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Cocaine-evoked synaptic plasticity: persistence in the VTA triggers adaptations in the NAc

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Addictive drugs hijack mechanisms of learning and memory that normally underlie reinforcement of natural rewards and induce synaptic plasticity of glutamatergic transmission in the mesolimbic dopamine (DA) system. In the ventral tegmental area (VTA), a single exposure to cocaine efficiently triggers NMDA receptor–dependent synaptic plasticity in DA neurons, whereas plasticity in the nucleus accumbens (NAc) occurs only after repeated injections. Whether these two forms of plasticity are independent or hierarchically organized remains unknown. We combined *ex vivo* electrophysiology in acute brain slices with behavioral assays modeling drug relapse in mice and found that the duration of the cocaine-evoked synaptic plasticity in the VTA is gated by mGluR1. Overriding mGluR1 *in vivo* made the potentiation in the VTA persistent. This led to synaptic plasticity in the NAc, which contributes to cocaine-seeking behavior after protracted withdrawal. Impaired mGluR1 function in vulnerable individuals could represent a first step in the recruitment of the neuronal network that underlies drug addiction.

Cocaine, one of the most addictive drugs of abuse, can induce synaptic plasticity of glutamatergic transmission in the mesolimbic DA system of rodents[1–3]. Within hours of a single cocaine injection, excitatory inputs onto DA neurons of the VTA are strengthened, which can be monitored by an increased AMPA/NMDA ratio[4]. This drug-evoked potentiation is in part mediated through an exchange of GluR2-containing and GluR2-lacking AMPA receptors, leading to excitatory postsynaptic currents (EPSCs) that are sensitive to polyamines and have a rectifying current-voltage relationship[5,6]. This plasticity is triggered by all of the addictive drugs tested so far and lasts about 5 d[4,7,8]. If cocaine is self-administered repetitively for 2 weeks, plasticity in the VTA becomes persistent, and can be detected even months after withdrawal[9].

In the NAc, cocaine-evoked plasticity also occurs, but on a slower timescale and with a steeper induction threshold. A single cocaine injection is not sufficient to trigger changes in synaptic transmission. However, after 5 d of consecutive injections, AMPA/NMDA ratios are depressed[10]. During withdrawal from both passive exposure and self-administration, this long-term depression (LTD)-like plasticity in the NAc transforms into a potentiation through an insertion of AMPA receptors (AMPARs)[10,11]. Biochemical and electrophysiological investigations suggest that the inserted receptors are GluR1 homomeric channels[12,13]. Recently, it has been observed that the *in vivo* inhibition of these channels by a polyamine toxin substantially reduces cue-induced cocaine seeking after withdrawal[11]. This behavioral phenomenon, termed incubation of craving, becomes apparent following protracted withdrawal and is believed to mimic relapse in humans[14]. Similarly, cocaine-seeking was blocked by injections of antisense oligonucleotides of GluR1 mRNA into the NAc[15]. GluR2-lacking AMPARs seem to be involved in a receptor redistribution that contributes to the remodeling of neuronal networks underling addictive behaviors[16].

Taken together, a single injection of cocaine causes a switch-like, rapid, but transient, potentiation of excitatory inputs in the VTA, whereas several injections are required to induce plasticity in the NAc. We manipulated the persistence of the plasticity in the VTA via an mGluR1-dependent mechanism and used genetically modified mice lacking NMDA receptor (NMDAR) selectively in DA neurons of the midbrain to test the effects of cocaine on the enduring forms of plasticity in the NAc and on the incubation of craving.

RESULTS

mGluR1-dependent reversal of plasticity *in vivo*

In vitro, cocaine-evoked plasticity in the VTA can be reversed rapidly via mGluR1 activation by either synaptic glutamate or exogenous agonists[5]. Mechanistically, such mGluR-LTD exchanges the GluR2-lacking AMPARs that are inserted during cocaine-evoked plasticity with newly synthesized GluR2-containing receptors[17]. We therefore asked whether functional mGluR1 in the VTA are required for the endogenous reversal of cocaine-evoked plasticity *in vivo*. To this end, we interfered with mGluR1 function by using a dominant-negative peptide that precludes the binding of mGluR1 and Homer1b/c (TAT-mGluRct)[18]. This peptide specifically inhibits mGluR-dependent synaptic plasticity in the hippocampus[19]. The peptide was TAT- and fluorescein-conjugated and stereotactically delivered bilaterally to the

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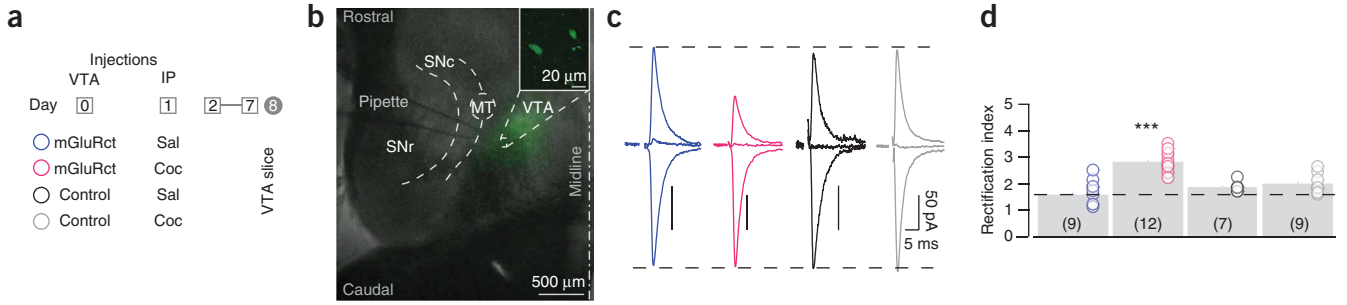

