

Cocaine Dysregulates Dynorphin Modulation of Inhibitory Neurotransmission in the Ventral Pallidum in a Cell-Type-Specific Manner

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Cocaine-driven changes in the modulation of neurotransmission by neuromodulators are poorly understood. The ventral pallidum (VP) is a key structure in the reward system, in which GABA neurotransmission is regulated by opioid neuropeptides, including dynorphin. However, it is not known whether dynorphin acts differently on different cell types in the VP and whether its effects are altered by withdrawal from cocaine. Here, we trained wild-type, D1-Cre, A2A-Cre, or vGluT2-Cre: Ai9 male and female mice in a cocaine conditioned place preference protocol followed by 2 weeks of abstinence, and then recorded GABAergic synaptic input evoked either electrically or optogenetically onto identified VP neurons before and after applying dynorphin. We found that after cocaine CPP and abstinence dynorphin attenuated inhibitory input to VP_{GABA} neurons through a postsynaptic mechanism. This effect was absent in saline mice. Furthermore, this effect was seen specifically on the inputs from nucleus accumbens medium spiny neurons expressing either the D1 or the D2 dopamine receptor. Unlike its effect on VP_{GABA} neurons, dynorphin surprisingly potentiated the inhibitory input on VP_{vGluT2} neurons, but this effect was abolished after cocaine CPP and abstinence. Thus, dynorphin has contrasting influences on GABA input to VP_{GABA} and VP_{vGluT2} neurons and these influences are affected differentially by cocaine CPP and abstinence. Collectively, our data suggest a role for dynorphin in withdrawal through its actions in the VP. As VP_{GABA} and VP_{vGluT2} neurons have contrasting effects on drug-seeking behavior, our data may indicate a complex role for dynorphin in withdrawal from cocaine.

Key words: cocaine; dynorphin; electrophysiology; GABA; ventral pallidum; vGluT2 neurons

Significance Statement

The ventral pallidum consists mainly of GABAergic reward-promoting neurons, but it also encloses a subgroup of aversion-promoting glutamatergic neurons. Dynorphin, an opioid neuropeptide abundant in the ventral pallidum, shows differential modulation of GABA input to GABAergic and glutamatergic pallidal neurons and may therefore affect both the rewarding and aversive aspects of withdrawal. Indeed, abstinence after repeated exposure to cocaine alters dynorphin actions in a cell-type-specific manner; after abstinence dynorphin suppresses the inhibitory drive on the “rewarding” GABAergic neurons but ceases to modulate the inhibitory drive on the “aversive” glutamatergic neurons. This reflects a complex role for dynorphin in cocaine reward and abstinence.

Introduction

Failure to withdraw from chronic use of drugs is a major problem that underlies the high number of individuals suffering from drug addiction in the world. During withdrawal, many changes

occur in the reward system that engrave the irresistible urge to use the drugs (Kalivas and Volkow, 2005; Lüscher, 2016; Scofield et al., 2016; Dong et al., 2017). These changes are thought to underlie the dysphoric and aversive symptoms people with drug addiction experience during withdrawal and encompass synaptic and genetic changes in many brain regions, and particularly in the nucleus accumbens (NAc; Conrad et al., 2008; Gipson et al., 2013; Barrientos et al., 2018).

The main target of NAc projection neurons is the ventral pallidum (VP; Zahm and Heimer, 1990; Heimer et al., 1991). The VP is part of the basal forebrain that is deeply involved in encoding of the hedonic features of reward (Smith and Berridge, 2005; Tindell et al., 2006; Tachibana and Hikosaka, 2012; Richard et al., 2018),

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and is important in mediating cue-induced relapse to cocaine and alcohol (Stefanik et al., 2013; Prasad and McNally, 2016; Heinsbroek et al., 2017). Neurotransmission in the VP is mostly GABAergic (Kupchik and Kalivas, 2013) and arises mainly from two populations of medium spiny neurons (MSNs) in the NAc; those expressing the D1 (D1-MSNs) or the D2 (D2-MSNs) dopamine receptors (Zahm and Heimer, 1990; Bock et al., 2013; Kupchik et al., 2015; Creed et al., 2016; Matsui and Alvarez, 2018). Interestingly, these two inputs coexpress two different opioid neuropeptides; dynorphin (in D1-MSNs) and enkephalin (in D2-MSNs). In a previous study we have described a potential role for enkephalin in withdrawal from cocaine (Kupchik et al., 2014), showing that enkephalin modulates GABA neurotransmission in the VP through a presynaptic mechanism and that this modulation is weakened after withdrawal from cocaine. However, the role of VP dynorphin in regulating neurotransmission in the VP and in withdrawal from cocaine is still not known.

Dynorphin is a selective agonist of the κ -opioid receptor (KOR; Chavkin et al., 1982) and had been repeatedly linked to dysphoria and anhedonia, particularly in the context of withdrawal from drugs (Shippenberg et al., 2007; Chavkin and Koob, 2016). Both dynorphin and the KOR are abundant in the VP. Interestingly, KORs seem to be primarily postsynaptic in the VP and in close proximity to the presynaptic dynorphin (Arvidsson et al., 1995; Allen Institute for Brain Science, 2004; Lein et al., 2007). This is in contrast to the μ -opioid receptors, the target of enkephalin, who are mainly presynaptic in the VP (Olive et al., 1997). Postmortem investigation revealed that dynorphin levels in the VP of individuals who suffered from addiction to cocaine are increased by >300% (Frankel et al., 2008). Also, dynorphin mRNA levels in NAc D1-MSNs (which project to the VP) are increased after cocaine self-administration (Hurd et al., 1992). This increase in dynorphin signaling may be related to the dysphoric symptoms of withdrawal rather than the hedonic effect of the drug because KOR phosphorylation levels in the VP increase also after injection of the stressful agent corticotropin-releasing factor (Land et al., 2008).

In this study, we use whole-cell patch-clamp recordings combined with optogenetics and an animal model of withdrawal from cocaine to characterize the dynorphinergic modulation of GABA neurotransmission in the VP in health and determine whether and how this modulation is altered after repeated cocaine exposure followed by abstinence. Moreover, we examine whether dynorphin affects differently D1-MSN and D2-MSN inputs to the VP and whether glutamate-expressing cells in the VP (VP_{vGluT2}; Geisler et al., 2007), who were recently suggested to encode aversive behavior (Faget et al., 2018; Tooley et al., 2018), are affected differently by dynorphin.

Materials and Methods

Animals and housing. Experimentally naive C57BL/6J mice of both sexes (overall 41 males, 35 females) aged 8–10 weeks were group-housed (4/cage) throughout the experiments (males separately from females). A

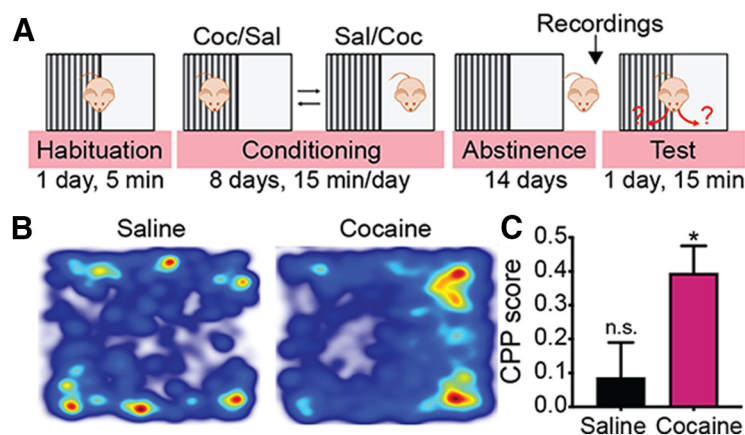


Figure 1. CPP paradigm. **A**, Timeline of the CPP experiments (from left to right). Mice were habituated to the arena on the first day and then received eight alternating intraperitoneal injections of either cocaine (15 mg/kg) or saline, one injection per day. Control (cocaine-naïve) mice received only saline injections. Cocaine was paired with one of the sides and saline was given on the other side. After conditioning mice went through 14 d of abstinence from cocaine and then were either used for electrophysiological recordings or tested for their preference of the cocaine-paired side. **B**, Representative movement heatmaps of a control saline mouse (left) and a cocaine-abstinent mouse (right) in a CPP test. **C**, Cocaine mice ($n = 10$) showed a significant preference for the cocaine-paired side in the CPP test: $CPP\ score = \frac{time\ in\ paired\ zone - time\ in\ unpaired\ zone}{time\ in\ paired\ zone + time\ in\ unpaired\ zone}$. * $p < 0.05$ compared with saline mice ($n = 8$), unpaired t test. n.s., not significant.

