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ARTICLE

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Cocaine induces input and cell-type-specific synaptic plasticity in ventral pallidum-projecting nucleus accumbens medium spiny neurons

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Cocaine use and abstinence induce long-term synaptic alterations in the excitatory input to nucleus accumbens (NAc) medium spiny neurons (MSNs). The NAc regulates reward-related behaviors through two parallel projections to the ventral pallidum (VP)—originating in D1 or D2-expressing MSNs (D1-MSNs$_{\rightarrow VP}$; D2-MSNs$_{\rightarrow VP}$). The activity of these projections depends on their excitatory synaptic inputs, but it is not known whether and how abstinence from cocaine affects the excitatory transmission to D1-MSNs$_{\rightarrow VP}$ and D2-MSNs$_{\rightarrow VP}$. Here we examined different forms of cocaine-induced synaptic plasticity in the inputs from the basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) to NAc D1-MSNs$_{\rightarrow VP}$ and putative D2-MSNs$_{\rightarrow VP}$ (pD2-MSNs$_{\rightarrow VP}$) in the core and shell subcompartments of the NAc. We used the whole-cell patch-clamp technique to record excitatory postsynaptic currents from D1-tdTomato mice injected with ChR2 in either the BLA or the mPFC and retrograde tracer (RetroBeads) in the VP. We found that cocaine conditioned place preference (CPP) followed by abstinence potentiated the excitatory input from the BLA and mPFC to both D1-MSNs$_{\rightarrow VP}$ and pD2-MSNs$_{\rightarrow VP}$. Interestingly, while the strengthening of the inputs to D1-MSNs$_{\rightarrow VP}$ was of postsynaptic origin and manifested as increased AMPA to NMDA ratio, in pD2-MSNs$_{\rightarrow VP}$ plasticity was predominantly presynaptic and was detected as changes in the paired-pulse ratio and coefficient of variation. Lastly, some of the changes were sex-specific. Overall our data show that abstinence from cocaine changes the excitatory inputs to both D1-MSNs$_{\rightarrow VP}$ and pD2-MSNs$_{\rightarrow VP}$ but with different mechanisms. This may help understand how circuits converging into the VP change after cocaine exposure.

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INTRODUCTION

Evidence from animal models indicates that long-term plasticity plays a role in the behavioral effects of cocaine [1–5]. A hallmark of this plasticity is the increase in the ratio between the current flowing through AMPA receptors (AMPARs) and NMDA receptors (A/N ratio) in inputs to nucleus accumbens (NAc) projection neurons [6–13]. Though the NAc projection neurons comprise of two distinct populations—medium spiny neurons (MSNs) expressing the D1 dopamine receptor (D1-MSNs) or MSNs expressing the D2 dopamine receptor (D2-MSNs) [14, 15], the increase in the A/N ratio has been repeatedly reported to occur primarily in D1-MSNs [8, 9, 12, 16]. Since D1 and D2-MSNs differentially modulate reward-related behaviors—D1-MSNs promote and D2-MSNs attenuate drug seeking [12, 17]—plasticity at either of them can be functionally meaningful and represent a different mechanism of how drugs recruit neural circuits within the reward system.

The ventral pallidum (VP) is the main target of NAc projections [18, 19] and is necessary for reward-related behaviors [20, 21]. Both D1-MSNs and D2-MSNs project to the VP [15, 22–25] and previous studies have shown differential effects of cocaine exposure on each of these inputs in the VP [26, 27]. However, it is possible that these two circuits that converge into the VP differ also upstream of the VP, at the excitatory inputs to different VP-projecting NAc MSNs. Since the increase in A/N ratio after cocaine exposure was reported for the input to NAc D1-MSNs but not D2-MSNs [9, 12, 16], it is possible that exposure to cocaine induces plasticity only at inputs to VP-projecting D1-MSNs (D1-MSNs$_{\rightarrow VP}$) without affecting inputs to VP-projecting D2-MSNs (D2-MSNs$_{\rightarrow VP}$). Such finding would suggest changes in the balance between D1-MSN and D2-MSN inputs to the VP after cocaine exposure. Importantly, previous research has mostly focused on postsynaptic plasticity and often did not look for plasticity of presynaptic origin in the inputs to the NAc. It thus remains to be determined whether other forms of synaptic plasticity take place following cocaine exposure and abstinence in the inputs to D1-MSNs$_{\rightarrow VP}$ and D2-MSNs$_{\rightarrow VP}$.

A recent study suggests that repeated cocaine injections potentiate the input from the basolateral amygdala (BLA) on D1-MSNs$_{\rightarrow VP}$ in the NAc shell (NAshell) [24], but it is not known whether this is a long-term change that persists also after abstinence. It is also not known whether other inputs to VP-

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projecting NAc MSNs change after abstinence, whether these effects are restricted to the NAshell, whether plasticity can be seen also in inputs to VP-projecting D2-MSNs and whether plasticity is sex-specific. In this work we examine synaptic plasticity of presynaptic and postsynaptic origins at the excitatory synapses that the BLA and the medial prefrontal cortex (mPFC) make on VP-projecting NAshell and NAc core (NAcore) D1- and putative D2-MSNs (pD2-MSNs) of male and female mice after abstinence from repeated cocaine exposure. We show significant changes in inputs from both BLA and mPFC to D1-MSNs$_{\rightarrow VP}$ and pD2-MSNs$_{\rightarrow VP}$, with postsynaptic plasticity occurring primarily on D1-MSNs$_{\rightarrow VP}$ and presynaptic plasticity occurring primarily on pD2-MSNs$_{\rightarrow VP}$.

MATERIALS AND METHODS

Experimental model and subject details

All mice were experimentally naïve Drd1a-tdTomato BAC transgenic mice (Stock #016204, The Jackson Laboratories) crossed with wild-type C57BL/6J mice (Envigo, Israel) and bred in-house. Males and females, aged 10–12 weeks were group-housed and under a 12 h reverse light cycle (lights off at 8:00 a.m.) with food and water *ad libitum*. All procedures were approved by the Research Animal Care Committee of the Hebrew University.

Stereotaxic injections

Mice were anesthetized with isoflurane and fixed in a stereotaxic frame (Kopf, Model 940). Two sets of bilateral holes were drilled into the skull. One set served to microinject the viral construct (AAV2-hSyn-hChR2 (H134R)-EYFP, 5.7×10^{12} vg/ml, generated by Karl Deisseroth and sold by University of North Carolina Viral Core) through a 33 ga NanoFil syringe (World Precision Instruments; 300 nl per hemisphere, 100 nl/min, needle retracted 5 min after injection terminated) into the BLA or the mPFC. The second set of holes served to microinject the retrograde tracer red RetroBeads™ (Lumafluor, Durham, NC) through a 30 ga syringe (Hamilton: 300 nl per hemisphere, 300 nl/min, needle retracted 10 min after injection terminated) into the subcommissural VP. Injections coordinates in millimeters relative to Bregma (anterior/posterior, medial/lateral, dorsal/ventral): BLA = −1.4, ±3.38, −5.14; mPFC = +1.9, ±0.3, −2.35; VP = +0.4, ±1.15, −5.15.

Cocaine conditioned place preference

After two weeks of acclimation to the reverse light cycle and recovery from surgery, mice were trained in the unbiased cocaine conditioned place preference (CPP) procedure as described previously [23] (Fig. 1A). On the first day, mice were allowed to freely explore both sides of a 30 cm × 30 cm arena, divided in two, each side with a different wall pattern and floor texture (Fig. 1A). On the following days, experimental mice received one daily injection of either cocaine (15 mg/kg, i.p.) or saline such that cocaine was always given in one side of the box (the "cocaine-paired" side) and saline in the other. Cocaine-paired sides were counterbalanced for pattern and side. Cocaine/saline injections alternated daily until each mouse received 4 of each. Control mice received 8 injections of saline. Then, mice were left in their cages for 14 days (abstinence) before electrophysiological recordings began or the CPP test was performed. In the test, mice were allowed to move freely between the two sides of the arena for 15 min. Movement was tracked using MediaRecorder (Noldus, the Netherlands), analyzed using Optimouse software [28] and CPP scores were calculated off-line as the ratio between the difference in time spent between the cocaine-paired and unpaired sides and the total time [CPP score = (time in paired zone − time in unpaired zone)/(time in paired zone + time in unpaired zone)].

