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Glutamate Receptors on Dopamine Neurons Control the Persistence of Cocaine Seeking

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SUMMARY

Cocaine strengthens excitatory synapses onto midbrain dopamine neurons through the synaptic delivery of GluR1-containing AMPA receptors. This cocaine-evoked plasticity depends on NMDA receptor activation, but its behavioral significance in the context of addiction remains elusive. Here, we generated mice lacking the GluR1, GluR2, or NR1 receptor subunits selectively in dopamine neurons. We report that in midbrain slices of cocaine-treated mice, synaptic transmission was no longer strengthened when GluR1 or NR1 was abolished, while in the respective mice the drug still induced normal conditioned place preference and locomotor sensitization. In contrast, extinction of drug-seeking behavior was absent in mice lacking GluR1, while in the NR1 mutant mice reinstatement was abolished. In conclusion, cocaine-evoked synaptic plasticity does not mediate concurrent short-term behavioral effects of the drug but may initiate adaptive changes eventually leading to the persistence of drug-seeking behavior.

INTRODUCTION

A major hypothesis in the addiction field is that cocaine-induced neuroadaptations in the mesocorticolimbic dopamine (DA) system and associated limbic structures contribute to persistent compulsive drug use and relapse (Kauer and Malenka, 2007; Thomas et al., 2008). In particular, glutamatergic synapses on DA neurons in the ventral tegmental area (VTA) undergo plastic changes after cocaine administration (Saal et al., 2003; Ungless et al., 2001). By increasing synaptic strength (Ungless et al., 2001) and facilitating long-term potentiation (Liu et al., 2005), cocaine augments the responsiveness of DA neurons to glutamate. Cocaine-induced synaptic strengthening in DA neurons in the VTA is associated with changes in AMPA receptor (AMPAR) subunit composition (Bellone and Lüscher, 2006). Thus, incorporation of GluR1 subunits, most likely by forming highly conductive, calcium permeable GluR1 homomeric AMPARs, drives the drug-induced synaptic strengthening, while the insertion of GluR2 containing receptors reverts it (Mameli et al., 2007). The synaptic recruitment of GluR1 subunits and the resulting synaptic potentiation requires the activation of NMDA receptors (NMDARs) (Dong et al., 2004). Although synaptic strengthening of DA neurons by a single (Ungless et al., 2001; Bellone and Lüscher, 2006) or repeated (Borgland et al., 2004) cocaine injection may eventually lead to enduring neuroplasticity (Thomas et al., 2008), its relationship to drug-induced behavioral effects remains elusive.

Therefore, a fundamental but unresolved question is how drug-induced synaptic changes in DA neurons relate to the development of reinforcement and landmarks of addictive behavior in rodents such as behavioral sensitization and drug-seeking behavior (Sanchis-Segura and Spanagel, 2006). While it is clear that synaptic potentiation of excitatory synapses on DA neurons of the VTA is too short-lasting (days) to serve as a neural substrate for persistent addictive behavior, it may be essential for early cocaine-induced behavioral responses and for triggering long-term adaptations that underlie addiction (Lüscher and Bellone, 2008; Thomas et al., 2008). In support of a behavioral link, glutamate antagonists injected into the VTA reduce locomotor sensitization (Carlezon and Nestler, 2002) and attenuate cocaine reinforcement measured by conditioned place preference (CPP) (Harris and Aston-Jones, 2003). Furthermore, cocaine exposure increases GluR1 subunit expression in the VTA (Fitzgerald et al., 1996; Churchill et al., 1999; Grignaschi et al., 2004; but see also Lu et al., 2002), and virally mediated GluR1 upregulation in the VTA increases drug-induced locomotor sensitization and CPP (Carlezon et al., 1997). These results led to the hypothesis that cocaine-induced locomotor sensitization and reinforcement