12 h light/dark cycle was maintained at all times, with lights off at 8:00 A.M. Mice were either wild-type (Envigo) or transgenic: D1-Cre (MMRRC, stock #029178-UCD, breeding hemizygous \times wild-type), A2A-Cre (MMRRC stock #036158-UCD, breeding hemizygous \times wild-type) and a cross between vGluT2-IRES-Cre (Jackson Laboratories, strain #016963, breeding homozygous \times homozygous) and Ai9 (Jackson Laboratories strain #007905, breeding homozygous \times homozygous) named vGluT2-Cre: Ai9.

Drugs. Cocaine hydrochloride (Tamar Marketing) was dissolved in sterile saline and filtered before use. Dynorphin A and NorBNI (Abcam) were first dissolved in water and then added to aCSF solution containing also CNQX (Alomone Labs).

Behavioral procedures. Behavioral procedures started after 2 weeks of acclimation to the reverse light cycle, when the mice were ~ 10 weeks old. For cocaine CPP, all mice were trained in the unbiased cocaine conditioned place preference (CPP) paradigm; a 30 cm \times 30 cm arena was divided in two, each side with different wall patterns and floor texture (Fig. 1). On the first day, all mice were allowed to explore the arena freely. Then, experimental mice received one daily injection of either cocaine (in the paired side, 15 mg/kg, i.p.; Mu et al., 2011; Turner et al., 2018) or saline (in the unpaired side). Each side of the box served as a cocaine-paired side for one-half of the mice. Cocaine/saline injections alternated daily until each mouse received four injections of each. Then, mice were left in their cages for 14 d before being tested for preference of the cocaine-paired side or before electrophysiological recordings began. During the test (15 min), mice were placed in the CPP box with free access to both paired and unpaired sides. Mouse movement was tracked (EthoVision XT 11.5, Noldus) and quantified off-line. CPP score was calculated using the following equation: $CPP\ score = \frac{time\ in\ paired\ zone - time\ in\ unpaired\ zone}{time\ in\ paired\ zone + time\ in\ unpaired\ zone}$. As shown (Fig. 1 B, C), cocaine mice demonstrated a strong preference for the drug-paired side [Saline mice ($n = 8$), CPP score: 0.09 ± 0.29 ; Cocaine mice ($n = 10$), CPP score: 0.40 ± 0.25 ; unpaired t test comparing cocaine to saline mice: $t_{(16)} = 2.38$, $p = 0.03$]. For food CPP we used the same protocols as for the cocaine CPP, but mice received high-fat, high-sugar food pellets (D12451, 45% kcal fat, 4.73 kcal g^{-1} ; Research Diets) as the reward and allowed to stay in the box 30 min in each conditioning session. For the cocaine intraperitoneal protocol, mice received daily injections of cocaine (15 mg/kg, i.p.) for 5 consecutive days and returned to their home

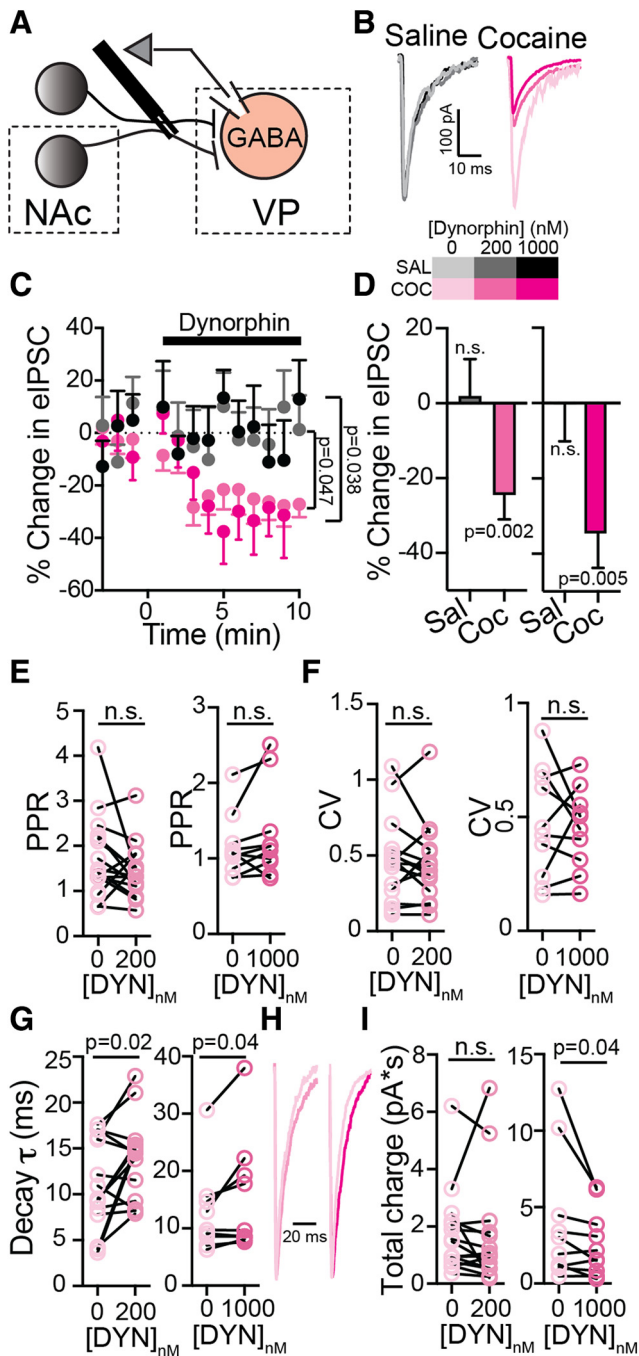


Figure 2. Dynorphin suppresses GABA neurotransmission to VP_{GABA} neurons after abstinence from cocaine. Data presented as mean \pm SEM. **A**, Schematic drawing of the recording setup. Stimulating electrode was placed rostral to the VP, along the presumed path of NAc and possibly other GABAergic projections, to evoke GABA neurotransmission in the VP. Synaptic events were recorded from neurons that were identified as GABA neurons based on their physiology (see Materials and Methods). **B**, Representative eIPSC traces from saline (grayscale) and cocaine (magenta scale) mice. **C**, **D**, Time course (**C**) and average (**D**) of the effect of 200 and 1000 nM dynorphin on eIPSCs in saline (gray) and cocaine (magenta) mice. Dynorphin suppressed eIPSCs in both concentrations in cocaine, but not saline mice (time courses, two-way, repeated-measures ANOVA, group main effects: $F_{(1,25)} = 4.35$ for 200 nM dynorphin and $F_{(1,25)} = 5.08$ for 1000 nM dynorphin; averages: one-sample t test, comparing to 0% change). **E**, Dynorphin did not affect the PPR of the eIPSCs (paired t test, $t_{(15)} = 1.44$, $p = 0.17$ for 200 nM dynorphin, $t_{(9)} = 1.54$, $p = 0.16$ for 1000 nM dynorphin) after cocaine CPP and abstinence. **F**, Dynorphin did not affect the CV of the eIPSCs (paired t test, $t_{(15)} = 0.65$, $p = 0.53$ for 200 nM dynorphin, $t_{(9)} = 0.33$, $p = 0.75$ for 1000 nM dynorphin) after cocaine CPP and abstinence. **G**, Dynorphin significantly increased the decay time-constant of the eIPSCs (paired t test, $t_{(15)} = 2.57$, $p = 0.02$ for 200 nM dynorphin, $t_{(9)} = 2.39$, $p = 0.04$ for 1000 nM dynorphin) after cocaine

cage after every injection. All procedures were approved by the Research Animal Care Committee of the Hebrew University.

Viral injections. Mice were anesthetized with isoflurane and fixed in a stereotaxic frame (Kopf, Model 940). Bilateral holes were drilled in the skull and 300 nl of virus were microinjected through a 30 G NanoFil syringe (World Precision Instruments; 100 nl/min, needle retracted slowly 5 min after injection terminated). The viral construct (AAV2-EF1a-DIO-hChR2(H134R)-EYFP, generated by Karl Deisseroth and sold by University of North Carolina Viral Core) was injected into the nucleus accumbens (injection coordinates in mm relative to bregma, anterior/posterior: +1.8; medial/lateral: ± 1 ; dorsal/ventral: -4.6) of D1-Cre or A2A-Cre mice.

Slice preparation. As described previously (Kupchik et al., 2015), mice were anesthetized (150 mg/kg ketamine HCl), decapitated, and sagittal or coronal slices (200 μ m) of the VP were prepared (VT1200S Leica vibratome). Slices were transferred to a vial containing aCSF (in mM: 126 NaCl, 1.4 NaH₂PO₄, 25 NaHCO₃, 11 glucose, 1.2 MgCl₂, 2.4 CaCl₂, 2.5 KCl, 2.0 NaPyruvate, 0.4 ascorbic acid, bubbled with 95% O₂ and 5% CO₂) and a mixture of 5 mM kynurenic acid and 100 μ M MK-801. Slices were stored at room temperature (22–24°C) until recording.