Slice preparation

Mice were decapitated after being deeply anesthetized with 150 mg/kg ketamine HCl. Then, coronal slices (200 µm) of the NAc, VP and either the BLA or the mPFC were prepared (VT1200S vibratome, Leica). Slices were stored in a vial containing artificial cerebrospinal fluid (in mM: 126 NaCl, 1.4 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 2.5 KCl, 2.0 Na-pyruvate, 0.4 ascorbic acid, bubbled with 95% O_2 and 5% CO_2) and a mixture of 5 mM kynurenic acid and 50 µM d-AP5, at room temperature (22–24 °C) until recording. VP, mPFC and BLA slices were used to confirm injection accuracy, NAc slices were used for whole-cell patch-clamp recordings.

Whole-cell recordings

All recordings were collected at 32 °C (TC-344B, Warner Instruments). Slices were left to rest for at least 10 min after being transferred from the vials to the recording bath to allow washout of kynurenic acid and AP5 that were present in the vials. The NAc, VP, BLA, and mPFC were identified using a mouse brain atlas [29]. Neurons were visualized with a BX51WI microscope (Olympus). Inhibitory synaptic transmission was blocked with picrotoxin (0.1 mM, catalog # P-325 Alomone Labs). MultiClamp 700B (Molecular Devices) was used to record excitatory postsynaptic currents (EPSCs) in whole-cell configuration. Glass microelectrodes (1.6–2 MΩ) were filled with internal solution (in mM: 128 cesium methanesulfonate, 10 HEPES potassium, 1 EGTA, 1 $MgCl_2$, 10 NaCl, 2.0 Mg-ATP, 0.3 Na-GTP, 1 QX-314, and 0.3 mM spermine for AMPAR current rectification recordings at pH 7.2–3 and ~280 mOsm). VP-projecting MSNs were identified by the fluorescence of red RetroBeads (Fig. 1E). D1-MSNs were identified by the fluorescence of tdTomato and neurons lacking such fluorescence were assumed to be putative D2-MSNs. Recordings in NAcore neurons were performed in a radius of up to 300 µm from the center of the anterior commissure while NAshell recordings concentrated in the medial NAshell, approximately 700 µm medially or ventromedially to the anterior commissure. Recordings started at least 10 min after whole-cell configuration was achieved to allow for diffusion of the internal solution to remote dendrites. Data were acquired at 10 kHz and filtered at 2 kHz using AxoGraph X software (AxoGraph Scientific). To evoke EPSCs we used a 470 nm LED light source (Mightex Systems; 0.1–1 ms in duration) directed at the slice through the 60× objective. The stimulation intensity was set to evoke 50% of maximal EPSC at −80 mV. Recordings were collected every 20 s. Series resistance (Rs), measured using a − 2 mV hyperpolarizing step (10 ms) applied with each stimulus, and holding current were monitored online. Recordings with unstable Rs, or with Rs higher than 20 MΩ were discarded.

For each cell, paired pulses (100 ms interval) were first recorded at −80 mV. This allowed us to ensure response stability and to calculate the paired-pulse ratio—the ratio between the peaks of the second and the first pulses. We also calculated from the first response of the pair the coefficient of variation of the peak using the Microsoft Excel standard deviation formula for a sample (STDEV) divided by the cell mean (20–25 events per cell). Then, membrane potential was gradually increased until +40 mV. To allow for stabilization of cell parameters, recordings resumed 5 min after reaching +40 mV. First, we recorded the total current mediated by both AMPA and NMDA receptors. Then, NMDA current was blocked by bath application of d-AP5 (50 µM, catalog # ab120003, Abcam). Recording resumed 2 min following bath application. To calculate the NMDA current, we subtracted the AMPAR current from the total current at +40 mV. For the AMPA and NMDA currents we measured, apart from the peak amplitude, the time constant of the decay (by fitting an exponential) and the total charge (by calculating the area under the synaptic current curve) using the Axograph X software.

A separate set of cells was used for the evaluation of AMPAR current rectification. AMPAR current was recorded at −70 mV, 0 mV and +40 mV. Membrane potential was gradually increased and recordings started 5 min after the new holding potential was reached. To calculate the rectification index, we first calculated the predicted AMPA current at +40 mV based on the currents recorded at −70 mV and 0 mV. Assuming there is no rectification, the slope between the currents at −70 mV and 0 mV is equal to the slope between currents at −70 mV and 0 mV -

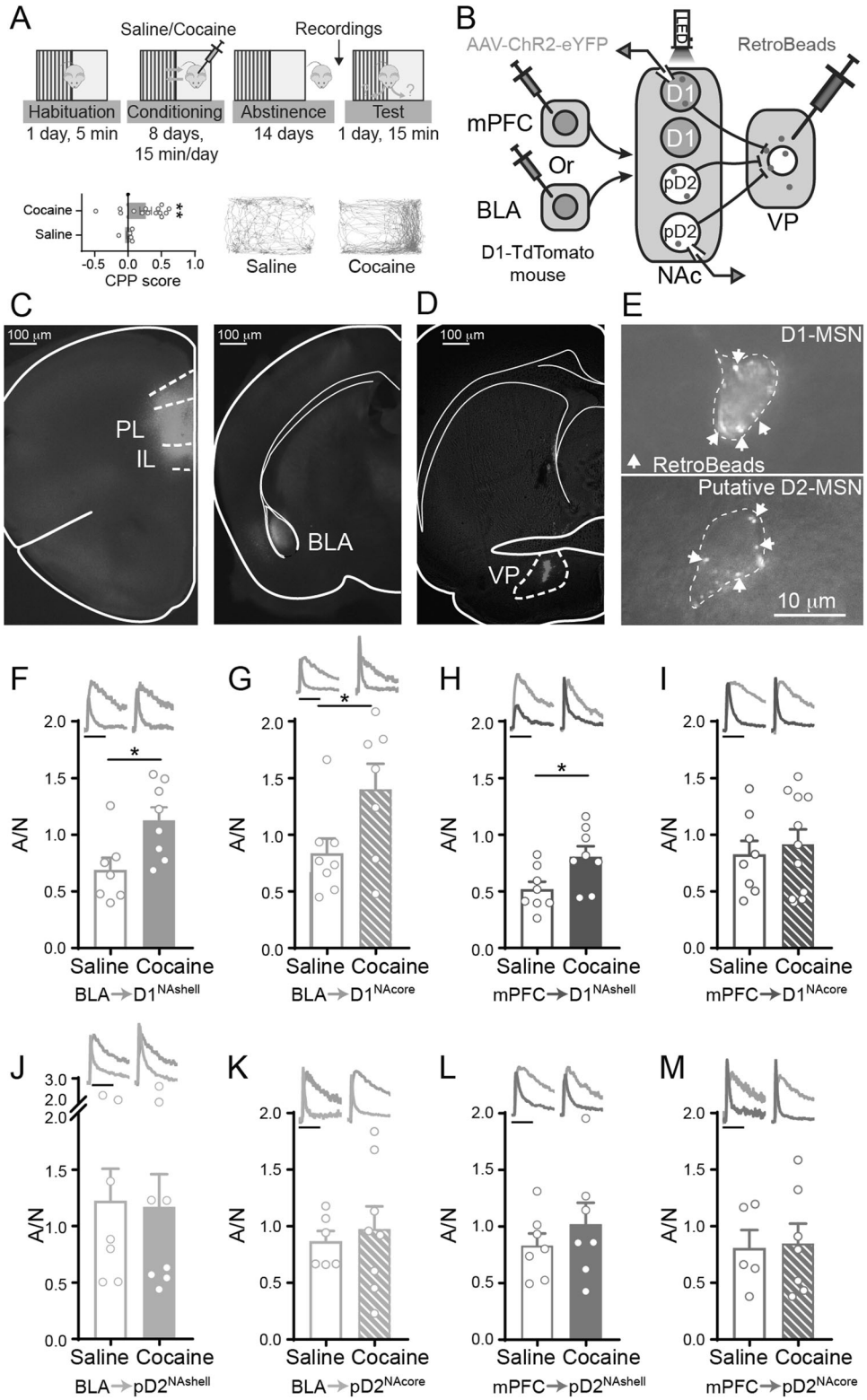
$$\frac{\hat{I}_{+40\ mV} - I_{0\ mV}}{V_{+40\ mV} - V_{0\ mV}} = \frac{I_{0\ mV} - I_{-70\ mV}}{V_{0\ mV} - V_{-70\ mV}} \quad (1)$$

where $\hat{I}_{+40\ mV}$ represents the predicted current at a holding potential of +40 mV, $I_{0\ mV}$ and $I_{-70\ mV}$ represent the measured currents at 0 and −70 mV, respectively, and V represents the holding membrane potential. From Eq. 1 we can calculate the predicted AMPA current at +40 mV –

$$\hat{I}_{+40\ mV} = \frac{11}{7} I_{0\ mV} - \frac{4}{7} I_{-70\ mV} \quad (2)$$