Figure 1 Disruption of Homer 1b/c–mGluR interaction in the VTA renders cocaine-evoked plasticity persistent. (**a**) Saline (blue and black circle) or cocaine (red and gray circle) were injected intraperitoneally (IP) 24 h after stereotactic delivery of TAT-mGluRct or TAT control into the VTA. Acute midbrain slices were then prepared at day 8. (**b**) Confocal image obtained 8 d after injection of TAT-mGluRct (0.6 µl at 1 µM). The fluorescence signal is superimposed on the transmitted light image. The image is shown at 40× magnification. MT, medial terminal nucleus of the accessory optical tract. SNc, substantia nigra compacta; SNr, substantia nigra reticulata. (**c**) Examples of AMPAR-EPSCs obtained at −70, 0 and +40 mV. (**d**) Bar graph of averaged rectification indexes with superimposed scatter plot (symbols as above). Dashed line in the bar graph indicates the value of a linear *I-V* curve (70/40 = 1.75). *** indicates $P < 0.001$.

VTA of mice. To ensure that the peptide did not spread beyond the VTA, but remained present in DA neurons for the duration of the experiment (**Fig. 1a**), we visualized the fluorescence 8 d after the injection (**Fig. 1b**) and made recordings from fluorescent-labeled DA neurons. Although the TAT peptide was probably also loaded into GABA neurons, the overwhelming majority of the fluorescent neurons were DA neurons, as judged by electrophysiological criteria (see Online Methods). We also tested whether the dominant-negative peptide reduced currents evoked by the potent agonist of mGluRs S-3,5-dihydroxyphenylglycine (DHPG) and whether it blocked mGluR-LTD *in vitro*, which was indeed the case (**Supplementary Fig. 1**). We then measured EPSCs at –70, 0 and +40 mV to calculate the rectification index ($EPSC_{-70mV}/EPSC_{+40mV}$). In mice in which TAT-mGluRct was delivered, rectification indices measured 7 d after a single cocaine injection were significantly higher than those of control mice or after the stereotactic injection of a TAT control peptide (TAT peptide delivery × intraperitoneal injection interaction: $F_{1,31} = 14.14$, $P = 0.0007$; **Fig. 1c,d**). In the presence of the dominant-negative peptide, cocaine-evoked plasticity was intact, which demonstrates that mGluR-Homer binding is not required for induction (for example, NMDA dependence[4]) or expression (for example, PICK1-dependent AMPAR redistribution[5]; **Supplementary Fig. 1**). Taken together, these findings suggest that the disruption of Homer 1b/c–mGluR interaction in the VTA renders the cocaine-evoked plasticity persistent.

To confirm this result pharmacologically, we blocked mGluR1 with daily systemic (intraperitoneal) injections of the antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA) following a single injection of cocaine (**Fig. 2a**). At a dose of 0.25 mg per kg of body weight, AIDA selectively inhibits mGluR1 receptors[20]. Because we established that 7 d is normally enough for a full reversal of cocaine-evoked plasticity, we cut slices on day 8 and found that the current-voltage relationship (*I-V*) was rectifying (**Fig. 2b**; for corresponding AMPA/NMDA ratio see **Supplementary Fig. 2**), which was not the case if one injection of cocaine was followed by daily injections of saline (**Fig. 2b**). As further controls, we ensured that saline or AIDA alone had no effect on the rectification index. In contrast, with seven injections of cocaine the EPSCs were rectifying and the rectification index was significantly elevated compared with control values ($P < 0.001$). Notably, the increased rectification index following seven injections of cocaine was similar to what we observed previously 24 h after a single injection, arguing that the plasticity may already be saturated by a single injection of cocaine[8]. To determine the temporal requirement of mGluR1 function, we broke down the AIDA treatment and compared an immediate treatment (days 2 and 3) with a late treatment (days 5 and 6) and prepared slices at day 8. Only the latter was efficient in maintaining synaptic plasticity, arguing that mGluR1 must be activated during a narrow time window to reverse the cocaine-evoked plasticity (**Supplementary Fig. 3**). Taken together, one injection of cocaine leads to persistent plasticity when followed by daily injections of AIDA, suggesting that the endogenous reversal of cocaine-evoked plasticity depends on functional mGluR1 receptors *in vivo*.