Whole-cell recordings. All recordings were collected at 32°C (TC-344B, Warner Instruments). Ventral pallidal neurons were visualized with an Olympus BX51WI microscope. Excitatory synaptic transmission was blocked with CNQX (10 μ M). MultiClamp 700B (Molecular Devices) was used to record IPSCs in whole-cell configuration. Glass microelectrodes (1.3–2 M Ω) were filled with internal solution (in mM: 68 KCl, 65 D-gluconic acid potassium salt, 7.5 HEPES potassium, 1 EGTA, 1.25 MgCl₂, 10 NaCl, 2.0 MgATP, and 0.4 NaGTP, pH 7.2–7.3, 275 mOsm). In most experiments (see Figs. 2–6) we targeted “classic” GABAergic cells (Kupchik and Kalivas, 2013). Cholinergic and accumbens-like VP neurons were avoided based on their size and physiology (Bengtson and Osborne, 2000; Kupchik and Kalivas, 2013). VP_{vGluT2} neurons were, if at all, patched in negligible numbers (except for Fig. 7, where they were specifically targeted) based on their 5–10% prevalence in the medio-caudal VP, where we performed our experiments [observed by others (Faget et al., 2018) and us (data not shown)]. Recordings started no earlier than 10 min after the cell membrane was ruptured. Data were acquired at 10 kHz and filtered at 2 kHz using AxoGraph X software (AxoGraph Scientific). To evoke IPSCs electrically, a bipolar stimulating electrode was placed ~ 200 – 300 μ m anterior of the cell to maximize chances of stimulating NAc afferents (although other GABAergic afferents were most likely activated as well). For the optogenetic experiments (see Fig. 6), we used a 470 nm LED light source (Mightex Systems) directed to the slice through the objective. The stimulation 1 ms pulse intensity was set to evoke 50% of maximal IPSC. Recordings were collected every 20 s. Series resistance (R_s), measured with a -2 mV depolarizing step (10 ms) given with each stimulus, and holding current were monitored online. Recordings with unstable R_s , or when R_s exceeded 20 M Ω were aborted.

Data analysis. Statistics were performed using GraphPad Prism 8.0. Data on evoked neurotransmission (see Figs. 2, 4–7) are given as percentage change and not in raw numbers because of the high variability of the evoked IPSC amplitudes within groups. In graphs showing the time-dependent effect of dynorphin on GABA currents (see Figs. 2C, 3A, D, 4C, 5A, 6C, D, 7C, F, I) the two-way repeated-measures ANOVA was performed on minutes 1–10 and accompanying average bar graphs were calculated from the same time points. Parametric statistics (Student’s t test, one-sample t test, one-way or two-way repeated-measures ANOVA) was used unless otherwise stated. Nonparametric statistics (Kolmogorov–Smirnov tests) were used in Figures 3, 5, and 7 to compare cumulative plots. The cumulative probability curve depicted in Figures 3, 5 and 7 is

CPP and abstinence. **H**, Representative traces showing the effect of 200 and 1000 nM dynorphin on the decay of the eIPSC. **I**, Dynorphin (only at 1000 nM) significantly decreased the total charge of the eIPSCs (paired one-tailed t test, $t_{(15)} = 0.39$, $p = 0.35$ for 200 nM dynorphin, $t_{(9)} = 1.89$, $p = 0.04$ for 1000 nM dynorphin). Number of cells was between 9 and 22 from 4 to 11 mice for all conditions. n.s., not significant.

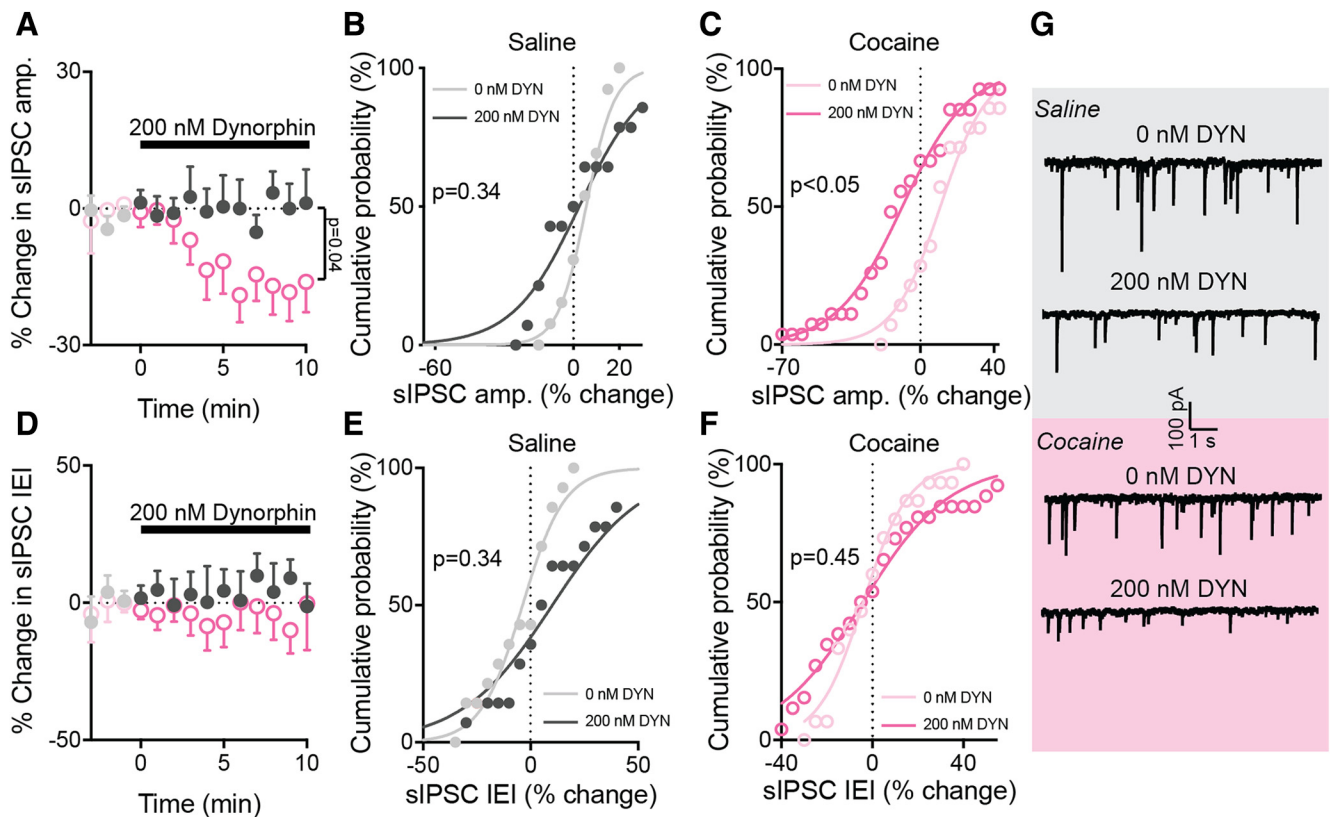


Figure 3. Dynorphin reduces the amplitude of spontaneous GABA currents in VP_{GABA} neurons of cocaine-withdrawn mice. **A–C**, Dynorphin (200 nM) decreased the amplitude of sIPSCs in VP_{GABA} neurons after cocaine CPP and abstinence (magenta) but not in saline mice (gray). This is seen both in the time course of the effect of dynorphin (**A**; main group effect, two-way repeated-measures ANOVA: $F_{(1,30)} = 4.26, p = 0.04$) and in the cumulative probability plots (Kolmogorov–Smirnov test). Lines are fitted sigmoidal curves running from 0 to 100. **D–F**, Dynorphin (200 nM) did not affect the frequency of sIPSCs, measured as IEL. **G**, Representative traces, depicting the increased suppressive effect of dynorphin on sIPSC amplitude after withdrawal from cocaine. Number of cells was between 8 and 28 from 3 to 11 mice for all conditions.

a fitted sigmoidal curve running from 0 to 100, computed by GraphPad Prism 8.0. Data in Results and figure legends are presented as mean \pm SD. Error bars in graphs represent SEM.

