reducing the probability of GABA release, but rather by diminishing neurotransmitter content within presynaptic vesicles containing GABA. Indeed, we previously showed that the quantal size for optically stimulated GABAergic transmission at EPN-to-LHb synapses was reduced during cocaine withdrawal, as expected in case of reduced vesicular content (Meye *et al.*, 2016). In that regard, it is notable that in the current study we did not find an effect of cocaine withdrawal on the amplitude of mIPSCs when recording from lateral LHb neurons. A possible explanation for this may be that the suboptimal vesicle filling may be masked when synaptic activity is greatly reduced (i.e. lower demand on synapses), as in the case of TTX-induced abolishing of synaptic activity when recording mIPSCs. In accordance with this scenario, we previously showed that in cocaine withdrawal, long persistent stimulation trains of EPN-to-LHb synapses compromised GABA signaling more readily, suggesting an activity-dependence of the deficit (Meye *et al.*, 2016).

Since many EPN-to-LHb synapses corelease GABA and glutamate (Shabel *et al.*, 2014; Root *et al.*, 2018), it is relevant to speculate on the repercussions of altered GABA vesicle filling on glutamate packaging. A previous study proposed a scenario of single vesicles storing both GABA and glutamate at EPN-to-LHb synapses (Shabel *et al.*, 2014). In contrast, a recent study suggests that while GABA and glutamate vesicles co-occur in the same EPN-to-LHb axon terminals, they likely exist as two separate vesicular populations (Root *et al.*, 2018). Especially in the latter scenario it is feasible that perturbed GABA vesicle filling at EPN-to-LHb synapses would not necessarily directly impact on the vesicular filling of glutamate in these synapses. Accordingly, several studies have found evidence that altered GABA signaling can occur without concomitant glutamatergic changes at EPN-to-LHb synapses (Shabel *et al.*, 2014; Meye *et al.*, 2016).

Presumed working mechanisms of glutamine to rescue GABA neurotransmission

Here, we demonstrated that glutamine incubation counteracts the cocaine-withdrawal mediated deficits in GABA$_B$R-mediated signaling at EPN-to-LHb synapses. Glutamine likely exerts its effect by entering the presynaptic EPN nerve terminals via glutamine transporters expressed on the membrane and resulting, after two enzymatic conversion steps, in the biosynthesis of GABA (Edwards, 2007; Roth & Draguhn, 2012). Indeed, incubation of brain slices with glutamine produces a strong increase in the synthesis of GABA (Battaglioli & Martin, 1991), which can prevent impairments of GABA vesicle filling (Wang *et al.*, 2013). The current finding that glutamine incubation rescues GABA$_B$R neurotransmission at EPN-to-LHb synapses during cocaine withdrawal is therefore in accordance with an overall scenario in which GABA vesicles in EPN-to-LHb boutons are sub-optimally packed with neurotransmitter (increasingly so when synaptic activity is higher and more demanding). Nevertheless, questions remain with regards to the exact causes for this deficit. Our earlier work showed that reductions in VGAT protein at EPN-to-LHb synapses plays a pivotal role in the GABA$_A$R neurotransmission deficit during cocaine withdrawal (Meye *et al.*, 2016). One pertinent question is then how the application of glutamine, which presumably acts upstream of VGAT, can overcome this deficit. Several possibilities exist in this regard.