We have previously shown that the positive mGluR1-modulator Ro 67-7674 leads to the disappearance of cocaine-evoked plasticity by 24 h after one intraperitoneal injection[5]. We therefore tested whether a positive modulation of mGluR1 receptors could reverse cocaine-evoked plasticity with a more robust induction by seven injections of cocaine, each given 1 h after Ro 67-7476 (**Fig. 2a**). Indeed seven injections of cocaine when paired with Ro 67-7476 yielded a linear *I-V* curve (**Fig. 2c**). As a control, Ro 67-7476 injected together with saline had no effect on rectification, whereas seven injections of cocaine along with saline, similar to the result above, led to significant rectification ($P = 0.031$). Thus, positive modulation of mGluR1 causes rapid

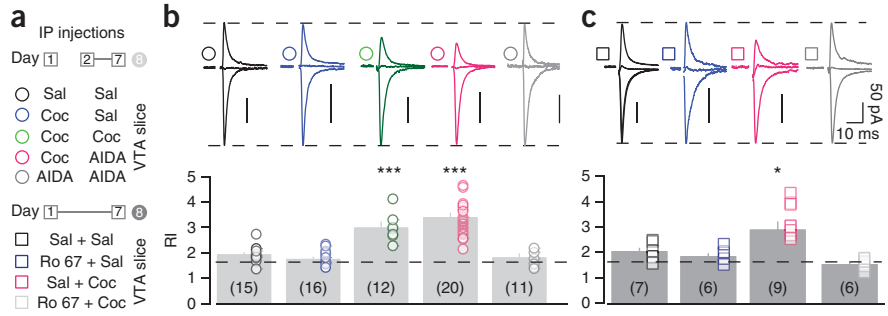

Figure 2 Bi-directional modulation of mGluR1 controls the persistency of cocaine-evoked plasticity in VTA. (**a**) Experimental protocols used for intraperitoneal injections of AIDA (top) and Ro 67-7476 (Ro 67, bottom). (**b**) AMPAR-EPSCs obtained at −70, 0 and +40 mV and respective mean rectification index for the experimental group treated with AIDA ($F_{4, 69} = 33.6$, *** indicates $P < 0.001$). (**c**) Corresponding graphs using Ro 67-7476, the positive modulator of mGluR1 (saline/cocaine × saline/ Ro 67-7476 interaction: $F_{1, 23} = 5.2$, $P = 0.031$).

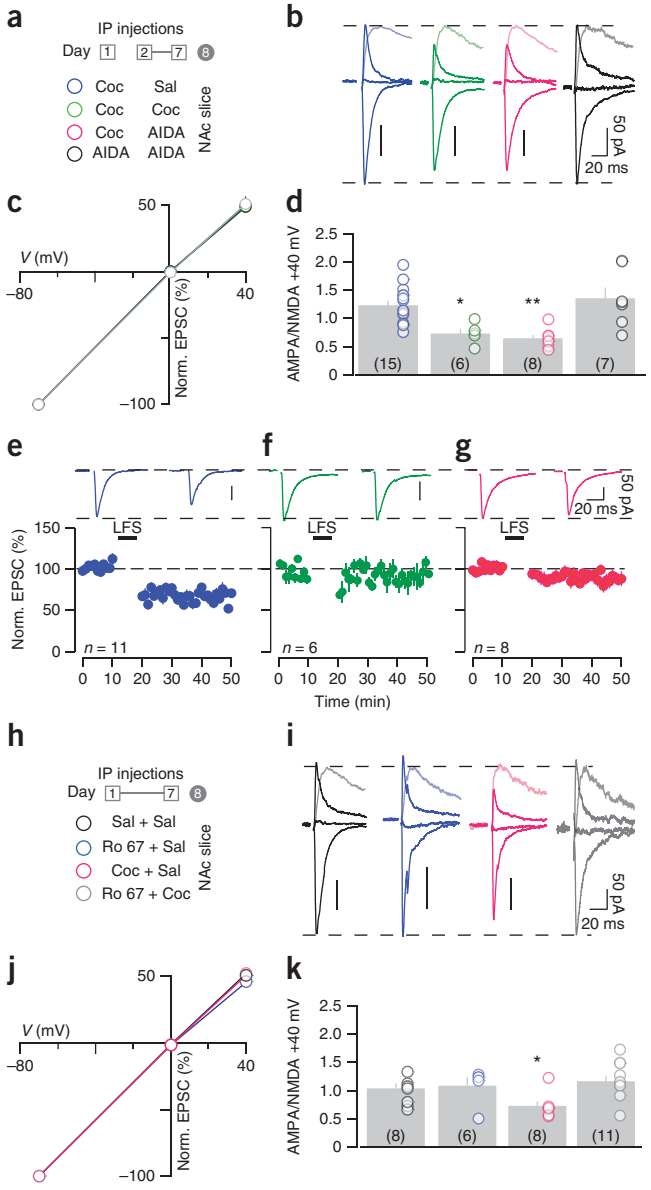

Figure 3 Modulation of mGluR1 controls cocaine-evoked plasticity in the NAc. (**a**) Experimental protocol. (**b**) AMPAR-EPSCs obtained at −70, 0 and +40 mV and NMDAR-EPSCs (shaded trace) obtained at +40 mV in medium spiny neurons of the NAc shell. (**c**) *I-V* plot for AMPAR-EPSCs. (**d**) Averaged AMPA/NMDA ratios obtained at +40 mV for each experimental group ($F_{3, 31} = 9.23$, * indicates $P < 0.05$, ** indicates $P < 0.01$). (**e**–**g**) LFS-LTD in the different experimental groups. (**h**) Experimental protocol. (**i**) AMPAR-EPSCs and NMDAR-EPSCs obtained as above in slices from mice treated with the mGluR1 enhancer Ro 67-7476 as indicated in **h**. (**j**). *I-V* plots of AMPAR-EPSCs. (**k**) Averaged AMPA/NMDA ratios obtained at +40 mV (saline/cocaine × saline/ Ro 67-7476 interaction: $F_{1, 33} = 4.34$, $P = 0.045$).

reversal of cocaine-evoked plasticity in the VTA *in vivo*, even in response to repeated cocaine injections.