Then, the rectification index (RI) is the ratio between the observed ($I_{+40\ mV}$) and the predicted ($\hat{I}_{+40\ mV}$) current at a holding potential of +40 mV –

$$RI = \frac{I_{+40\ mV}}{\hat{I}_{+40\ mV}}$$



Quantification and statistical analysis

All statistical analyses were performed using GraphPad Prism 7.03 (GraphPad Software Inc.). Parametric statistics (Student's *t* test and two-way ANOVA) was used with p values < 0.05 considered significant. Statistical tests are indicated in the figure legends. Bar graphs represent mean ± SEM. Each dot represents data from one cell. All groups consist 4–11 cells from 3–6 mice.

RESULTS

The excitatory input from the mPFC and BLA on VP-projecting D1-MSNs is stronger after cocaine CPP and abstinence

The mPFC and BLA provide significant input to both NAc D1-MSNs and D2-MSNs [24, 30, 31], but it is not known whether these inputs specifically to VP-projecting MSNs ($MSNs_{\rightarrow VP}$) show plasticity after

Fig. 1 The excitatory input from the mPFC and BLA on VP-projecting D1-MSNs is stronger after cocaine CPP and abstinence. A Top—Cocaine-induced conditioned place preference included 1 day of habituation to the arena, 8 days of intraperitoneal injections of either cocaine (Coc) or saline (Sal) (alternating daily in separate compartments) and 14 days of abstinence. Then, mice were either tested for side preference or sacrificed for the physiological experiments. Bottom left—mice that received cocaine showed significant preference for the cocaine-paired side. Saline-injected mice did not show preference for either side (one-sample Student's *t* test comparing to a CPP score of 0, $p = 0.009$ for cocaine-injected mice). Bottom right—Representative trajectory traces during the test for cocaine and saline mice. **B** Schematic representation of the recording setup. D1-TdTomato mice (expression of TdTomato is indicated in light red) were injected with AAV-ChR2-eYFP (in green) to either the mPFC or the BLA and with red RetroBeads (red dots) to the VP. Ex vivo electrophysiological recordings were performed on identified VP-projecting D1 or pD2-MSNs in the NAshell and NAcore. **C** Coronal sections with ChR2 injection sites in the mPFC (left) and BLA (right). Prelimbic cortex; PL, infralimbic cortex; IL. **D** Coronal section of the subcommissural VP showing the injection site of the red RetroBeads. **E** Images of D1-MSN$_{\rightarrow VP}$ with TdTomato expression and RetroBeads (top) and pD2-MSN$_{\rightarrow VP}$ containing only RetroBeads (bottom). Cocaine CPP and abstinence increased A/N ratio in BLA→NAc synapses on D1-MSNs$_{\rightarrow VP}$ both in the NAshell (**F**) and in the NAcore (**G**). Cocaine CPP and abstinence increased A/N ratio in mPFC→NAc synapses in NAshell D1-MSNs$_{\rightarrow VP}$ (**H**) but not in NAcore D1-MSNs$_{\rightarrow VP}$ (**I**). In pD2-MSNs$_{\rightarrow VP}$, cocaine CPP and abstinence did not alter A/N ratio in both BLA→NAc synapses (**J**, **K**) and mPFC→NAc synapses (**L**, **M**) in the NAshell (**J**, **L**) and NAcore (**K**, **M**). Representative traces of AMPA (in color) and NMDA (in gray) receptor currents are shown above each bar. In each comparison currents were normalized to NMDA peaks. Scale bar: 100 ms. For all groups cell number ranged from 5 to 11 and data were collected from 3–6 mice. Data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$. All points are shown.

abstinence from cocaine (but before being re-introduced to the CPP box after abstinence) (Fig. 1A). To examine this, we microinjected AAV-ChR2-eYFP (ChR2) to either the BLA or the mPFC and the retrograde tracer red RetroBeads to the VP of D1-TdTomato mice (Fig. 1B–D). Thus, we could activate optogenetically either of the two afferents while recording AMPA and NMDA receptor-mediated currents from identified D1-MSNs$_{\rightarrow VP}$ or pD2-MSNs$_{\rightarrow VP}$ in the core and shell subcompartments of the NAc (Fig. 1E) [pD2-MSNs were defined as NAc neurons not expressing tdTomato, based on the observation that MSNs make ~95% of the neurons in the NAc [32, 33], the small overlap between D1-expressing and D2-expressing MSNs [15, 32, 34] and the physiological differences between MSNs and NAc interneurons [33]].

To evaluate the AMPA/NMDA (A/N) ratio we stimulated the specific input optogenetically and recorded the postsynaptic current at a holding potential of +40 mV once without blockers and then with the NMDA blocker AP5 to yield the AMPA current. NMDA currents were derived from the subtraction of the AMPA current from the total current. Our data show that the A/N ratio in BLA → D1-MSNs$_{\rightarrow VP}$ synapses in NAshell (Fig. 1F) and NAcore (Fig. 1G) were elevated after cocaine CPP and abstinence (unpaired two-tailed Student's *t* tests: $t_{(13)} = 2.70$, $p = 0.02$ for BLA → D1-MSNs$_{\rightarrow VP}$ in NAshell; $t_{(13)} = 2.25$, $p = 0.04$ for BLA → D1-MSNs$_{\rightarrow VP}$ in NAcore). This was accompanied by increase in the amplitude of the AMPA but not NMDA currents (Table S1). A similar elevation of A/N was seen also in mPFC→D1-MSNs$_{\rightarrow VP}$ synapses in NAshell (Fig. 1H) but not NAcore (Fig. 1I) ($t_{(14)} = 2.53$, $p = 0.02$ for mPFC→D1-MSNs$_{\rightarrow VP}$ in NAshell; $t_{(17)} = 0.48$, $p = 0.64$ for mPFC→D1-MSNs$_{\rightarrow VP}$ in NAcore). The mPFC input to D1-MSNs$_{\rightarrow VP}$ in the NAshell also showed an increase in the AMPA and NMDA peak amplitudes and charge conducted (Table S2).

Conversely, we did not observe changes in BLA or mPFC inputs to pD2-MSNs$_{\rightarrow VP}$ in the NAcore or NAshell (Fig. 1J–M; Unpaired two-tailed Student's *t* tests: $t_{(13)} = 0.13$, $p = 0.90$ for BLA → pD2-MSNs$_{\rightarrow VP}$ in NAshell; $t_{(12)} = 0.46$, $p = 0.66$ for BLA → pD2-MSNs$_{\rightarrow VP}$ in NAcore; $t_{(12)} = 0.86$, $p = 0.41$ for mPFC→pD2-MSNs$_{\rightarrow VP}$ in NAshell; $t_{(10)} = 0.17$, $p = 0.87$ for mPFC→pD2-MSNs$_{\rightarrow VP}$ in NAcore). In addition, we did not detect any changes in the time constants of the currents (Tables S1 and S2). These data resemble earlier data showing that glutamatergic inputs to the NAc strengthen specifically on D1-MSNs after cocaine exposure [8, 9, 12, 16] and suggest that this is also true specifically for MSNs that project to the VP.