Results

Cocaine CPP and abstinence causes strong dynorphin-induced suppression of GABA neurotransmission onto VP_{GABA} neurons

The current study aims to reveal a possible role for dynorphin in mediating long-lasting changes occurring in the VP after abstinence from cocaine. To achieve that, we first examined whether dynorphin regulates evoked GABA neurotransmission in the VP. We stimulated electrically the GABAergic input to the VP and recorded from unlabeled VP neurons (Fig. 2A). As the proportion of glutamatergic neurons in the VP is low [only 29 cells per $10^7 \mu\text{m}^3$ tissue (Hur and Zaborszky, 2005); 7% of neurons in our hand (data not shown)] and cholinergic neurons are scarce in the VP and can be identified by their morphological and physiological parameters (Bengtson and Osborne, 2000), we assume that $>95\%$ of patched unlabeled neurons are in fact classical GABAergic ventral pallidal neurons (VP_{GABA} ; see Materials and Methods). In cocaine-naïve mice (injected with saline), dynorphin did not affect the amplitude of the electrically-evoked IPSC (eIPSC) both at 200 and 1000 nM (Fig. 2B–D; average percentage change from baseline was 1.95 ± 31.09 and -0.29 ± 27.92 for 200 and 1000 nM dynorphin, respectively; one-sample t test comparing to 0% change from baseline: $t_{(9)} = 0.20, p = 0.85$ and $t_{(7)} = 0.03, p = 0.98$ for 200 and 1000 nM dynorphin). In contrast, in mice that underwent cocaine CPP and abstinence dynorphin attenuated

eIPSCs in the VP (Fig. 2B–D); eIPSCs were decreased by $24.5 \pm 6.5\%$ and $34.6 \pm 9.2\%$ with 200 and 1000 nM dynorphin, respectively (two-way, repeated-measures ANOVA, main group effects comparing cocaine and saline groups within each dynorphin concentration, $F_{(1,25)} = 4.35, p = 0.047$ for 200 nM dynorphin and $F_{(1,25)} = 5.08, p = 0.038$ for 1000 nM dynorphin; one-sample t tests comparing to baseline: $t_{(16)} = 3.74, p = 0.002$ and $t_{(8)} = 3.77, p = 0.005$ for 200 and 1000 nM dynorphin, respectively).

We have previously shown that enkephalin, an opioid neuropeptide that binds δ - and μ -opioid receptors, inhibits eIPSCs in the VP via a presynaptic mechanism (Kupchik et al., 2014). To examine whether this is the case also for dynorphin we used a dual approach: (1) examination of the characteristics of the evoked IPSCs, including the paired-pulse ratio (PPR) and the coefficient of variation (CV), both indicators of presynaptic changes, and the decay time constant, which reflects postsynaptic mechanisms; and (2) examination of the frequency and amplitude of the spontaneous IPSCs (sIPSCs). In contrast to enkephalin, our convergent approach suggests that the effect of dynorphin in the VP after cocaine CPP and abstinence is more likely to be mediated by a postsynaptic mechanism. First, dynorphin application did not change the PPR (Fig. 2E; paired t test: $t_{(15)} = 1.44, p = 0.17$ and $t_{(9)} = 1.54, p = 0.16$ for 200 and 1000 nM dynorphin, respectively). Lack of change in the PPR is often interpreted as lack of change in the probability of synaptic release, but this interpretation should be taken with caution, as G-protein-coupled receptors, including the KOR (the target of dynorphin), were shown to have a presynaptic site of action without changing the PPR (Li et

al., 2012). Second, application of dynorphin did not affect the CV of eIPSCs (Fig. 2F; paired t test: $t_{(15)} = 0.65$, $p = 0.53$ and $t_{(9)} = 0.33$, $p = 0.75$ for 200 and 1000 nM dynorphin, respectively), again advocating against a presynaptic mechanism of action. In contrast, we found that dynorphin application on slices of cocaine-withdrawn mice increased the decay time constant of the eIPSCs (Fig. 2G,H; paired t test: $t_{(15)} = 2.57$, $p = 0.02$ and $t_{(9)} = 2.39$, $p = 0.04$ for 200 and 1000 nM dynorphin, respectively), suggesting a postsynaptic site of action for dynorphin. Note that longer decay time constants allow for more charge to flow through the channels. Thus, the dynorphin-induced prolongation of the eIPSC decay promotes, while the dynorphin-induced suppression of the eIPSC peak attenuates, charge flow. When examining whether dynorphin inhibited the total charge (calculated as the area under the eIPSC curve) despite the increase in the decay time constant, we found that only 1000 nM dynorphin was sufficient to decrease significantly the total charge in mice after cocaine CPP and abstinence (Fig. 2I; paired t test: $t_{(15)} = 0.39$, $p = 0.35$ and $t_{(9)} = 1.89$, $p = 0.04$ for 200 and 1000 nM dynorphin, respectively).

To supplement the observations on the eIPSCs, we also examined the effect of dynorphin on the amplitude and frequency of spontaneous IPSCs. To achieve this, we calculated the dynorphin-induced change in each recorded cell and then compared the averages (Fig. 3A,D,G) and cumulative probabilities of these changes on sIPSC amplitude (Fig. 3B,C) and inter-event interval (IEI; Fig. 3E,F) at a concentration of 200 nM. We found that 200 nM dynorphin caused a decrease in the average sIPSC amplitude (two-way, repeated-measures ANOVA, main group effect comparing cocaine and saline groups: $F_{(1,30)} = 4.26$, $p = 0.047$) and a leftward shift in the sIPSC amplitude plot toward negative values (Kolmogorov–Smirnov test, $D = 0.45$, $p = 0.045$), but only after cocaine CPP and abstinence. In contrast, dynorphin had no effect on the IEI (average sIPSC IEI: two-way, repeated-measures ANOVA, no main group effect comparing cocaine and saline groups: $F_{(1,30)} = 0.32$, $p = 0.57$; IEI cumulative plots: Kolmogorov–Smirnov test, $D = 0.28$, $p = 0.45$). Because changes in sIPSC amplitude reflect postsynaptic mechanisms, these results, together with the analysis of eIPSCs (Fig. 2), support the hypothesis that after cocaine CPP and abstinence dynorphin inhibits the amplitude of the GABAergic input to VP_{GABA} neurons through a postsynaptic mechanism.

The effect of dynorphin on eIPSCs is strongest in a cocaine CPP and abstinence model

We next wanted to examine whether the effects of dynorphin on the eIPSCs seen in mice after cocaine CPP and withdrawal are more likely to be generated by the mere exposure to cocaine or by the CPP training. We thus used two more groups of mice. The first group (Fig. 4A) received five intraperitoneal injections of cocaine, whereas another group (Fig. 4B) was trained on CPP using high-fat-high-sugar food as the reward. In both groups, recordings were performed 14 d after the last exposure to reward. We found that dynorphin application (1000 nM) on slices from mice that underwent food CPP did not affect eIPSC amplitude or PPR (Fig. 4C–E; average $0.05 \pm 18.2\%$ of baseline; one-sample t test compared with baseline: $t_{(13)} = 0.01$, $p = 0.99$ and $t_{(13)} = 0.08$, $p = 0.94$ for eIPSC amplitude and PPR, respectively). In contrast, in the mice that received the five intraperitoneal cocaine injections dynorphin showed some nonsignificant suppression of the eIPSC (average $-7.56 \pm 13.2\%$ of baseline; one-sample t test compared with baseline: $t_{(13)} = 0.126$, $p = 0.23$), although this was much less pronounced than the effect of dynorphin in

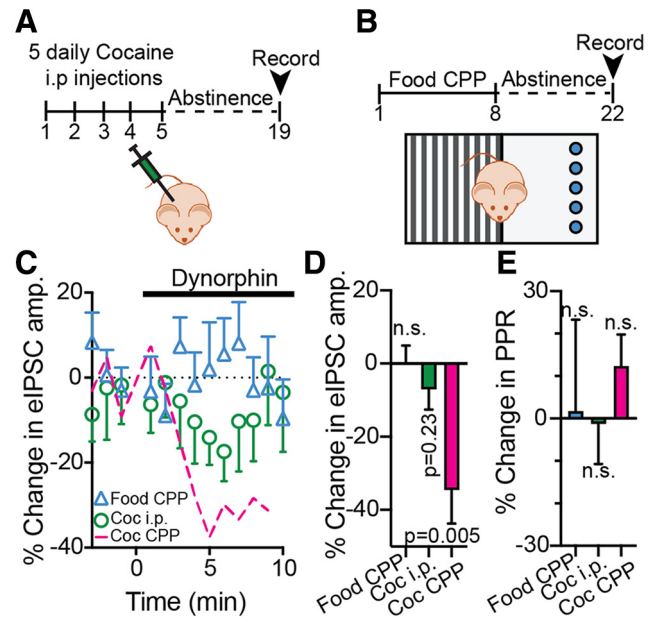


Figure 4. The dynorphinergic changes in the VP are most pronounced in the cocaine CPP and abstinence paradigm. **A**, Schematic drawing of the cocaine intraperitoneal injection protocol. Mice received five daily intraperitoneal cocaine injections, followed by 14 d of abstinence. **B**, Schematic drawing of the food CPP protocol. Mice were trained for CPP just as described above for cocaine, but with high-fat, high-sugar (HFHS) food pellets as the reward (blue circles). After 8 d of conditioning, mice went through 14 d of abstinence from the HFHS food. **C, D**, Time course (**C**) and average (**D**) of the effect of 1000 nM dynorphin on eIPSCs in the VP in the food CPP mice (blue triangles), cocaine intraperitoneal injection mice (green circles) and cocaine CPP mice (magenta line; same data as in Fig. 2, given here for comparison). It can be seen that dynorphin induced a transient small suppression of the eIPSC in the cocaine intraperitoneal mice, but this effect was weak and did not reach significance (one-sample t tests compared with 0% change from baseline). **E**, Dynorphin did not affect the PPR in any of the groups (one-sample t test compared with 0% change from baseline). n.s., not significant.