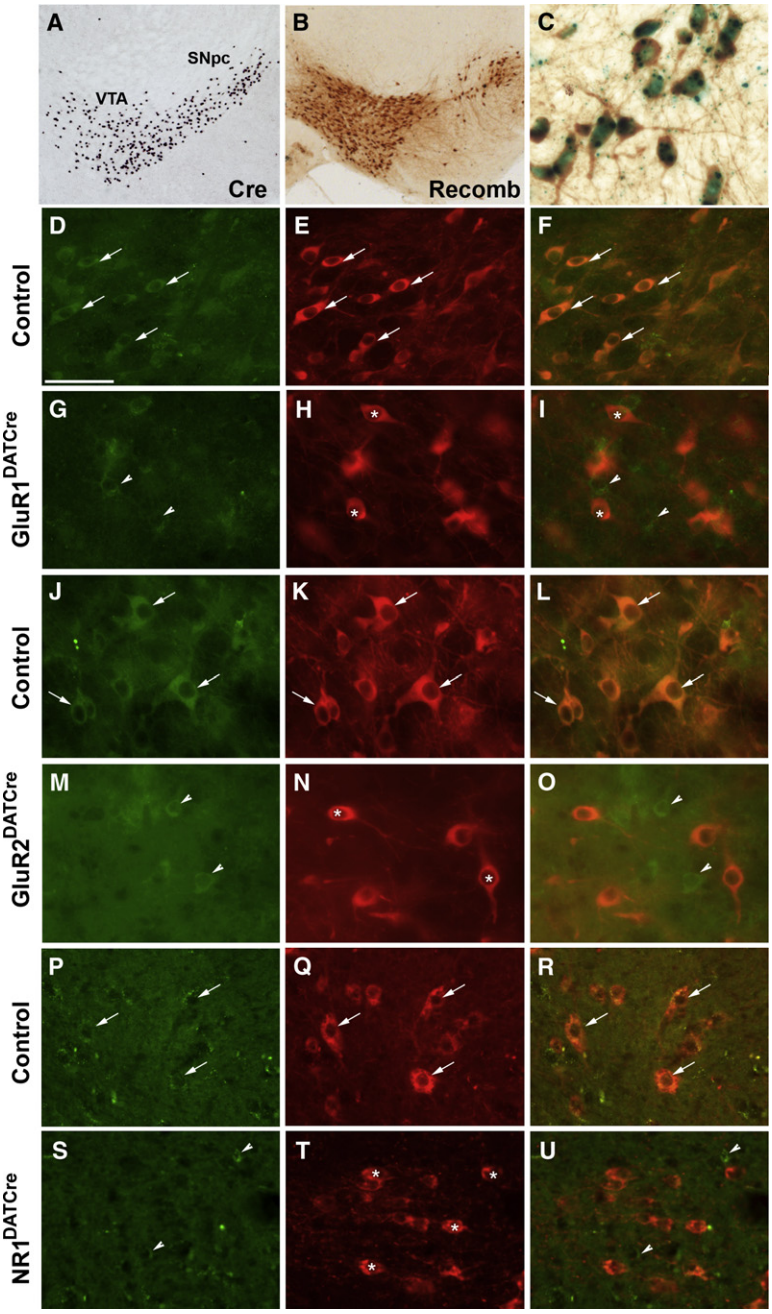

Figure 1. Targeting of the Mutation to Dopaminergic Cells

(A) Immunohistochemistry showing abundant Cre-positive cells in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc) but not in surrounding areas.

(B) Micrograph from a Z/EG^{DATCre} mouse, showing EGFP immunoreactivity, which indicates recombination. Recombination can be seen in structures known to harbor dopaminergic cells while other structures show no signs of recombination.

(C) High-power micrograph from a ROSA26^{DATCre} mouse. Brown reaction product indicates immunoreactivity for tyrosine hydroxylase, and blue reaction product indicates presence of β-galactosidase, which is expressed only after recombination. Note that recombination has occurred in all TH-positive cells.

(D–U) Expression of GluR1, GluR2, and NR1 in dopaminergic neurons of transgenic mice. (D–I) Representative coronal sections from the VTA showing immunofluorescence for GluR1 (D and G) and TH (E and H) in control (D–F) and GluR1^{DATCre} (G–I) mice, respectively. In sections from control mice, GluR1 colocalize with TH, while GluR1 is only expressed in TH-negative neurons (e.g., arrowheads) in GluR1^{DATCre} mice. (F and I) Overlay of the images in (D) and (E) and (G) and (H). (J–O and P–U) Corresponding analysis for GluR2 and NR1 in GluR2^{DATCre} mice (M–O) with controls (J–L) and NR1^{DATCre} mice (S–U) with controls (P–R), respectively. Note that the deletion was efficient and specific in all cases.

are mediated by changes in AMPAR composition and also depend on NMDARs in DA neurons of the VTA. However, direct experimental evidence for the behavioral significance of the cocaine-induced synaptic changes involving GluR1, GluR2, and NMDARs in DA neurons is lacking.