Cocaine CPP and abstinence increases the rectification of AMPA receptor-mediated currents in mPFC inputs to NAshell D1-MSN$_{\rightarrow VP}$

Surface expression of calcium-permeable GluA2-lacking AMPARs is often elevated in the NAc following cocaine self-administration and prolonged withdrawal [9, 35–37]. This has also been observed in animal models of non-contingent cocaine injections [37–39] although not consistently [40]. The recruitment of GluA2-lacking AMPARs was also linked in several studies to changes in A/N ratio in the NAc [35, 37, 39]. GluA2-lacking AMPARs are calcium-conducting inwardly-rectifying channels [41, 42]—they conduct less current in depolarized membrane potentials. This allows to identify their presence in the synapse by calculating the rectification index of the glutamatergic input. A change in the rectification index indicates rectified current and may imply the recruitment of GluA2-lacking AMPARs. Therefore, we next explored whether the increase in A/N ratio seen in BLA and mPFC synapses on D1-MSNs$_{\rightarrow VP}$ is accompanied by a change in the rectification index. In order to detect changes in rectification we added spermine to the internal solution and recorded AMPAR currents at holding potentials of +40 mV, 0 mV and −70 mV. The rectification index was calculated as the ratio between the observed and the predicted peak current at +40 mV [43, 44] (see Methods). Our data show that cocaine CPP and abstinence decreased the rectification index in the synapse between mPFC projections and D1-MSNs$_{\rightarrow VP}$ in the NAshell (Fig. 2C; Unpaired two-tailed Student's *t* test: $t_{(11)} = 2.47$, $p = 0.03$). However, it did not change the rectification in mPFC→D1-MSNs$_{\rightarrow VP}$ synapses in the NAcore (Fig. 2D; Unpaired two-tailed Student's *t* test: $t_{(10)} = 0.11$, $p = 0.91$) or in BLA→D1-MSNs$_{\rightarrow VP}$ synapses in the NAshell (Fig. 2A; Unpaired two-tailed Student's *t* test: $t_{(12)} = 0.56$, $p = 0.59$) and NAcore (Fig. 2B; Unpaired two-tailed Student's *t* test: $t_{(13)} = 1.01$, $p = 0.33$). It also did not alter the rectification index of AMPAR currents in the synapses of BLA and mPFC with pD2-MSNs$_{\rightarrow VP}$ (Fig. 2; Unpaired two-tailed Student's *t* tests: $t_{(12)} = 0.84$, $p = 0.42$ for BLA inputs to NAshell; $t_{(13)} = 0.64$, $p = 0.54$ for BLA inputs to NAcore; $t_{(10)} = 1.21$, $p = 0.25$ for mPFC inputs to NAshell; $t_{(9)} = 1.15$, $p = 0.28$ for mPFC inputs to NAcore). Thus, among the various synapses that showed an increase in the A/N ratio after cocaine CPP and abstinence, only the mPFC→D1-MSN$_{\rightarrow VP}$ synapse in the NAshell also showed rectification. This may indicate a different mechanism of long-term potentiation in the mPFC and BLA inputs to D1-MSNs$_{\rightarrow VP}$.

Note that the baseline rectification index in saline mice varied between the different synapses. Thus, in some synapses (mPFC→D1-MSNs$_{\rightarrow VP}$, mPFC→pD2-MSNs$_{\rightarrow VP}$ and BLA→D1-MSNs$_{\rightarrow VP}$, all in the NAcore) the rectification index was significantly lower than 1, indicating the presence of rectifying AMPARs (one-sample *t* test compared to 1, $p < 0.05$ for all three synapses). Nevertheless, cocaine did not affect the rectification in these synapses.

Presynaptic plasticity at the glutamatergic input to pD2-MSNs$_{\rightarrow VP}$ following cocaine CPP and abstinence

Changes in A/N ratio and in AMPAR current rectification are often considered manifestations of postsynaptic plasticity, yet