mice that underwent cocaine CPP and abstinence (average $-34.6 \pm 27.5\%$ of baseline; one-sample t test compared with baseline: $t_{(8)} = 3.77$, $p = 0.005$). Thus, cocaine exposure and abstinence seems to be more central to the dynorphinergic changes in the VP than the CPP training itself, although CPP training may amplify the cocaine-induced effects on dynorphin action in the VP.

The effects of dynorphin in the VP are mediated by KORs

Dynorphin is known as a selective agonist of KORs, but the VP expresses also δ - and μ -opioid receptors that may also be affected by dynorphin (Zhang et al., 1998). To examine this possibility, we repeated our experiments on cocaine-abstinent mice but in the presence of the selective and long-lasting KOR blocker nor-binaltorphimine (NorBNI).

Washing NorBNI alone on the slice did not have any effect on eIPSCs or sIPSCs both in saline and after cocaine CPP and abstinence (Fig. 5; eIPSCs, one-sample t test compared with 0% change from baseline: $t_{(6)} = 0.27$, $p = 0.80$ and $t_{(9)} = 0.10$, $p = 0.92$ in saline and cocaine mice, respectively; for sIPSC amplitude, Kolmogorov–Smirnov test: $D = 0.44$, $p = 0.35$ and $D = 0.15$, $p = 0.99$ in saline and cocaine mice, respectively; for sIPSC IEI, Kolmogorov–Smirnov test: $D = 0.22$, $p = 0.99$ and $D = 0.31$, $p = 0.57$ in saline and cocaine mice, respectively). This indicates that, in contrast to the tonic activation of μ -opioid receptors by enkephalin after abstinence (Kupchik et al., 2014; Heinsbroek et al., 2017), KORs maintain a normal basal level of activity after abstinence from cocaine. Moreover, NorBNI successfully pre-

vented dynorphin from inhibiting the eIPSCs (Fig. 5A–C; one-sample t test compared with 0% change from baseline: $t_{(9)} = 0.25$, $p = 0.81$ and $t_{(4)} = 0.85$, $p = 0.45$ in 200 and 1000 nM dynorphin, respectively), changing their decay time constant (Fig. 5D–G; paired t test: $t_{(7)} = 0.79$, $p = 0.46$) and affecting the amplitude or frequency of sIPSCs (Fig. 5H–K; Kolmogorov–Smirnov test: $D = 0.25$, $p = 0.85$ and $D = 0.39$, $p = 0.29$ for sIPSC amplitude and frequency, respectively) in mice that underwent cocaine CPP and abstinence. This, together with our data so far, indicates that the effects of dynorphin on GABA transmission onto VP_{GABA} neurons are mediated by postsynaptic KORs.

Dynorphin suppresses GABA transmission from D1-MSNs and D2-MSNs in the VP in saline mice and after cocaine CPP and abstinence

The major portion of inhibitory input to the VP originates in the NAc (Zahm et al., 1985; Root et al., 2015). We and others recently showed that both populations of MSNs in the NAc, those expressing the D1 (D1-MSNs) or the D2 (D2-MSNs) dopamine receptor, provide input to the VP (Kupchik et al., 2015; Creed et al., 2016; Heinsbroek et al., 2017; Pardo-Garcia et al., 2019). These two different inputs may have different roles in encoding drug-induced effects and therefore may be affected differently by dynorphin. To examine this, we activated selectively D1-MSN or D2-MSN terminals in the VP with optogenetics and recorded from VP_{GABA} neurons (Fig. 6A). Our data show that 1000 nM dynorphin successfully suppressed GABA transmission from both D1-MSN and D2-MSN terminals, and that this suppression was seen both in saline mice and in mice that underwent cocaine CPP and abstinence (Fig. 6B–D; one-sample t test compared with 0% change from baseline, D1-MSN input: $8.83 \pm 13.8\%$ suppression, $t_{(12)} = 2.3$, $p = 0.04$ and $-25.00 \pm 17.2\%$ suppression, $t_{(12)} = 5.24$, $p = 0.0002$ for saline and cocaine mice, respectively; D2-MSN input: $16.9 \pm 16.6\%$ suppression, $t_{(9)} = 3.22$, $p = 0.01$ and $-19.8 \pm 26.3\%$ suppression, $t_{(9)} = 2.38$, $p = 0.04$ for saline and cocaine mice, respectively). Interestingly, cocaine CPP and abstinence strengthened the dynorphin-induced suppression of D1-MSNs (Fig. 6C, inset; unpaired t test: $t_{(24)} = 2.64$, $p = 0.014$) but not of D2-MSNs (Fig. 6D, inset; unpaired t test: $t_{(18)} = 0.29$, $p = 0.78$). There was no change in the PPR or CV in both inputs (data not shown). Dynorphin also did not change on average the eIPSC decay time-constant in D1-MSN and D2-MSN inputs (Fig. 6E,F; paired t test: $t_{(12)} =$