Bi-directional control of plasticity in the NAc

We next used the modulation of mGluR1 as a tool to test the link between cocaine-evoked potentiation in the VTA and cocaine-evoked depression in the NAc. We started by testing the effect of the mGluR1 antagonist AIDA. Similar to the experiments described above, a first dose of cocaine was followed by six daily injections of either saline, cocaine or AIDA, and on day 8 we prepared coronal brain slices containing the NAc (**Fig. 3a**). In medium spiny neurons of the NAc shell, a day after the last injection, the *I-V* curves were linear in all conditions (**Fig. 3b**,**c**). A single injection of cocaine was not sufficient to induce changes in the AMPA/NMDA ratio, but transmission was significantly decreased with seven injections, consistent with previous reports[10] (**Fig. 3b**,**d**). Also consistent with previous reports, the decrease of AMPA/NMDA ratio elicited by cocaine occluded the induction of low-frequency stimulation (LFS)-induced LTD, suggesting that the two phenomena share underlying mechanisms[21] (**Fig. 3e**,**f**).

AIDA injections had no effect on synaptic transmission (**Fig. 3b**,**d**), but lowered the threshold for cocaine-evoked plasticity in the NAc; a single injection of cocaine was sufficient to significantly decrease the AMPA/NMDA ratio and occlude the synaptically induced LTD if that injection of cocaine was followed by six injections of AIDA (**Fig. 3b**,**d**,**g**). Conversely, when we applied seven injections of cocaine with Ro 67-7476 (**Fig. 3h**), the decrease of AMPA/NMDA ratio in the NAc was abolished (**Fig. 3i**,**k**).

To ensure that effects on plasticity in the NAc were the results of a local intervention at the level of the VTA, we carried out a series of experiments (**Fig. 4a**) using stereotactic injections in the VTA of the TAT-conjugated dominant-negative peptide (**Fig. 1a**). We observed that with this selective, local disruption of mGluR1 function in neurons of the VTA, a single injection of cocaine was sufficient to trigger the depression in the NAc. The control peptide followed by one injection of cocaine or saline had no effect on the AMPA/NMDA ratios (**Fig. 4b–d**). Similarly one saline injection after TAT-mGluRct delivery also had no effect (**Fig. 4b**,**d**). We next tested whether overriding mGluR1 in the VTA had an effect on enduring forms of plasticity, such as the insertion of GluR2-lacking AMPARs that can be observed a month after withdrawal. To this end, we measured the rectification index in medium spiny neurons of the NAc 35 d after the last cocaine injection (**Fig. 4e**). Ten daily injections followed by this protracted withdrawal period led to strongly rectifying EPSCs compared with controls ($t_{14} = 2.19$, $P < 0.05$; **Fig. 4f**,**h**). A similarly high rectification index was observed after only one cocaine injection if the mouse was pre-treated with a stereotactic injection of TAT-mGluRct ($t_{16} = 3.4$, $P < 0.01$; **Fig. 4f**,**h**). Thus, our data suggest that persistent plasticity in the VTA triggers a synaptic depression in the NAc and that a swift reversal of this plasticity may prevent synaptic alterations in the NAc. Taken together, interfering selectively with mGluR function in neurons of the VTA controls early forms and enduring forms of cocaine-evoked plasticity in the NAc.

VTA plasticity modulates cocaine seeking after withdrawal

It has been suggested that insertion of GluR2-lacking AMPARs contributes to the development of incubation of craving[11]. We hypothesized that preventing cocaine-evoked plasticity in the NAc would impair drug-seeking behavior after protracted withdrawal. We therefore tested this behavioral phenomenon in *Grin1*$^{loxP/loxP}$*; Slc6a3-creERT2* mice (*Grin1* is also known as *NR1* and *Slc6a3* is also known as *DAT*; we refer to these mice as NR1$^{DAT\text{-}CreERT2}$ mice). In this mouse, NMDARs are ablated in DA neurons during adulthood after tamoxifen-triggered recombination and we recently found that a single injection of cocaine no longer induces a synaptic potentiation in the VTA[22]. Following food shaping, NR1$^{DAT\text{-}CreERT2}$ and control mice were trained for 8 d in 4-h sessions to lever press for intravenous cocaine self-administration that was associated with a light cue. NR1$^{DAT\text{-}CreERT2}$ mice acquired stable lever pressing for cocaine similarly to controls (**Fig. 5a**; for lever presses on the inactive lever see **Supplementary Fig. 4**). After 35 d of