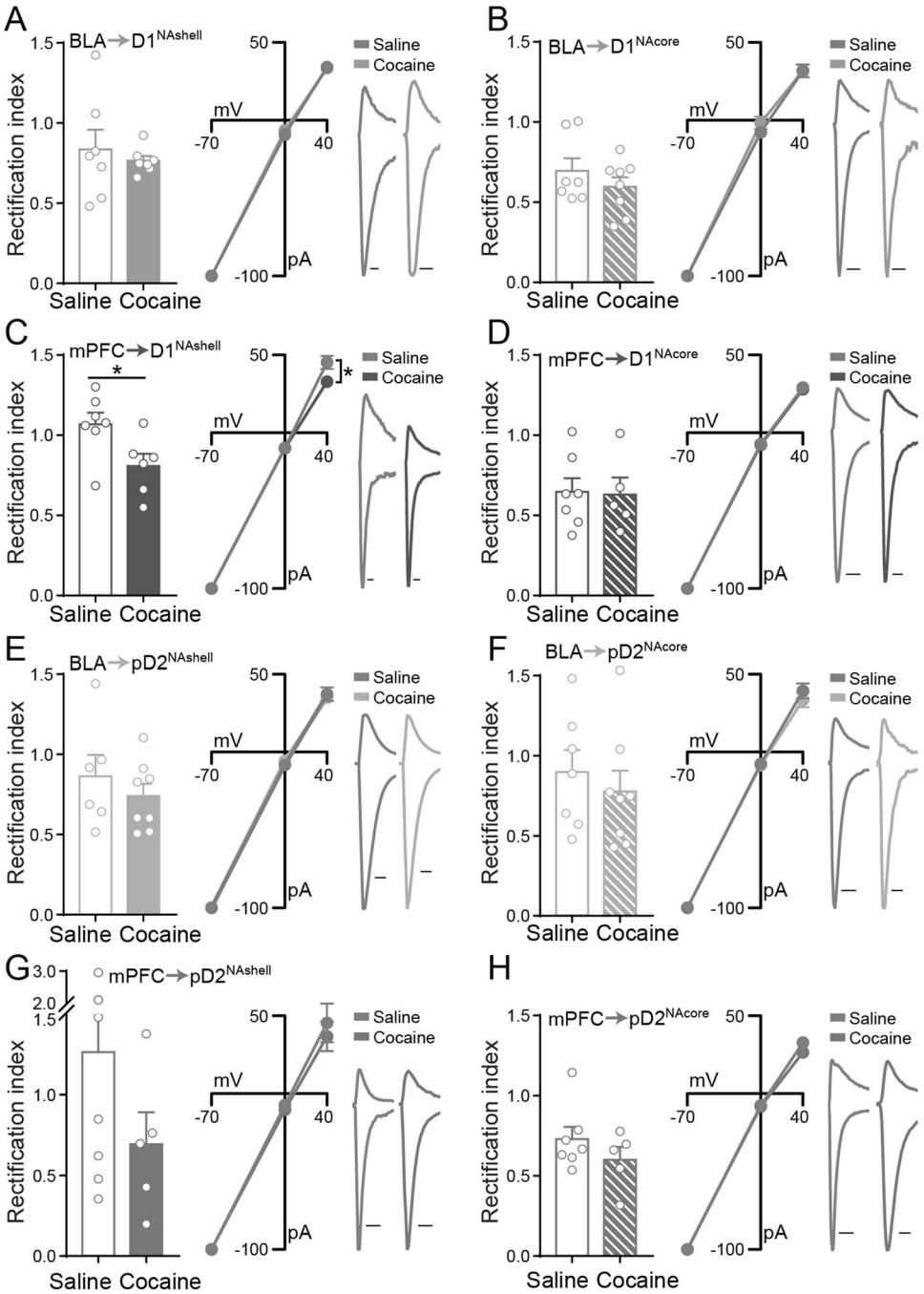


Fig. 2 Cocaine CPP and abstinence increases the rectification of AMPA receptor-mediated currents in mPFC→NAshell D1-MSN$_{→VP}$ neurons. A–H Left: Rectification index calculated as the observed amplitude of AMPAR current at holding potential of +40 mV divided by the predicted amplitude at holding potential of +40 mV. Middle: Current–voltage (*IV*) curve of evoked AMPAR currents obtained at holding potentials of −70 mV, 0 mV, and 40 mV. Right: Representative traces. All currents were normalized to the peak amplitude at −70 mV. Scale bars: 10 ms. **A**, **B** Cocaine CPP and abstinence had no significant effect on the rectification index of AMPAR currents in BLA→NAc synapses on D1-MSNs$_{→VP}$ in the NAshell (**A**) or NAcore (**B**). Cocaine CPP and abstinence changed the rectification index in the mPFC input to NAshell D1-MSNs$_{→VP}$ (**C**) but not to NAcore D1-MSNs$_{→VP}$ (**D**). The rectification index of AMPAR currents in the BLA (**E**, **F**) and mPFC (**G**, **H**) inputs to NAc pD2-MSNs$_{→VP}$ was not altered by cocaine CPP and abstinence in the NAshell (**E**, **G**) and the NAcore (**F**, **H**). For all groups cell number ranged from 5 to 8 and data were collected from 3–5 mice. Data are presented as mean ± SEM, *$p < 0.05$. All points are shown.

they do not rule out plasticity at the presynaptic terminal. Hence, we further explored whether cocaine CPP and abstinence induced alterations in the presynaptic release of neurotransmitter from mPFC or BLA inputs to VP-projecting MSNs. To achieve this, we stimulated ChR2-expressing terminals from either the BLA or mPFC in a paired-pulse paradigm (100 ms interval) and recorded from MSNs at a holding potential of −80 mV. From the elicited currents we then calculated two measurements, changes in which are considered to reflect presynaptic plasticity: (1) The paired-pulse ratio (PPR; the peak of the second evoked current divided by the first) [45]; and (2) The coefficient of variation (CV) of the peak of the first evoked current [46, 47]. Decrease in both measures indicates an increase in release probability.

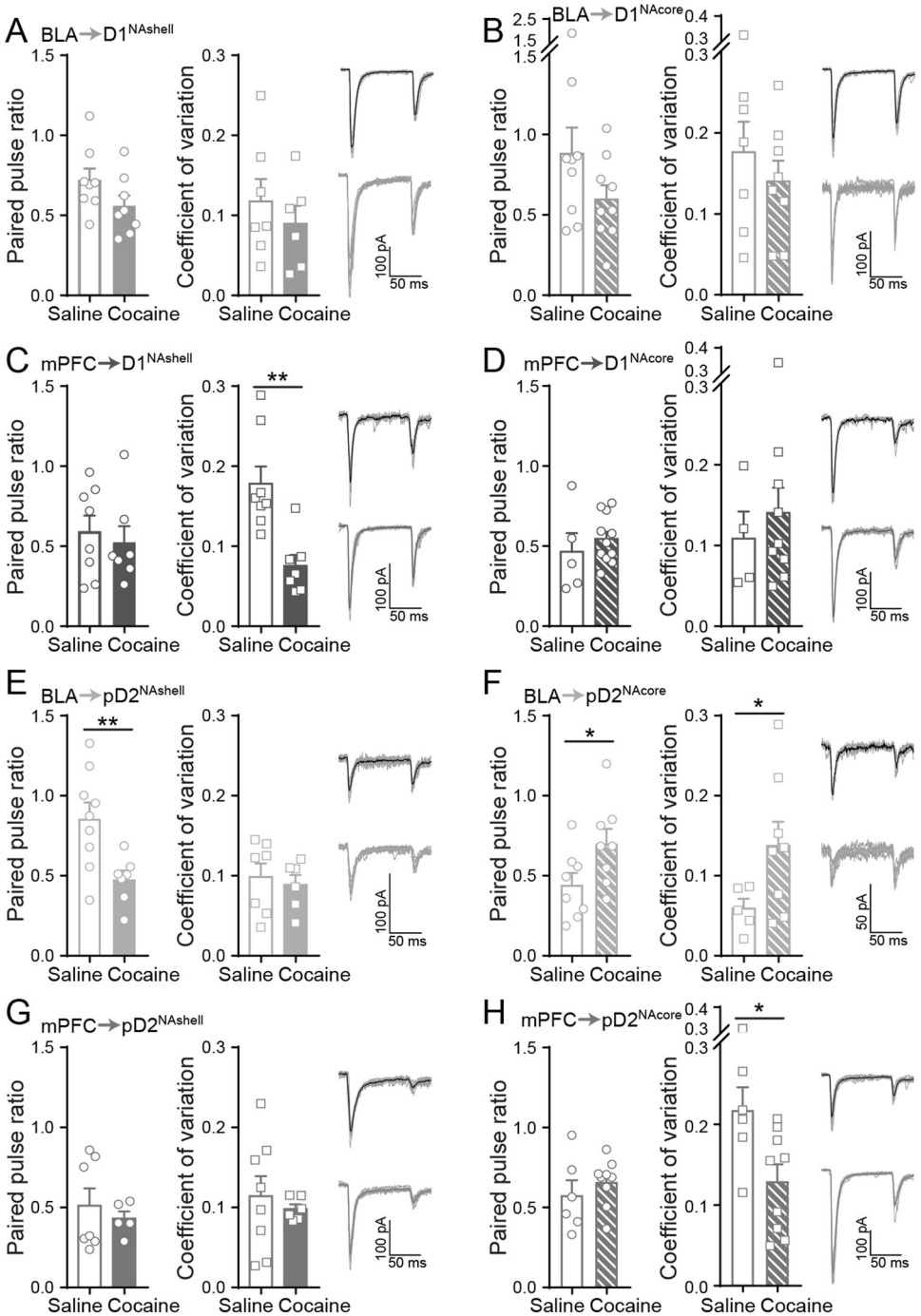


Fig. 3 Cocaine CPP and abstinence induce presynaptic plasticity primarily at the glutamatergic input to pD2-MSNs$_{\rightarrow VP}$. A–H Left: Paired-pulse ratio (PPR). Middle: coefficient of variation (CV). Right: Representative traces of the evoked currents, average trace in black (saline) or color (cocaine). Cocaine CPP and abstinence did not affect the PPR and CV of BLA→NAc synapses on D1-MSNs$_{\rightarrow VP}$ in the NAshell (**A**) and the NAcore (**B**). In mPFC→NAc synapses on D1-MSNs$_{\rightarrow VP}$, cocaine CPP and abstinence did not affect the PPR but decreased the CV in the NAshell (**C**) and did not affect both the PPR and CV in the NAcore (**D**). Cocaine CPP and abstinence decreased the PPR but not the CV in BLA→NAc synapses on pD2-MSNs$_{\rightarrow VP}$ in the NAshell (**E**), and increased both the PPR and the CV in BLA→NAc synapses on pD2-MSNs$_{\rightarrow VP}$ in the NAcore (**F**). Cocaine CPP and abstinence did not affect the PPR and CV in mPFC-NAc synapses on pD2-MSNs$_{\rightarrow VP}$ in the NAshell (**G**), but decreased the CV without altering the PPR in the NAcore (**H**). For all groups cell number ranged from 5 to 11 and data were collected from 3–5 mice. Data are presented as mean ± SEM, *$p < 0.05$, **$p < 0.01$. All points are shown.