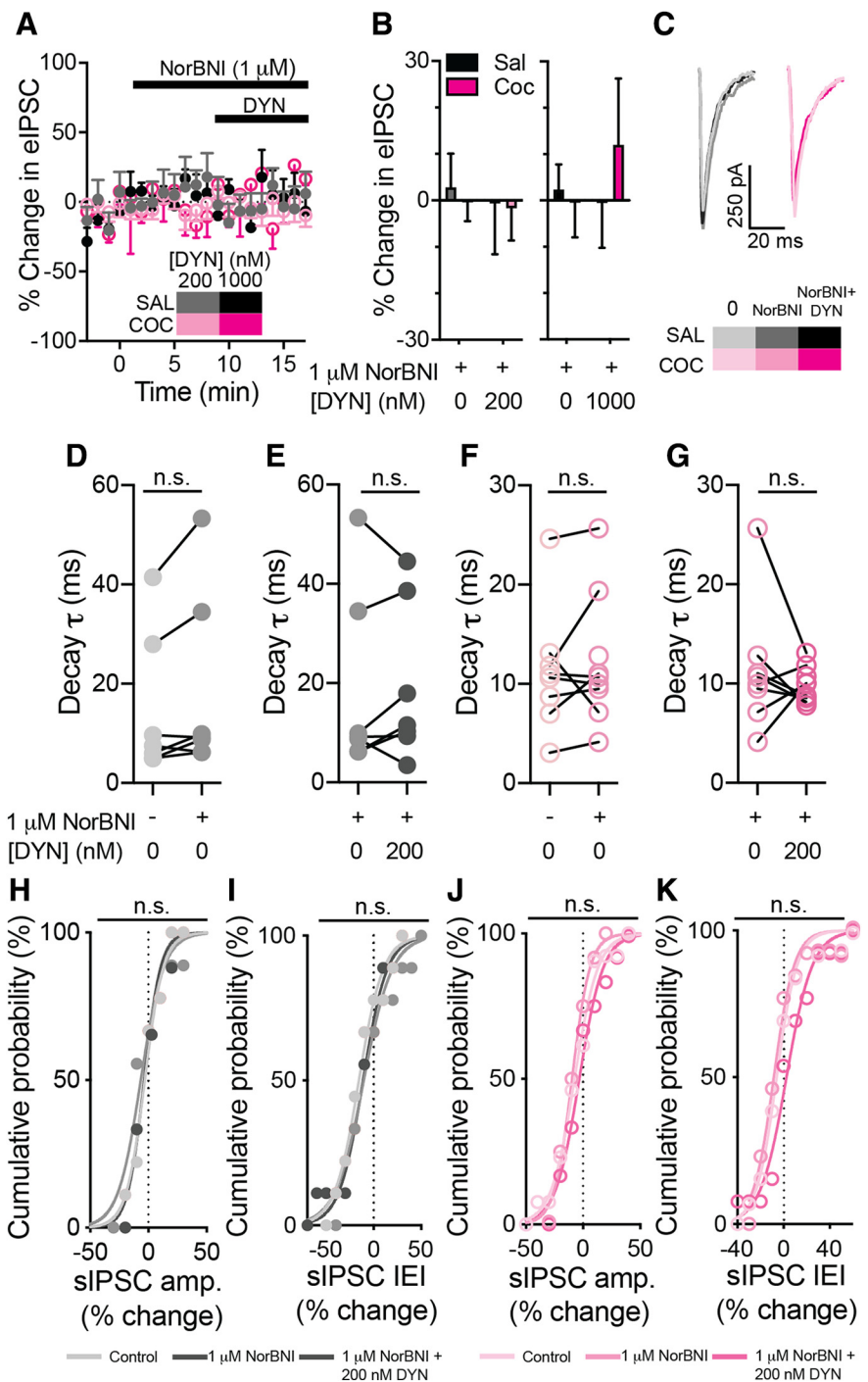


Figure 5. The effects of dynorphin are mediated by KORs. All experiments were performed by recording baseline synaptic release, then adding the KOR antagonist NorBNI (1000 nM) and finally a mixture of NorBNI and 200 or 1000 nM dynorphin. Data presented as mean \pm SEM. **A**, Time course of experiment. NorBNI alone, as well as 200 or 1000 nM dynorphin given together with NorBNI, did not affect the amplitude of eIPSCs in either saline or cocaine mice. **B**, Summary of experimental results presented in **A**. None of the experimental groups was different from 0 (one-sample t test). $n = 4$ –13 cells from 3 to 5 mice in all groups. **C**, Representative traces. **D–G**, NorBNI did not affect, and prevented the effects of dynorphin on, the eIPSC decay time-constant in saline ($n = 7$) or cocaine ($n = 8$) mice (effect examined using paired t test). **H–K**, NorBNI did not affect, and prevented the effects of dynorphin on the sIPSC amplitude and IEI in saline ($n = 9$) and cocaine-withdrawn ($n = 13$) mice. Lines are fitted sigmoidal curves running from 0 to 100. n.s., not significant.

1.06 , $p = 0.31$ and $t_{(9)} = 2.18$, $p = 0.056$ for D1-MSN and D2-MSN inputs, respectively); although note that 8 of 10 cells showed an increase in the decay time-constant of D2-MSN-induced eIPSCs. Overall, dynorphin inhibits both D1-MSN and D2-MSN

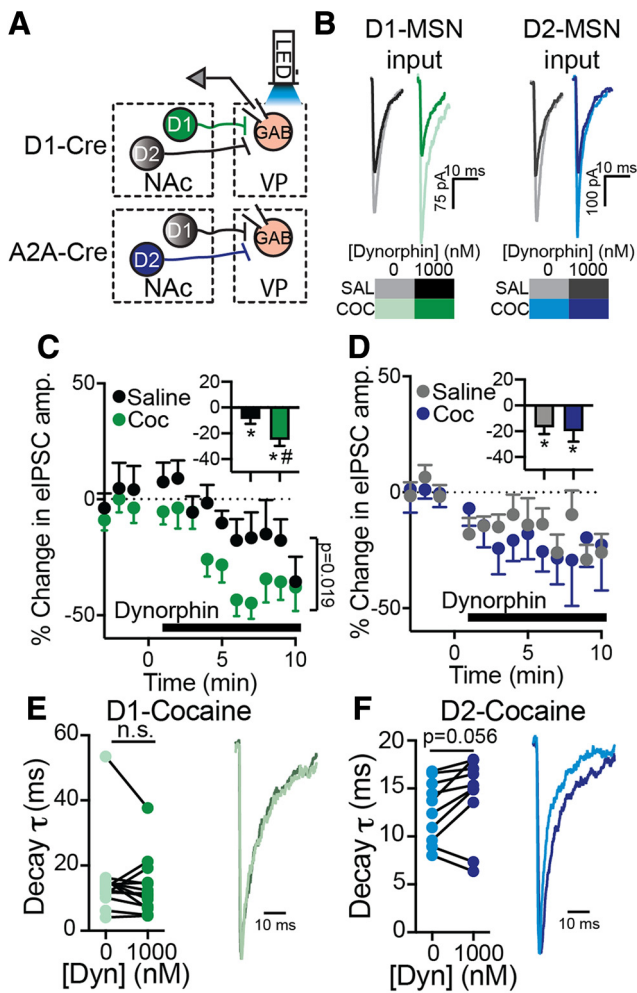


Figure 6. Dynorphin suppresses GABA neurotransmission from D1-MSN and D2-MSN terminals in the VP in saline and cocaine-withdrawn mice. **A**, Schematic representation of recording conditions. Chr2 was expressed selectively in D1-MSNs (using D1-Cre mice) or D2-MSNs (using A2A-Cre mice) and the terminals were activated by 470 nm LED light in the VP while recording from VP_{GABA} neurons (GAB). **B**, Representative traces of optogenetically-evoked IPSCs recorded from VP_{GABA} neurons while activating terminals of D1-MSNs (green) or D2-MSNs (blue). **C**, Time course and average effect (inset) of 1000 nM dynorphin on D1-MSN GABA neurotransmission in the VP of saline mice (black) or mice that underwent cocaine CPP and withdrawal (green). Dynorphin suppressed GABA neurotransmission in both groups, but the suppression was stronger in the cocaine group (two-way, repeated-measures ANOVA, group main effects: $F_{(1,24)} = 6.29$; averages in insets: $*p = 0.04$ and $p = 0.0002$ for saline and cocaine mice, respectively, one-sample t test, comparing to 0% change; $\#p = 0.01$ comparing to saline). **D**, Dynorphin (1000 nM) suppressed GABA neurotransmission from D2-MSN terminals in the VP in both saline mice (gray) and after cocaine CPP and abstinence (blue; inset, averages: $*p = 0.01$ and $p = 0.04$ for saline and cocaine groups, respectively, one-sample t test comparing to 0% change). The effect was comparable between groups. **E**, Dynorphin (1000 nM) did not affect the decay time-constant of D1-MSN currents (paired t test, $t_{(12)} = 1.06$, $p = 0.31$). Right, Representative traces. **F**, Dynorphin (1000 nM) did not change the decay time-constant of D2-MSN currents (paired t test, $t_{(9)} = 2.18$, $p = 0.056$). Data collected from 10 to 13 cells in each group from 4 to 6 mice. n.s., not significant.

inputs to the VP and cocaine CPP and abstinence seems to enhance this suppression selectively in D1-MSN input to the VP.

Dynorphin effect on VP_{vGluT2} neurons is cocaine-dependent and different from its effect on VP_{GABA} neurons

Dynorphin is usually linked to the expression of avoidance (Land et al., 2008; Chavkin and Koob, 2016), whereas the activity of the VP is mostly linked to reward seeking (Hubner and Koob, 1990; Smith and Berridge, 2005; Tindell et al., 2005; Tachibana and

Hikosaka, 2012; Richard et al., 2016, 2018). Recent studies have identified a subgroup of neurons in the VP that, in contrast to the GABAergic majority, express the vesicular glutamate transporter 2 (vGluT2), thus making these cells glutamatergic (Geisler et al., 2007). Behavioral examinations revealed that VP_{vGluT2} neurons, in contrast to VP_{GABA} neurons, encode for aversive behavior (Faget et al., 2018; Tooley et al., 2018). As dynorphin and VP_{vGluT2} neurons both seem to encode aversion we examined whether dynorphin regulates GABA input to VP_{vGluT2} neurons differently than the general VP_{GABA} population and whether this regulation is impaired after cocaine CPP and abstinence (Fig. 7A,B). Our data show that in contrast to its effect on VP_{GABA} neurons (Fig. 2), dynorphin potentiated the evoked inhibitory input to VP_{vGluT2} neurons in drug-naïve mice by $74.86 \pm 10.8\%$ (Fig. 7C–E; two-way, repeated-measures ANOVA, main group effect comparing cocaine and saline groups: $F_{(1,113)} = 49.47$, $p < 0.0001$; unpaired t test comparing dynorphin effect between saline and cocaine mice: $t_{(9)} = 8.93$, $p < 0.0001$). Moreover, although cocaine CPP and abstinence enhanced the effect of dynorphin in the VP_{GABA} population (Fig. 2), it abolished the effect of dynorphin on VP_{vGluT2} neurons. We were not able to see any effect of dynorphin on the PPR, CV or decay time-constant of the eIPSC (data not shown), as well as effects on the amplitude or frequency of sIPSCs (Fig. 7F–K; Kolmogorov–Smirnov tests; saline mice: $D = 0.5$, $p = 0.33$ and $D = 0.29$, $p = 0.96$ for sIPSC amplitude and frequency, respectively; cocaine mice: $D = 0.3$, $p = 0.67$ and $D = 0.17$, $p = 0.99$ for sIPSC amplitude and frequency, respectively). Thus, we cannot determine whether the effect of dynorphin on the inhibitory input to VP_{vGluT2} neurons is based on a presynaptic or postsynaptic mechanism.