It is feasible that the application of glutamine itself drives the upregulation of VGAT levels at EPN-to-LHb synapses in mice in cocaine withdrawal. VGAT levels themselves are indeed subject to modulation, and can for instance be down- or upregulated as a function of neuronal activity (De Gois *et al.*, 2005; Edwards, 2007; Takamori, 2016). Moreover, VGAT is physically coupled, and functionally linked, to the GABA synthetizing enzyme isoform GAD65, suggesting that the effects of glutamine application on GABA biosynthesis (Battaglioli & Martin, 1991) could also regulate VGAT (Jin *et al.*, 2003). In an alternative scenario the effects of glutamine may occur without normalizing VGAT perturbations. In this case, a glutamine-induced increase in the GABA concentration in boutons such as those of the EPN in the LHb may result in more optimal vesicle filling despite a persistent reduction in VGAT protein on GABA vesicles. This requires that VGAT-mediated vesicle filling is not already engaged at maximal capacity and is still sensitive to increases in the concentration of cytosolic GABA. In support of this, VGAT has a rather low affinity for GABA (Edwards, 2007) and cytosolic GABA levels may even be reduced during cocaine withdrawal (Meshul *et al.*, 1998). Future studies are needed to further address the mechanisms of the presynaptic deficit in EPN-to-LHb synapses during cocaine withdrawal.

Relevance of GABA$_A$R and GABA$_B$R neurotransmission at EPN-to-LHb synapses

One of the major findings of this study is that both GABA$_A$R- and GABA$_B$R-dependent currents are diminished at lateral LHb neurons during cocaine withdrawal. This raises questions of when these two different kinds of inhibitory neurotransmission are engaged in the LHb, and what functions they serve.

As we show in this study, synaptically evoked GABA$_B$R currents at EPN-to-LHb synapses occur mainly with stimulation trains of multiple pulses, and particularly at higher frequency stimulation. Interestingly, a study in rhesus macaques showed that EPN neurons (globus pallidus interna in primates) that project to the LHb fired at 33 Hz on average during baseline conditions (Hong & Hikosaka, 2008). Another recent *in vivo* electrophysiology study in mice

showed that EPN neurons projecting to the LHb exhibit average basal firing rates exceeding 10 Hz (Stephenson-Jones *et al.*, 2016). Given that we have found frequency-dependent $GABA_BR$ responses when stimulating EPN-to-LHb projections with 5–20 Hz, it is likely that there is already a basal contribution of $GABA_BR$ signaling at EPN-to-LHb synapses *in vivo*.

The function of these distinct forms of inhibition in the LHb may also be partially different. We and others have previously shown that fast ionotropic $GABA_AR$ currents at EPN-to-LHb synapses play an important role in partly masking concomitant AMPA receptor-mediated glutamatergic transmission at these synapses (Shabel *et al.*, 2014; Meye *et al.*, 2016). This suggests that $GABA_AR$ currents at EPN-to-LHb synapses regulate the impact of phasic excitatory input. Instead, recent studies have shown that $GABA_BRs$ in the LHb play a profound role in modulating the overall excitability of LHb neurons, acting via their downstream GIRK channels (Lecca *et al.*, 2016; Tchenio *et al.*, 2017). Our findings therefore suggest that the diminished $GABA_AR$- and $GABA_BR$-currents at EPN-to-LHb synapses during cocaine withdrawal will result in stronger (unmasked) glutamatergic synaptic input and higher overall LHb neuron excitability, respectively. Together with other mechanisms that occur during cocaine withdrawal like AMPA receptor insertion and reduced K+-currents (Meye *et al.*, 2015), diminished $GABA_AR$- and $GABA_BR$-dependent inhibition of habenular neurons by the EPN therefore likely contributes to the LHb hyperactivity linked to the emergence of negative cocaine withdrawal symptoms.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that may represent a potential conflict of interest.

Data accessibility

Data can be obtained by contacting the corresponding author.

Author contributions

F.J.M. and M.M. designed the experiments. D.T., A.N.P., and F.J.M. performed the electrophysiological experiments. D.T., A.N.P, and F.J.M. analyzed the data. F.J.M. and M.M. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Abbreviations

ChR2, ChannelRhodopsin-2; CoChR, Chloromonas oogama ChannelRhodopsin; EPN, entopeduncular nucleus; IPSC, inhibitory postsynaptic current; LHb, lateral habenula; PPR, paired-pulse ratio; Q, glutamine; VGAT, vesicular GABA transporter.

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