Most synapses between the mPFC or BLA and D1-MSNs$_{\rightarrow VP}$ did not show presynaptic plasticity after cocaine CPP and abstinence (Fig. 3A, B, D; unpaired two-tailed Student's t tests; mPFC to NAcore: $t_{(14)} = 0.79$, $p = 0.44$ for PPR and $t_{(14)} = 0.61$, $p = 0.55$ for CV; BLA to NAcore: $t_{(16)} = 1.56$, $p = 0.14$ for PPR and $t_{(17)} = 0.82$, $p = 0.43$ for CV; BLA to NAshell: $t_{(14)} = 1.64$, $p = 0.12$ for PPR and $t_{(14)} = 0.76$, $p = 0.46$ for CV). The only exception was the mPFC→D1-MSN$_{\rightarrow VP}$ synapse in the NAshell. This synapse showed a decrease in the CV (but not PPR) after cocaine CPP and abstinence (unpaired two-tailed Student's t tests: $t_{(14)} = 3.86$, $p = 0.002$ for CV and $t_{(13)} = 0.48$, $p = 0.64$ for PPR), indicating presynaptic potentiation (Fig. 3C). This, together with the increase

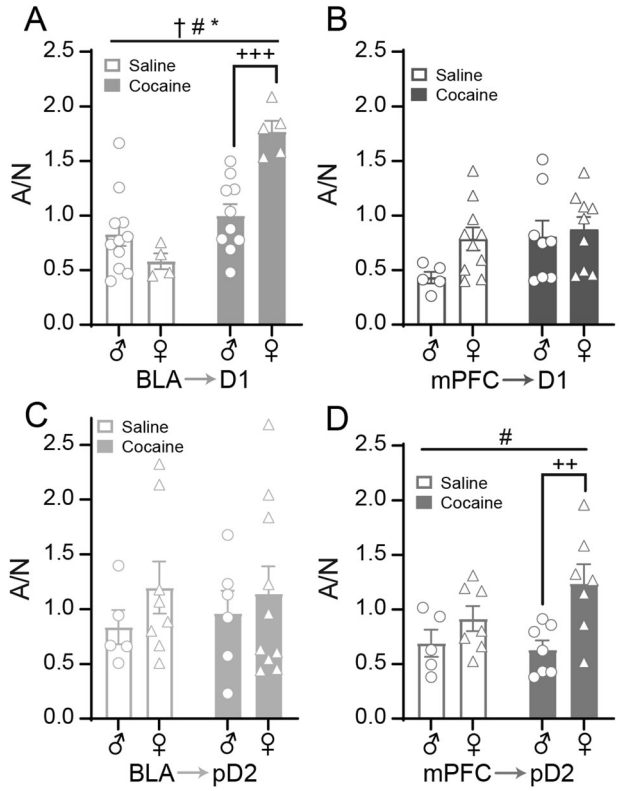


Fig. 4 Sex-specific effects of cocaine CPP and abstinence on the A/N in mPFC and BLA inputs to VP-projecting NAc MSNs. Sex-specific effects of cocaine on A/N ratio in the BLA→D1-MSN$_{→VP}$ synapse (**A**), mPFC→D1-MSN$_{→VP}$ synapse (**B**), BLA→pD2-MSN$_{→VP}$ synapse (**C**) and mPFC→pD2-MSN$_{→VP}$ synapse (**D**). ♂- males. ♀- females. NAcore and NAshell were pooled together. There were no sex-specific effects in the mPFC→D1-MSN$_{→VP}$ (**B**) and BLA→pD2-MSN$_{→VP}$ (**C**) synapses. In the BLA→D1-MSN$_{→VP}$ synapse (**A**) there was a Two-way ANOVA main effect both for the drug group (*) and the sex (#). Also, there was an interaction drug group × sex (†) and Sidak's post-hoc multiple comparisons analyses revealed that after cocaine CPP and abstinence the A/N was significantly higher in females compared to males. There was also a main sex effect in the mPFC→pD2-MSN$_{→VP}$ synapse and Sidak's post-hoc multiple comparisons analyses revealed that after cocaine CPP and abstinence the A/N was significantly higher in females compared to males. For all groups cell number ranged from 4 to 11 and data were collected from 3–5 mice. Data are presented as mean ± SEM. *, #, † - $p < 0.05$. ++, $p < 0.01$. +++, $p < 0.001$. All points are shown.

in A/N ratio in the same synapse (Fig. 1H), supports a long-term potentiation of the mPFC input specifically to VP-projecting D1-MSNs in the NAshell after cocaine CPP and abstinence. Overall, the mPFC and BLA inputs to D1-MSNs$_{→VP}$ do not seem to show presynaptic plasticity after cocaine CPP and abstinence, except for the mPFC→D1-MSN$_{→VP}$ in the NAshell, which also shows postsynaptic potentiation. This may indicate that cocaine induces mostly postsynaptic changes in the D1-MSNs$_{→VP}$ and not in the incoming terminals to these cells.

The data in Fig. 1 show no changes in A/N ratio on pD2-MSNs$_{→VP}$, implying lack of plasticity in the inputs to these neurons after cocaine exposure, as also suggested before [9, 12]. Examination of presynaptic plasticity challenges this concept. Our data show significant presynaptic plasticity in the BLA input to pD2-MSNs$_{→VP}$. In the NAshell, the BLA input to pD2-MSN$_{→VP}$ shows presynaptic potentiation (decreased PPR; unpaired two-tailed Student's *t* test: $t_{(14)} = 3.02$, $p = 0.009$ for PPR and $t_{(14)} = 0.44$, $p = 0.67$ for CV; Fig. 3E). In the NAcore, the BLA input to pD2-MSN$_{→VP}$ shows presynaptic depression (increased PPR and CV; unpaired two-tailed Student's *t* tests: $t_{(14)} = 2.15$, $p = 0.049$ for PPR and $t_{(14)} = 2.17$, $p = 0.048$ for CV; Fig. 3F). In contrast, the mPFC input to NAcore (but not NAshell) pD2-MSNs$_{→VP}$ is potentiated (decreased CV) (unpaired two-tailed Student's *t* tests: $t_{(13)} = 2.57$, $p = 0.02$ and $t_{(13)} = 0.85$, $p = 0.41$ for CV and PPR in NAcore respectively; $t_{(13)} = 0.54$, $p = 0.60$ and $t_{(10)} = 0.62$, $p = 0.55$ for CV in PPR in NAshell respectively; Fig. 3G–H), thus indicating that the mPFC input to the NAc does not only potentiate on NAshell D1-MSNs$_{→VP}$ but also on NAcore pD2-MSNs$_{→VP}$. Overall, our data show substantial presynaptic changes in the excitatory inputs to VP-projecting pD2-MSNs after cocaine CPP and abstinence. Particularly, the BLA input to NAcore pD2-MSNs$_{→VP}$ is weakened while the mPFC input to the same neurons is potentiated.

Sex-specific plasticity in the BLA and mPFC input to MSNs$_{→VP}$ after cocaine CPP and abstinence

Recent studies highlighted both the BLA and mPFC, as well as the NAc, as regions that show sex-specific changes related to drug- or natural reward-seeking behavior and drug memories [48–54]. Thus, we examined here whether cocaine CPP and abstinence alters the inputs from the mPFC and the BLA to NAc MSNs$_{→VP}$ in a sex-specific manner. We indeed found that both inputs showed sex-specific plasticity, albeit on different MSN cell types.

The A/N of the BLA input to D1-MSNs$_{→VP}$, which was comparable between control males and females, became significantly higher in females after cocaine CPP and abstinence (Fig. 4A) (Two-way ANOVA, main sex effect—$F_{(1,26)} = 4.2$, $p = 0.049$; main drug-group effect, $F_{(1,26)} = 28.6$, $p < 0.0001$; interaction, $F_{(1,26)} = 16.3$, $p < 0.001$; post-hoc Sidak's multiple comparisons test, males vs females after cocaine CPP and abstinence—$t_{26} = 4.45$, $p < 0.001$). This was not seen in pD2-MSNs$_{→VP}$ (Fig. 4C). In contrast, the A/N of the mPFC input became higher in females after cocaine CPP and abstinence only in pD2-MSNs$_{→VP}$ but not in D1-MSNs$_{→VP}$ (Fig. 4B, D) (For pD2-MSNs—two-way ANOVA, main sex effect—$F_{(1,22)} = 9.69$, $p = 0.005$; post-hoc Sidak's multiple comparisons test, males vs females after cocaine CPP and abstinence—$t_{22} = 3.37$, $p = 0.006$). Thus, the BLA and mPFC inputs to VP-projecting MSNs change after cocaine CPP and abstinence in a sex-specific manner. This may imply that the involvement of the VP in cocaine CPP and abstinence is, like that of the NAc, mPFC, and BLA, sex-specific.