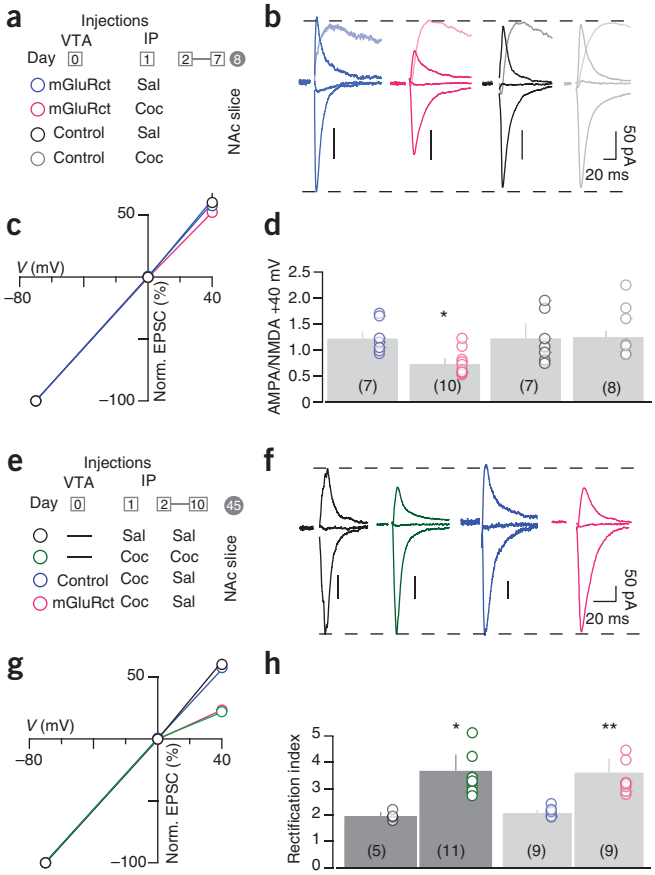

Figure 4 Early and enduring synaptic plasticity in the NAc after a single injection of cocaine. (**a**) Disruption of mGluR1 function selectively in the VTA through stereotactic injection of TAT-mGluRct (see **Fig. 1a**) and preparation of NAc slices at day 8. (**b**) AMPA- and NMDA-EPSCs obtained at –70, 0 and +40 mV. (**c**) *I-V* plots of AMPA-EPSCs. (**d**) Averaged AMPA/NMDA ratio obtained at +40 mV (TAT peptide delivery × intraperitoneal injection interaction: $F_{1,26}$, 4.24, $P = 0.05$). (**e**) Experimental protocol as described in **a**, but preparation of NAc slices was performed after 35 d of withdrawal. (**f**) AMPA- and NMDA-EPSCs obtained at –70, 0 and +40 mV. (**g**) *I-V* relationship of AMPA-EPSCs. (**h**) Averaged rectification indices.

withdrawal, the mice were tested for cue-induced cocaine seeking. This test session lasted 90 min, during which presses on the active lever under a fixed ratio schedule 1 triggered conditioned stimulus presentation without cocaine delivery. The incubation of cocaine-seeking behavior was significantly reduced in NR1$^{DAT\text{-}CreERT2}$ mice (factor genotype: $F_{1,14} = 5.126$, $P < 0.05$; factor lever: $F_{1,14} = 61.855$, $P < 0.001$; genotype × lever interaction: $F_{1,14} = 2.30$, $P > 0.05$; **Fig. 5b**).

Slices of the midbrain and the ventral striatum were then prepared from the very same mice within 48 h to measure AMPA/NMDA ratios and rectification indices. In the VTA of NR1$^{DAT\text{-}CreERT2}$ mice, NMDA EPSCs were absent and AMPAR EPSCs showed a linear rectification index value. In control mice, the AMPA/NMDA ratio and rectification index were high ($t_{11} = 2.61$, $P < 0.05$; **Fig. 5c,d**), confirming the presence of VTA plasticity following cocaine self-administration even after a protracted withdrawal period, a finding that was made previously in rats[9]. Moreover, in slices of the NAc, rectification was present in control mice, but not in NR1$^{DAT\text{-}CreERT2}$ mice ($t_{27} = 5.68$, $P < 0.001$; **Fig. 5c,d**). Plotting the rectification index as a function of the behavioral score revealed a significant correlation (weighted regression: $F_{1,14} = 13.7$, $P = 0.003$, $r^2 = 0.533$; **Fig. 5e**). Taken together, preventing the induction of cocaine-evoked plasticity in DA neurons of the VTA abolished early and enduring plasticity in the NAc and attenuated cue-induced cocaine seeking after prolonged withdrawal.

DISCUSSION

We found that mGluR1 receptors on DA neurons limit the persistence of the cocaine-evoked potentiation in the VTA. Moreover, overriding mGluR1 function caused enduring adaptations in the VTA that set the stage for the synaptic plasticity in the NAc, eventually leading to the insertion of GluR1 homomeric AMPARs and shaping cue-induced cocaine seeking.

Although these observations are compatible with a hierarchical organization of cocaine-evoked synaptic plasticity in between the VTA and the NAc, we did not identify the nature of the signal that transfers the message. It is possible that enhanced excitation of projection neurons in the VTA may facilitate the coincident release of DA and glutamate in the NAc through a continuous enhanced release of DA. This may then shift the threshold for the induction of local plasticity in the NAc by affecting circuit excitability or by integrating biochemical signals such as intracellular calcium or CaMKII signaling[10,23].