Discussion

The VP receives accumbal input from both D1- and D2-MSNs (Kupchik et al., 2015; Creed et al., 2016; Heinsbroek et al., 2017). The role of D2-MSN terminals in the VP in motivation and drug addiction has been studied to some extent (Bock et al., 2013; Creed et al., 2016; Soares-Cunha et al., 2016; Heinsbroek et al., 2017; Gallo et al., 2018) but the role of the newly found D1-MSN terminals in the VP in addiction to cocaine is not yet known. Here we investigated the long-term changes induced by cocaine CPP and abstinence in the action of dynorphin, an opioid neuropeptide released from NAc D1-MSN axons (among other potential sources like the lateral hypothalamus, substantia nigra, and more; Khachaturian et al., 1982; Chou et al., 2001; Ho and Berridge, 2013; Thomas et al., 2018) in the VP. We found that dynorphin had cell-type-specific effects in the VP, with contrasting effects on VP_{GABA} and VP_{vGluT2} neurons in saline and cocaine mice (Fig. 7). In saline mice, dynorphin did not affect evoked GABA input to VP_{GABA} neurons but potentiated eIPSC amplitude in VP_{vGluT2} neurons. In contrast, after cocaine CPP and abstinence dynorphin suppressed eIPSCs in VP_{GABA} neurons, whereas it lost its potentiating effect on the inhibitory input to VP_{vGluT2} neurons. Dynorphin-induced suppression of eIPSCs in VP_{GABA} neurons was observed also when activating selectively the input from D1-MSNs and D2-MSNs with optogenetic tools, regardless of cocaine experience. Last, an examination of eIPSC parameters (PPR, CV, and decay time-constant) and the amplitude and frequency of sIPSCs indicated that the effects of dynorphin are most likely mediated by postsynaptic KORs. Overall, our data show that dynorphin regulates GABA input to the VP from different inputs and on different VP cell types, and that abstinence from cocaine causes drastic changes in the actions of dynorphin in the VP. These changes present a mechanism by which dynorphin in the VP may contribute to addictive behavior.

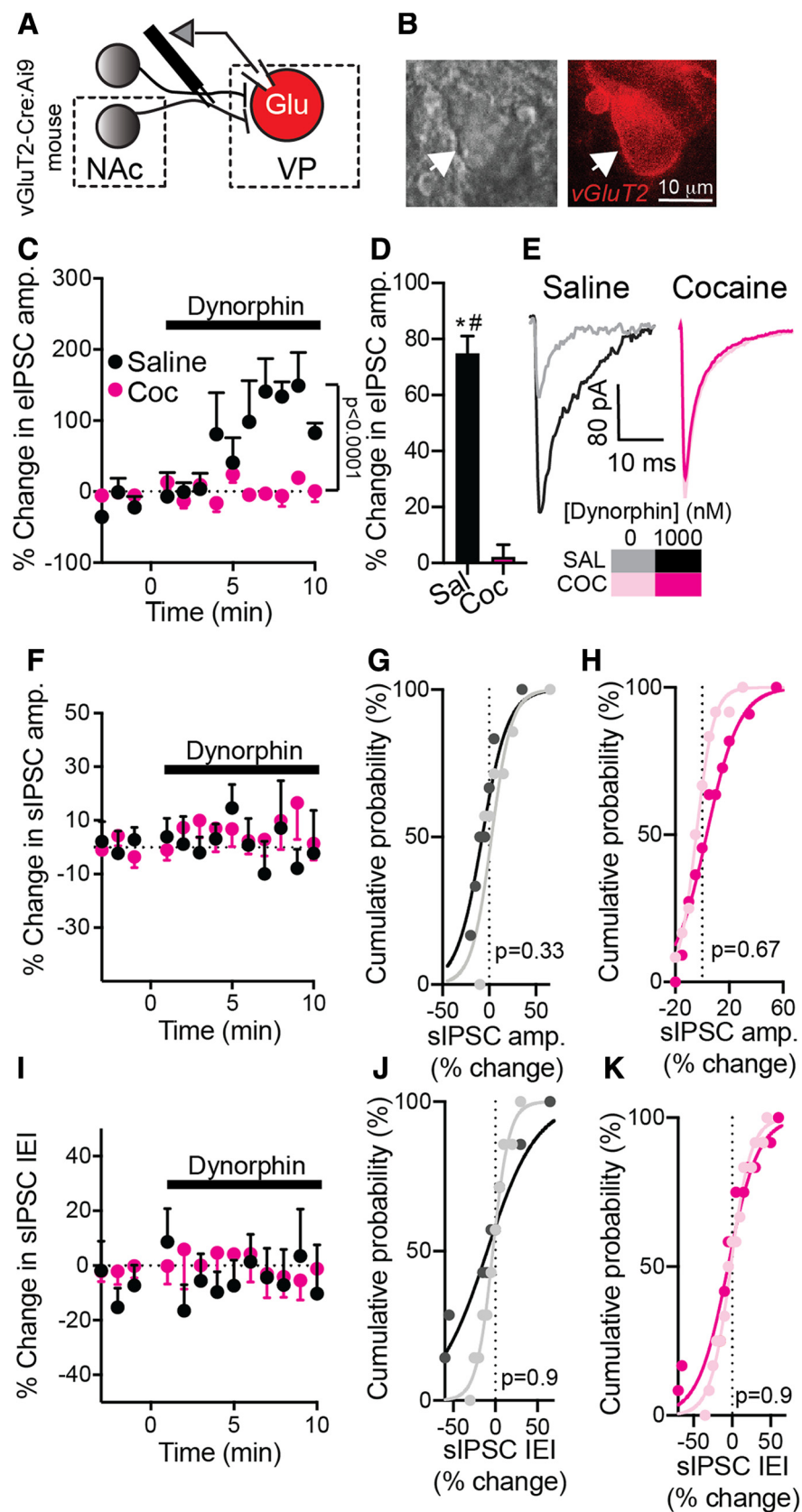


Figure 7. Cocaine CPP and abstinence abolishes dynorphin-induced potentiation of inhibitory input to VP_{vGluT2} neurons. **A**, Schematic representation of recording conditions. VP_{vGluT2} neurons (Glu) were identified in vGluT2-Cre: Ai9 mice (express tdTomato in vGluT2-expressing neurons) and patched while evoking synaptic transmission with electrical stimulations. **B**, Micrographs showing a VP_{vGluT2} neuron that was recorded from in bright field (left) and fluorescence (right). **C, D**, Time course (**C**) and average (**D**) effect of dynorphin (1000 nM) on eIPSCs in VP_{vGluT2} neurons. Dynorphin potentiated evoked GABA input to VP_{vGluT2}

Contrasting effects for dynorphin and enkephalin in the VP

The D1- and D2-MSN terminals in the VP release, in addition to GABA, also the opioid neuropeptides dynorphin and enkephalin, respectively (Zahm et al., 1985; Lu et al., 1998; Gerfen and Surmeier, 2011). Comparing our current data on dynorphin with our previous study on enkephalin in the VP (Kupchik et al., 2014), we find that these two neuropeptides act in different and complementary patterns. First, we show here that dynorphin modulates GABA neurotransmission in the VP through a postsynaptic mechanism while enkephalin acts presynaptically. Interestingly, this is in line with the pattern of expression of their respective receptors; dynorphin binds the KOR, which is expressed more robustly in the VP than in striatal neurons, whereas enkephalin acts on μ - and δ -opioid receptors, which are heavily expressed in striatal neurons (Mansour et al., 1994). Second, whereas withdrawal from cocaine blunts the effect of enkephalin on GABA transmission in the VP (Kupchik et al., 2014), cocaine CPP followed by abstinence potentiated the effect of dynorphin. Last, these two neuropeptides also differ in their regulation of GABA release from D1- and D2-MSN terminals in the VP after withdrawal from cocaine. In saline mice, both dynorphin and enkephalin depress GABA signaling from both D1 and D2-MSNs. However, after cocaine CPP and abstinence the effect of dynorphin on D1- and D2-MSN terminals persists (Fig. 6), whereas the enkephalinergic effect on D2-MSN terminals is abolished after withdrawal (Heinsbroek et al., 2017). Collectively, these findings draw an interesting picture, in which exposure to cocaine followed by a period of withdrawal preserves and even potentiates (for D1-MSNs) the postsynaptic effect of dynorphin on D1- and D2-MSN synapses on VP_{GABA} neurons, whereas it abolishes the presynaptic enkephalin-induced regu-

neurons of saline animals (time course, two-way repeated-measures ANOVA: $F_{(1,113)} = 49.5, p < 0.0001$; averages: $*p = 0.007$, one-sample *t* test comparing to 0% change, $\#p < 0.0001$ comparing to cocaine group). Abstinence from cocaine abolished this effect. **E**, Representative traces of evoked IPSCs in VP_{vGluT2} neurons corresponding to the data in **C** and **D**. **F–K**, Dynorphin did not affect the amplitude (**F–H**) or IEI (**J–K**) of sIPSCs recorded in VP_{vGluT2} neurons in either saline or cocaine mice (time courses, tested with two-way repeated-measures ANOVA; cumulative curves, with Kolmogorov–Smirnov test, lines are fitted sigmoidal curves running from 0 to 100). Data from 7 to 12 cells and 3–4 mice per group for all conditions.

lation of GABA release specifically from D2-MSN but not D1-MSN terminals. This may imply an increase in the influence of the dynorphinergic system in the VP after cocaine CPP and abstinence.