Examining the presynaptic measures (PPR and CV) did not show sex-specific changes after cocaine CPP and abstinence (Table S3). It therefore implies that the sex-specific changes occur in the MSNs themselves and not in the incoming terminals.

DISCUSSION

In this study we examined plasticity in the glutamatergic transmission from the mPFC and BLA to NAshell/NAcore D1- and pD2-MSNs that project to the VP after cocaine CPP and abstinence. Our results indicate that cocaine CPP and abstinence induce multiple forms of synaptic plasticity which are cell type- and projection-specific. Adding to previous studies, which depict plasticity mostly in D1-MSNs [8, 9, 12, 16], our data suggest that when focusing only on VP-projecting MSNs, the glutamatergic input is potentiated both at D1- and pD2-MSNs$_{→VP}$. The most striking result to emerge from the data is that while plasticity at D1-MSNs$_{→VP}$ occurred mostly at the postsynaptic site, in pD2-MSNs$_{→VP}$ the presynaptic site was more prone to changes following cocaine CPP and abstinence. Thus, the A/N ratio of both mPFC (to NAshell) and BLA (to both NAcore and NAshell) inputs is increased in D1-MSNs$_{→VP}$ but not pD2-MSNs$_{→VP}$ (Fig. 1) while the indicators of presynaptic plasticity, PPR and CV, change more on pD2-MSNs$_{→VP}$ than on D1-MSNs$_{→VP}$ (Fig. 3). To the best of our knowledge, this is the first demonstration of presynaptic plasticity at pD2-MSNs$_{→VP}$ following cocaine CPP and abstinence.

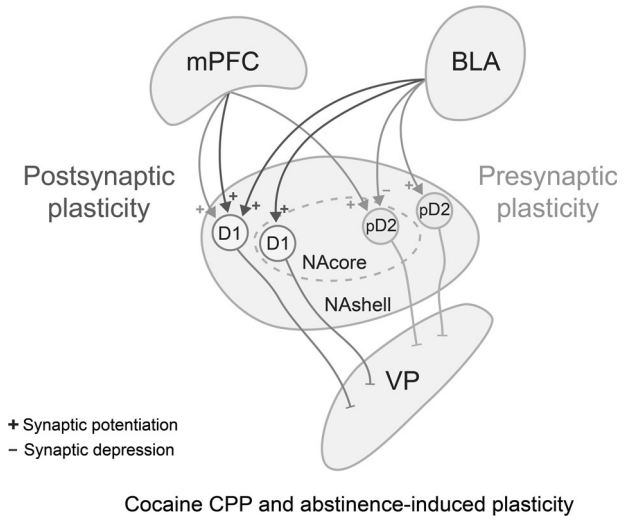


Fig. 5 Cocaine CPP and abstinence induce postsynaptic and presynaptic potentiation of the mPFC and BLA excitatory inputs to D1-MSNs$_{→VP}$ and pD2-MSN$_{→VP}$ respectively. Schematic summary of findings. In D1-MSNs$_{→VP}$—cocaine CPP and abstinence potentiated the inputs from the BLA to NAshell and NAcore D1-MSNs$_{→VP}$ and the inputs from the mPFC to NAshell D1-MSNs$_{→VP}$ in a postsynaptic manner. The input from the mPFC to NAshell D1-MSNs$_{→VP}$ was strengthened also through a presynaptic mechanism. In pD2-MSNs$_{→VP}$—cocaine CPP and abstinence potentiated the inputs from the BLA and mPFC to NAshell and NAcore pD2-MSNs$_{→VP}$ respectively but depressed the input from the BLA to NAcore pD2-MSNs$_{→VP}$, all through a presynaptic manner.

Potentiation of glutamatergic transmission to VP-projecting MSNs and the influence on the VP

Strengthening of excitatory transmission onto VP-projecting NAc MSNs is expected to make them more prone to activation, and therefore to increase their inhibitory drive on the VP. Our data show that cocaine CPP and abstinence mostly potentiated excitation on MSNs$_{→VP}$ (Fig. 5)—it potentiated mPFC input to NAshell D1-MSNs$_{→VP}$ and NAcore pD2-MSNs$_{→VP}$, BLA input to NAcore and NAshell D1-MSNs$_{→VP}$ and BLA input to NAshell pD2-MSNs$_{→VP}$. Only the BLA input to NAcore pD2-MSNs$_{→VP}$ showed depression after cocaine CPP and abstinence. This would suggest that the NAc input to the VP may become stronger in general after cocaine CPP and abstinence, but as the excitation on pD2-MSNs$_{→VP}$ also shows some depression, our findings may draw a picture where the incoming accumbal input to the VP is biased towards the D1-MSNs. A similar bias of activation of D1-MSNs$_{→VP}$ compared to D2-MSNs$_{→VP}$ by BLA input after repeated cocaine exposure was also suggested in a recent study [24]. Since D1-MSN input to the VP promotes, while D2-MSN input to the VP inhibits drug seeking [26, 27], an imbalance in the VP favoring D1-MSN input may promote drug seeking and may be generated upstream in the inputs to the NAc and not only locally in the VP. Thus, understanding the roles of the NAc→VP parallel pathways in drug reward and addiction may require a wider perspective on upstream and downstream circuits.

It is important to note that our study does not touch on all levels of complexity in this system. For example, D1-MSNs and D2-MSNs differ in the innervation of GABAergic and glutamatergic VP neurons [25] and in the neuropeptides they release in the VP [23, 55–58]. Thus, strengthening of the excitatory inputs to D1-MSNs$_{→VP}$ or D2-MSNs$_{→VP}$ may affect VP activity in many other ways that are still to be studied. In addition, recent works suggest that D1- and D2-MSNs activity might not be completely antagonistic, but rather work together to generate learning and behavior [59] in a way that depends on their activity patterns [60, 61]. With this in mind, the potentiation of the excitatory inputs to D1- and D2-MSNs$_{→VP}$ might reflect their cooperative roles in modulating and refining behavior.

Behavioral significance of plasticity in mPFC and BLA inputs to MSNs$_{→VP}$

The mPFC and the BLA are two major excitatory inputs to the NAc [62, 63]. Both these inputs have been shown to express synaptic plasticity in the NAc after abstinence or withdrawal from repeated exposure to drugs [9, 30, 64–69]. Although both these inputs excite NAc MSNs, they are considered to play different roles in motivated behavior—while the mPFC is important for the cognitive aspects of drug seeking and the persistence of addictive behavior [70–72] the BLA is thought to underlie the association between cues or context to reward exposure [65, 73, 74]. Thus, the potentiation of the mPFC inputs on VP-projecting MSNs may be responsible for the persistence of the memory of cocaine exposure after abstinence while the plasticity in the BLA input to the same neurons may encode the association of cocaine with the context (the cocaine-paired side of the CPP box). Supporting this thesis, the plasticity of the BLA input to D1-MSNs$_{→VP}$ was seen also before abstinence [24], as would be expected from plasticity that represents the learning process in the CPP protocol.

The fact that all recorded MSNs project to the VP suggests that the VP is involved in both the cognitive and the cue/context aspects of abstinence after cocaine CPP. This is corroborated by previous studies showing the importance of the VP in cocaine CPP and abstinence [23, 75–79] although it remains to be studied whether it is the input from NAc MSNs that determines the role of the VP in cocaine CPP.