If such hierarchical organization of cocaine-evoked plasticity is confirmed downstream of the NAc, it may be of relevance in the context of the anatomical organization of the striatum[24,25]. These tracing studies show that VTA and NAc are part of a reciprocal spiral

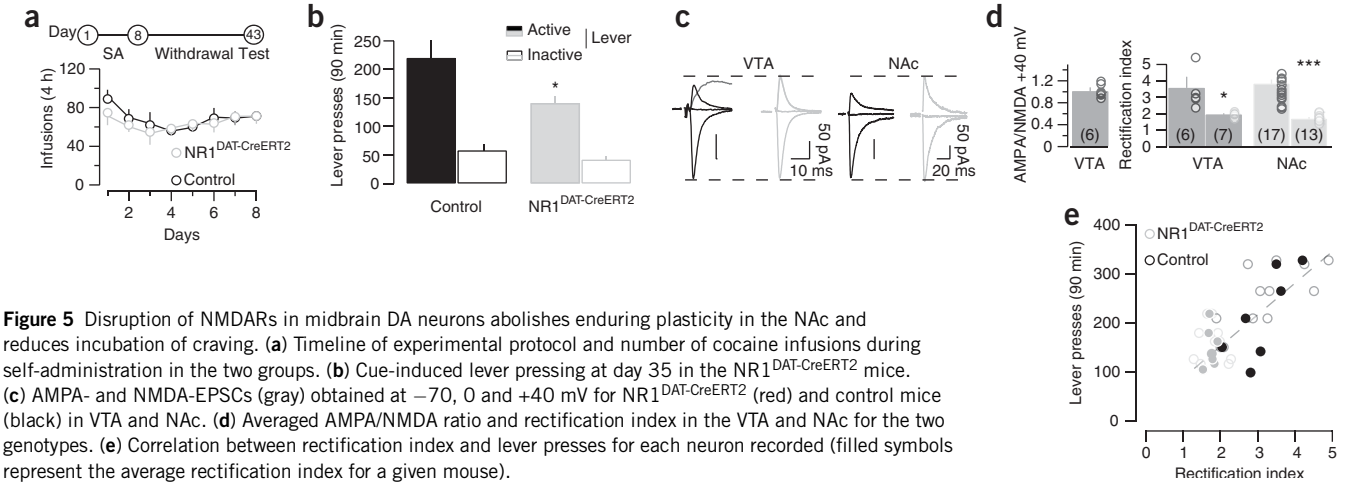

Figure 5 Disruption of NMDARs in midbrain DA neurons abolishes enduring plasticity in the NAc and reduces incubation of craving. (**a**) Timeline of experimental protocol and number of cocaine infusions during self-administration in the two groups. (**b**) Cue-induced lever pressing at day 35 in the NR1$^{DAT\text{-}CreERT2}$ mice. (**c**) AMPA- and NMDA-EPSCs (gray) obtained at –70, 0 and +40 mV for NR1$^{DAT\text{-}CreERT2}$ (red) and control mice (black) in VTA and NAc. (**d**) Averaged AMPA/NMDA ratio and rectification index in the VTA and NAc for the two genotypes. (**e**) Correlation between rectification index and lever presses for each neuron recorded (filled symbols represent the average rectification index for a given mouse).

in connectivity between the midbrain and the striatum. These findings implicate the dorsal components of the DA system in cocaine-seeking habits that are observed in addiction, which implies that the early drug effects on the VTA need to be transferred to the dorsal striatum via the NAc[2,26]. The hierarchical link of cocaine-evoked plasticity between VTA and NAc suggested here may represent the first leg of the spiraling connectivity underlying compulsive habits[27]. However, our work also clearly demonstrates that core components of addiction such as relapse can be modeled in rodents and do not require the recruitment of the dorsal striatum. It is possible that synaptic plasticity may emerge in more dorsal structures at later phases of the process.

Notably, recent studies suggest that the early cocaine-evoked plasticity in DA neurons of the VTA does not mediate concurrent short-term behavioral effects of the drug[22,28], although this idea was supported by earlier experiments. For example, local application of NMDAR antagonists in the VTA abolishes behavioral sensitization[29] and conditioned place preference (CPP)[30], as does genetic knockout of the AMPA receptor subunit GluR1 (ref. 31, but see ref. 32). Moreover, the viral overexpression of GluR1 enhances cocaine-induced behavioral sensitization in rats that have never been exposed to cocaine[33]. However, these approaches affect all cell types in the VTA. A selective deletion of the NMDAR subunit NR1 in the DA neurons of adult mice abolishes cocaine-evoked plasticity onto dopamine neurons of the VTA, but does not affect behavioral sensitization and the development of a CPP response[22]. In contrast, in the same mice, later drug-associated behaviors such as reinstatement of CPP are blocked. This finding remains controversial in the light of another study using a similar genetic approach, although differences in the CPP protocol may explain this discrepancy[28]. Altogether, these results raise the possibility that cocaine-evoked plasticity in the VTA may be important behaviorally for the late-stage drug-seeking behaviors, such as we observed. Because a recent report suggests that DA neurons projecting to the prefrontal cortex and the amygdala have reduced DAT expression[34], which may preclude recombination in the mutant mice, NAc projecting neurons are likely to mediate the bulk of the behavioral phenotype.