Dynorphin regulation of GABA neurotransmission in the VP

Our results show a clear link between the dynorphin activation of KORs in the VP and GABA neurotransmission. However, the underlying mechanisms are yet to be explored. Our data suggest that the modulation of IPSCs in the VP by dynorphin is mediated through a postsynaptic mechanism (Figs. 2, 3). This is in line with previous anatomical studies showing robust expression of KORs in VP neurons (Allen Institute for Brain Science, 2004), but to the best of our knowledge this is the first report of a dynorphin-induced postsynaptic modulation of synaptic neurotransmission. An abundance of studies show that KORs modulate the release of various neurotransmitters in different brain regions (Ogura and Kita, 2000; Mu et al., 2011; Li et al., 2012; Gilpin et al., 2014; Brooks and O'Donnell, 2017; Tejada et al., 2017; Kash and Li, 2018; Matzeu et al., 2018; Tejada and Bonci, 2019). In all of these studies the mechanism is presynaptic, even when the KORs show a postsynaptic effect on the passive electrical properties of the recorded neurons (Ogura and Kita, 2000).

Our data show that dynorphin has opposing effects on GABA neurotransmission in VP_{GABA} and VP_{vGluT2} neurons. Although the suppressive effect of dynorphin on GABA input to VP_{GABA} neurons joins many other studies showing that dynorphin inhibits neurotransmitter release, the potentiating effect of dynorphin on GABA neurotransmission in VP_{vGluT2} neurons is surprising. How could dynorphin exert opposing effects on GABA neurotransmission in these two neuronal populations? The answer may lie in the diversity of intracellular cascades that can be activated by KORs (Bruchas and Chavkin, 2010), most of which can influence $GABA_A$ receptor function. Interestingly, although some cascades are expected to diminish synaptic $GABA_A$ receptor number or function, others may enhance the efficacy of synaptic inhibition. For example, activation of KORs reduces Ca^{2+} influx into the cells (Rusin et al., 1997). Ca^{2+} is known to promote the activation of gephyrin, which recruits $GABA_A$ receptors to the synapse (Zacchi et al., 2014; Choi and Ko, 2015). Therefore, such cascade is expected to weaken the inhibitory synapse. In contrast, binding to KORs activates the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) pathway (Bruchas and Chavkin, 2010). ERK, in turn, may inhibit $GABA_A$ receptor internalization (Jurd and Moss, 2010) and promote the recruitment of $GABA_A$ receptors to the synapse through clustering gephyrin (Battaglia et al., 2018). Therefore, this cascade would potentiate the GABAergic synapse. This diversity in the possible outcomes of KOR activation may underlie the different effects of dynorphin in VP_{GABA} and VP_{vGluT2} neurons. At this point, the exact mechanisms governing either postsynaptic action of dynorphin in the VP is not known and clarifying the mechanisms in each cell type requires further investigation.

It should be noted that in this study we did not mimic the physiological release of dynorphin. Indeed, dynorphin release in the VP may be dynamic, depend on the rate of cellular activity and differ between different conditions or cell types. Thus, although we propose here a cellular mechanism of action for dynorphin in the VP, its *in vivo* influence on behavior is likely more complex and cannot be inferred directly from our data.

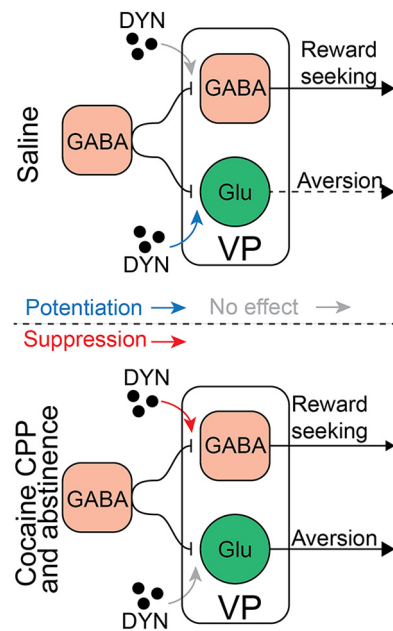


Figure 8. A possible mechanism of action for dynorphin in the VP before and after withdrawal from cocaine. Top, Effect of dynorphin on GABA neurotransmission on VP_{GABA} and VP_{vGluT2} neurons in cocaine-naïve animals. Dynorphin does not affect inhibitory input to VP_{GABA} cells but potentiates the inhibitory input onto VP_{vGluT2} neurons. Thus, release of dynorphin in the VP of drug-naïve mice is expected to decrease the activity of VP_{vGluT2} neurons. Because activation of VP_{vGluT2} neurons was shown before to drive aversive behavior, the action of dynorphin may translate to a decrease in aversion. In contrast, after cocaine CPP and abstinence (bottom) dynorphin suppresses the inhibitory input on VP_{GABA} neurons but ceases to modulate the inhibitory drive on VP_{vGluT2} neurons. Thus, spilling dynorphin in the VP after cocaine CPP and abstinence is expected to disinhibit VP_{GABA} neurons while not affecting VP_{vGluT2} neurons. In other words, it may shift the VP_{GABA}/VP_{vGluT2} balance in favor of the VP_{GABA} neurons. Because activation of VP_{GABA} neurons is known to promote reward-seeking behavior, the action of dynorphin in the VP after cocaine CPP and abstinence may favor reward-seeking behavior.

Potential influences of VP dynorphin on reward and aversion

A possible interpretation of our data is that dynorphin release in the VP may influence the production of drug-seeking and aversive behavior through its differential effects on VP_{GABA} and VP_{vGluT2} neurons (Figs. 2, 7, summarized in 8). Moreover, this influence may be altered by exposure to cocaine. This interpretation relies on recent studies showing that VP_{GABA} neurons promote reward-seeking behaviors, whereas VP_{vGluT2} neurons encode aversive states (Faget et al., 2018; Tooley et al., 2018; Wulff et al., 2019). In saline-treated mice, dynorphin did not affect the inhibitory input to VP_{GABA} neurons (Fig. 2) but potentiated the inhibitory input to VP_{vGluT2} neurons (Fig. 7). This may imply that in control mice the release of dynorphin promotes inhibition of VP_{vGluT2} neurons, which would be expected to result in decreased aversive behavior (Fig. 8). After cocaine CPP and abstinence, on the other hand, dynorphin acts in a completely different manner; its modulation of VP_{vGluT2} neurons is abolished but it now depresses the inhibitory input on VP_{GABA} neurons. In other words, dynorphin ceases to modulate the presumed “aversive pathway” but now promotes the activation of VP_{GABA} neurons, which drive reward seeking (Fig. 8). Note that both in control mice and after cocaine CPP and abstinence, dynorphin seems to shift the ratio between VP_{GABA} and VP_{vGluT2} activity in favor of the VP_{GABA} neurons. In saline mice, this is a result of increasing the inhibition onto VP_{vGluT2} neurons. In mice that underwent cocaine CPP and abstinence this effect is attributed to the disinhibition of VP_{GABA} neurons. Taking into account

the opposing behavioral influences of VP_{GABA} and VP_{vGluT2} neurons mentioned above, these data may imply that release of dynorphin in the VP may have an overall rewarding effect. Because the majority of the dynorphin in the VP presumably originates in NAc D1-MSNs, dynorphin release may allow the D1-MSN input to the VP to shift the VP_{GABA}/VP_{vGluT2} balance in favor of VP_{GABA} and thus possibly contribute to the promotion of drug-seeking behavior.

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