Source of synaptic plasticity

In this study, we observed synaptic plasticity in mPFC and BLA inputs to MSNs$_{→VP}$ following abstinence from cocaine-induced CPP. Therefore, it cannot be determined whether the plasticity we observed was induced by the exposure to cocaine or by the following abstinence. Indeed, Baimel et al. [24] found an increase in the excitatory input from the BLA to NAshell D1-MSNs$_{→VP}$ relative to non D1-MSNs (similar to what we found, Fig. 1F) 24 h after the last of 5 non-contingent cocaine injections and MacAskill et al. [16] found a similar potentiation of BLA input on D1-MSNs 3 days after the last of 5 non-contingent cocaine injections. In addition, Suska et al. [80] showed an increase in the release probability in the input from the mPFC to NAshell MSNs following only 1 day of withdrawal from repeated cocaine (i.p.) injections (although they did not identify D1- or D2-MSNs or VP-projecting MSNs). Thus, our finding of increased A/N ratio in the BLA input to NAshell D1-MSNs$_{→VP}$ (Fig. 1) and presynaptic potentiation of mPFC input to NAshell D1-MSNs$_{→VP}$ (Fig. 3) after 14 days of withdrawal may reflect long-term plasticity that resulted from the repeated exposure to cocaine and not from the abstinence period. More research needs to be done to further dissect the exact synaptic alterations that take place in the inputs to VP-projecting NAc MSNs at different time points after cocaine exposure.

Postsynaptic plasticity

Our data describe three forms of synaptic plasticity following cocaine CPP and abstinence in VP-projecting NAc MSNs—change in the probability of neurotransmitter release, change in the A/N ratio and change in the rectification of AMPAR-mediated currents. Changes in A/N ratio and AMPA rectification are usually considered to be postsynaptic and occur at the level of the recorded neuron. Therefore, these two processes may be linked, and indeed the co-occurrence of changes in the A/N ratio and in the rectification of AMPAR current was repeatedly reported in the NAc after both contingent or non-contingent cocaine exposure [9, 35, 37, 39]. Out of the synapses that we found to increase A/N ratio after cocaine CPP and abstinence, only the synapse of mPFC to NAshell D1-MSNs$_{→VP}$ also showed rectification of AMPAR

currents (Fig. 2). This indicates that an increase in A/N ratio may be achieved in different synapses by different mechanisms—it may be induced by recruitment of GluA2-lacking AMPARs (which would show rectification [81, 82] as in the mPFC-to-NAshell D1-MSNs$_{\rightarrow VP}$ synapse) or by other mechanisms. As there is substantial overlap between NAc MSNs receiving mPFC input and those receiving BLA input [83] it is highly probable that different spines of the same D1-MSN$_{\rightarrow VP}$ neuron show different forms of postsynaptic plasticity dependent on the source of input. How spines of the same MSN identify their input and drive input-specific plasticity remains to be solved.

Presynaptic plasticity

Presynaptic plasticity was determined as a change in either the PPR or the CV in a specific synapse (Fig. 3). These two measures are classic indicators of presynaptic plasticity [47, 84] although they may reflect different mechanisms of changes in the probability of synaptic release. Thus, while the CV reflects the level of instability of synaptic release, which can stem from a variety of mechanisms, the PPR reflects calcium-dependent facilitation of release driven by residual free calcium left in the terminals after the first pulse [45, 85]. It is therefore possible that some presynaptic changes in the probability of release will manifest in changes in one but not both parameters, as we observed here (Fig. 3). This may mean that the mechanisms underlying presynaptic plasticity are not the same for the mPFC and BLA inputs to VP-projecting MSNs.

The changes in the probability of neurotransmitter release in the inputs to VP-projecting MSNs may involve any of the known mechanisms of presynaptic plasticity in the NAc (For review see [86]). For example, withdrawal from cocaine decreases the extrasynaptic levels of glutamate in the NAc [87–90], which in turn suppresses the inhibition of synaptic release by presynaptic mGluR2/3 receptors [88, 91–94]. This may lead to presynaptic potentiation. Also, both mPFC and BLA terminals in the NAc synapse are in close apposition to dopamine axons [95, 96]. Therefore, cocaine exposure may activate presynaptic dopamine D1 receptors (D1Rs) on mPFC and BLA terminals in the NAc, which is known to potentiate glutamate release [69, 97, 98]. Another possible mechanism for presynaptic plasticity in mPFC and BLA inputs to VP-projecting NAc MSNs is through the cannabinoid CB1 presynaptic receptor. The CB1 receptor regulates glutamate release in the NAc [99] and specifically from mPFC and BLA inputs on both D1-MSNs and D2-MSNs [100]. Animal models of cocaine addiction and withdrawal show dysfunction of the endocannabinoid system and lower endocannabinoid tone in the NAc [101–103], potentially disinhibiting glutamate release as we found here. Lastly, changes in the CV of synaptic currents may reflect the generation and maturation of silent synapses [104–106]. Maturation of silent synapses in the NAc, including those of mPFC and BLA afferents [36, 68], have been mostly shown to contribute to the incubation of drug craving after cocaine self-administration, but this phenomenon occurs also in cocaine-induced locomotor sensitization [105] and, importantly, after abstinence from cocaine CPP [38]. Thus, the presynaptic plasticity we observe on VP-projecting MSNs may reflect maturation of silent synapses.

Different forms of plasticity in different synapses

Our results indicate that presynaptic and postsynaptic plasticity generally take place at separate synapses on VP-projecting MSNs. Whereas increase in A/N ratio occurred only in D1-MSNs$_{\rightarrow VP}$, changes in the probability of release were primarily detected in pD2-MSNs$_{\rightarrow VP}$. An exception to this dichotomy is the mPFC synapse on NAshell D1-MSNs$_{\rightarrow VP}$, which shows all three forms of plasticity—increase in A/N ratio, rectification of AMPA currents and increase in the presynaptic probability of release. This raises the possibility that the mPFC→D1-MSNs$_{\rightarrow VP}$ circuit in the NAshell undergoes maturation of silent synapses, as this process often involves recruitment of new GluA2-lacking AMPARs and thus increase in the A/N ratio and AMPA current rectification [35, 36, 38].

As activation of D1-MSNs$_{\rightarrow VP}$ and D2-MSNs$_{\rightarrow VP}$ has opposing influence on drug-seeking behavior [26, 27] it is possible that there are different functional implications for the presynaptic vs postsynaptic types of plasticity. This may stem from different patterns of connectivity of D1-MSNs and D2-MSNs in the VP [25], but also from the mere form of plasticity. A study that used a computational model to examine the consequences of pre- and postsynaptic plasticity showed that presynaptic plasticity allows for a better signal-to-noise ratio, improved response latencies and more dynamic learning [107].

In conclusion, the key finding that emerges from our data is that cocaine CPP and abstinence induce different forms of synaptic plasticity on VP-projecting NAc MSNs in a manner that depends on cell type, origin of input and cell location. This joins recent studies depicting complex changes in the NAc→VP pathways in animal models of cocaine reward and addiction [23, 24, 26, 27, 108]. Understanding how the NAc affects VP activity in a cell-type-specific manner, how different inputs to the NAc drive separate NAc→VP circuits, and how drug experience changes these circuits is crucial for better understanding of the neurobiological underpinnings of drug addiction.

Sex-specific changes in the inputs to MSNs$_{\rightarrow VP}$

The differences in the neurophysiological effects of drugs of abuse between males and females have been highlighted by various recent studies [50–53, 109], but knowledge is still limited. Our data show that such sex-specific effects occur not just generally in a brain region but also in specific cell types and projections. Our data also indicate that the sex-specific effects of cocaine CPP and abstinence are not necessarily linked to the cocaine-induced general effects that occur in the general population. Thus, the BLA input to D1-MSNs$_{\rightarrow VP}$ showed higher A/N after cocaine (Fig. 1F, G) and also a stronger increase in A/N in females (Fig. 4A) while the mPFC→pD2-MSN$_{\rightarrow VP}$ synapse, which showed higher A/N in females compared to males after cocaine CPP and abstinence (Fig. 4D), did not show a cocaine-induced change in A/N in the general population (Fig. 1L, M). Therefore, for the circuits converging into the VP through the NAc, examination of drug effects on each sex separately may lead to novel insights that may be missed when pooling both sexes together.

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AUTHOR CONTRIBUTIONS

Conceptualization, KI and YMK.; Methodology, KI and YMK; Formal analysis, KI and YMK; Investigation, KI, LAL and YMK; Writing—original draft, KI; Writing—review & Editing, KI and YMK; Visualization, KI; Supervision, YMK; Project Administration, KI, and YMK; Funding Acquisition, YMK, KI, and LAL.

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COMPETING INTERESTS

The authors declare no competing interests.

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