Our results may shed light on the mechanism underlying the progression from recreational use to compulsive abuse and relapse in drug addicts. They are consistent with the observation that self-administration of cocaine causes a more persistent plasticity in the VTA than passive injections[9]. Extending the observation that mGluR1-LTD rapidly reverses cocaine-evoked plasticity *in vitro*, we found that mGluR1-LTD in the VTA is required for the endogenous reversal of early cocaine-evoked plasticity in the VTA, as well as for adaptive gating of later cocaine-evoked plasticity in the NAc. Therefore, recruitment of mGluR1 functions as a protective mechanism to counteract drug exposure. However, there is a critical time window over which mGluR1 might control cocaine-evoked plasticity. Therefore, we think that it is unlikely that mGluR enhancement will be useful as a means to reverse previously established addiction in cocaine abusers, where persistent synaptic plasticity has already been relayed to other structures. However, our results do raise the possibility that individuals with deficient mGluR1-dependent LTD mechanisms may be particularly at risk of addiction. We suggest that the screening of genes controlling mGluR1 function may improve clinical efforts to assess individual vulnerability to drug addiction.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.M. carried out the electrophysiology experiments. B.H. performed the behavioral experiments. D.E. generated the mutant mice. J.R.P. bred the mice for the behavioral experiments and injected them with tamoxifen. C.L. designed the study with M.M. and C.C. and wrote the manuscript with the help of M.M. and R.S.

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ONLINE METHODS

Animals. $NR1^{DAT\text{-}CreERT2}$ mice were generated by crossing mice carrying an inducible Cre recombinase under the *Slc6a3* (*Dat*) promoter with mice carrying floxed alleles for NR1.1. The mutation was induced in 5-month-old mice by repeatedly intraperitoneally injecting 1 mg per kg of tamoxifen twice a day for 5 d. To ensure downregulation of NMDARs and a sufficient wash out period after tamoxifen treatment, we used two control and $NR1^{DAT\text{-}CreERT2}$ mice that were between 7 and 8 months old when the behavioral experiments started. They weighed 29.8 ± 1.4 g and 31.1 ± 0.8 g, respectively, on the day of surgery. Mice were single housed under constant temperature (21 ± 2 °C) and humidity conditions (50 ± 5%). All experiments took place during the light phase of the dark-light cycle, between 07:00 and 19:00. On three occasions during the food training phase, mice received limited access to food. They were otherwise given unrestricted access to food and water throughout the whole set of experiments. The mice's weight was monitored daily and kept above 85% of their initial weight. The experiments were conducted in accordance with the ethical European Union guidelines for the care and use of laboratory animals and were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe) and the Institutional Animal Care and Use Committee of the University of Geneva.

Drug treatment. C57BL/6 mice and Pitx3–green fluorescent protein (GFP) mice[35] (postnatal day 16–35) were injected intraperitoneally with 15 mg per kg cocaine, 0.9% saline, 0.25 mg per kg AIDA or 4 mg per kg Ro 67-7476 (injected 1 h prior to saline or cocaine injections) using a 26 gauge hypodermic needle (injection volume of 50–100 µl) to minimize stress. Unless specified, drugs were obtained from Tocris, spermine and picrotoxin were obtained from Sigma, and cocaine was obtained from the pharmacy of the Hopitaux Universitaires de Genève. Ro 67-7476 was a gift from F. Knoflach (F. Hoffman–La Roche). For the behavioral experiments, cocaine hydrochloride (Sigma-Aldrich, Chemie GmbH) was dissolved in saline. Ketamine and xylazine solutions were obtained from Pharmanovo GmbH. All solutions injected intravenously were first filtered through sterile filters (0.2 µm).

Stereotactic TAT-fused peptide delivery. Mice were anesthetized with ketamine (100 mg per kg) and xylazine (10 mg per kg) and placed in a stereotactic frame (myNeuroLab). The TAT sequence (YGRKKRRQRRR) was fused with *N*-carboxyfluorescein and a homologous sequence of the carboxy-terminal of Gp I mGluRs to prevent Homer 1b/c binding (ALTPPSPFR, Primm). As a control, the TAT sequence was fused only with *N*-carboxyfluorescein. TAT-fused peptides were injected (0.6 µl at the concentration of 1 µM) with a glass pipette (Drummond Scientific Company) bilaterally (−2.4 mm antero-posterior and 0.8 mm lateral from Bregma, and −4.4 mm from the surface).

Electrophysiology in acute brain slices. Horizontal slices from midbrain (250 µm thick) and coronal slices containing NAc (300 µm thick) were prepared following the experimental injections protocols described in the text. Slices were kept in artificial cerebrospinal fluid containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$ and 11 mM glucose, bubbled with 95% O_2 and 5% CO_2. Whole-cell voltage-clamp recording techniques were used (30–32 °C, 2–3 ml min^{-1}, submerged slices) to measure the holding currents and synaptic responses of DA neurons of the VTA and of medium spiny neurons of the NAc shell. The VTA is defined as the region medial to the MT (medial terminal nucleus of the accessory optical tract). DA neurons were identified either by the presence of a large hyperpolarization-activated (I_h) current immediately after obtaining a whole-cell configuration (80%) or by using slices form a Pitx3-GFP mouse strain (20%) that express GFP only in cells that express Pitx3, a transcription factor required for the development of DA neurons of the midbrain. The internal solution contained 130 mM CsCl, 4 mM NaCl, 2 mM $MgCl_2$, 1.1 mM EGTA, 5 mM HEPES, 2 mM Na_2ATP, 5 mM sodium creatine phosphate, 0.6 mM Na_3GTP and 0.1 mM spermine. Currents were amplified, filtered at 5 kHz and digitized at 20 kHz. The liquid junction potential was small (–3 mV) and traces were therefore not corrected. All experiments were carried out in the presence of picrotoxin (100 µM) and AMPAR EPSCs were pharmacologically isolated by application of the NMDA antagonist D,L(–)-2-amino-5-phosphonovaleric acid (D,L-AP5, 100 µM). The NMDAR component was calculated as the difference between the EPSCs measured in the absence and presence of D,L-AP5. The AMPAR to NMDAR ratio was calculated by dividing the peak amplitudes. The rectification index was calculated by dividing the amplitude of the AMPAR EPSC measured at −70 mV by the amplitude at +40 mV. The holding potential was −60 mV or −70 mV, and the access resistance was monitored by a hyperpolarizing step of −10 mV with each sweep, every 10 s. Experiments were discarded if the access resistance varied by more than 20%. Synaptic currents were evoked by stimuli (0.05–0.1 ms) at 0.1 Hz through bipolar stainless steel electrodes placed rostral to the VTA or, when recordings were performed in the NAc, at the prelimbic cortex−NAc border to stimulate preferentially cortical afferences. Where indicated, mGluR LTD in DA neurons of the VTA was induced by application of the Gp I mGluR agonist DHPG (20 µM for 5 min.), whereas NMDA-dependent LTD in medium spiny neurons of the NAc shell was induced by LFS (1 Hz at −40 mV for 10 min). Representative example traces are shown as the average of 10–20 consecutive EPSCs typically obtained at each potential or, in the case of plasticity protocols, during the last 5 min of the baseline and at least 20 min after the induction of plasticity.

Mouse behavior. All operant experiments were performed in mouse operant chambers model ENV-307W enclosed in light- and sound-attenuating cubicles (Med-Associates). Each chamber was equipped with two ultrasensitive retractable levers located on each side of a food pellet dispenser during the food-shaping procedure. During intravenous self-administration, the drug delivery PVC tubing was attached to a swivel (Instech Solomon) and connected to an infusion pump (PHM-100, Med-Associates) located outside the cubicle. Stimulus lights were located above each lever.

Food training. Sessions lasted 90 min and started with the presentation of the two levers. To guarantee unbiased lever training, the side of the initially active lever was alternated between each session. Active lever presses were reinforced by the delivery of a 16-mg sweetened pellet (Bio-Serv) under the following schedule: fixed ratio 1 (FR1) for eight reinforcements, FR2 for four reinforcements and FR4 for at least ten reinforcements with 80% accuracy on the active lever, which completed a cycle.

When such a cycle was achieved, the side of the active lever was switched. All mice underwent 16 daily food-training sessions. On three occasions, sessions were separated by a day of inactivity, during which mice had limited access to food. This happened between sessions 2 and 3, 9 and 10, and between sessions 14 and 15.

Intravenous self-administration. Following food shaping, mice were allowed 24 h before undergoing surgery. Mice were anesthetized with a ketamine (160 mg per kg) and xylazine (38 mg per kg) solutions and implanted with a catheter in the right jugular vein. On one end catheters were introduced for circa 1 cm toward the heart, while the other extremity was passed subcutaneously to an exit in the mid-scapular region. Catheters were (MIVSA, CamCaths) made of silicone elastomer tubing (outside diameter 0.63 mm × inside diameter 0.30 mm) attached to 26 gauge stainless steel tubing secured to a Bard mesh pad. Mice were given a minimum of 48 h recovery before intravenous self-administration sessions were initiated. Catheters were flushed daily before and after self-administration sessions with 30 µl of a heparine solution in saline (20 international units). At the beginning of the cocaine self-administration sessions, mice were placed into the operant chambers and the two levers were presented. Presses on the active lever under FR1 resulted in the infusion of 0.5 mg per kg per infusion of cocaine solution by activation of the infusion pump for 1.2 s. The side of the active lever was randomly assigned across mice on the first day of intravenous self-administration and remained constant thereafter. Each drug infusion was associated with a conditioned stimulus consisting of a 10-s flashing of stimulus lights located above the levers. To avoid accidental overdose, we gave the mice a 40-s time-out period following each infusion, during which active levers were recorded, but had no consequence. Each session lasted 4 h and mice received a total of eight sessions.

Incubation effect and cue-induced cocaine-seeking. At the end of the intravenous self-administration phase, mice were returned to their home cage, where they remained for 35 d. After this time, they were re-introduced to the operant chambers, where they were tested for cue-induced cocaine-seeking. The

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session lasted 90 min, during which, as in the self-administration condition, presses on the active lever under an FR1 triggered conditioned stimulus presentation and activation of the infusion pump. However, cocaine was no longer available and conditioned stimulus presentation lasted for 5 s only. Such a short conditioned stimulus presentation was preferred to prevent as much as possible extinction toward its repeated exposure.

Statistical analysis. Compiled data are expressed as mean ± s.e.m. The level of significance was taken at $P = 0.05$, as determined by the non-parametric Mann-Whitney or Wilcoxon tests. Data from food training and cocaine self-administration experiments were analyzed using repeated measures ANOVA to investigate the effect of day, genotype and lever when appropriate. Data from cue-induced cocaine seeking were analyzed using factorial ANOVA to investigate the effect of genotype and lever. Analyses were followed by Newman-Keuls tests when necessary.

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