RESULTS

Mice with Mutations Specific to DA Neurons

We used Cre/LoxP based conditional gene deletions in the mouse to evaluate the role of individual glutamate receptor subunits in the cocaine-induced plasticity in DA neurons and the functional importance of this plasticity for addiction-related behavior. To achieve specific gene deletion in DA neurons, we generated a mouse line expressing the Cre-recombinase controlled by the promoter of the dopamine transporter (DAT) (Parlato et al., 2006). The Cre construct, which is based on a bacterial artificial chromosome (BAC) harboring the DAT gene, was integrated into the genome by random insertion transgenesis. This approach has the advantage that the endogenous DAT gene is not compromised. In this specific line, it has previously been shown that Cre expression starts only postnatally and is preferentially localized to midbrain DA neurons (Parlato et al., 2006). Thus, hypothalamic DA neurons are not affected by recombination, likely due to the lower expression of DAT in these neurons. Moreover, compensatory mechanisms are not engaged during the prenatal development (Parlato et al., 2006). As expected, we found that Cre was expressed selectively in DA neurons of the midbrain (Figure 1A). Cre recombination, which was visualized by using the Z/EG and Rosa26-LacZ reporter lines, could be induced in all midbrain DA neurons examined (Figures 1B and 1C). However, we found no change in target abundance in other structures of the brain (see Figure S1 available online).

To determine the role of GluR1 in cocaine-induced synaptic plasticity, we generated mice lacking GluR1 in DA cells by crossing a mouse line with floxed GluR1 with the DATCre line (GluR1$^{fl/fl}$ × DATCre, abbreviated GluR1^{DATCre} mice). Since cocaine triggers a plasticity that is expressed by the insertion of GluR2-lacking AMPARs (Bellone and Lüscher, 2006) and induced

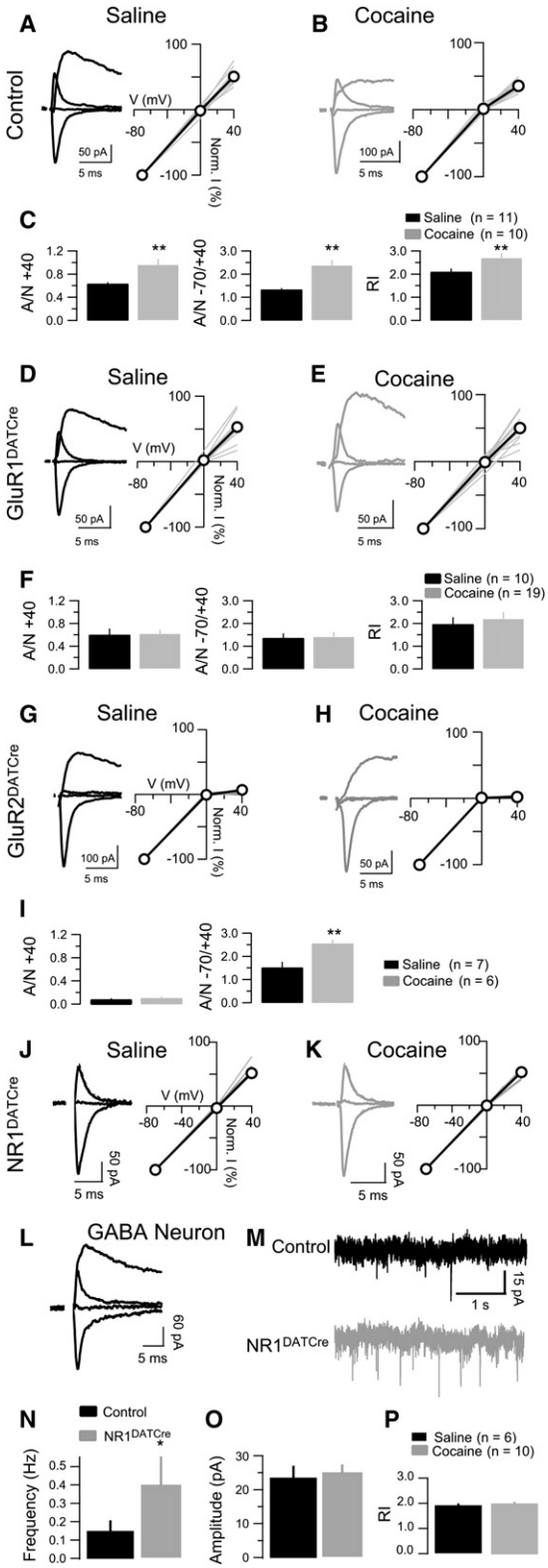

Figure 2. Effects on AMPAR- and NMDAR-Mediated EPSCs and Cocaine-Induced Synaptic Strengthening

(A) Representative traces from control mice of pharmacologically isolated AMPAR-mediated EPSCs recorded at −70, 0, and +40 mV and the mathematically subtracted NMDAR-mediated EPSC recorded at +40 mV and corresponding I-V plots of AMPAR-EPSCs. Only the control group for the GluR1^{DATCre} mice is shown. The control groups for GluR2^{DATCre} and NR1^{DATCre} mice are shown in Figure S2.

(B) Traces obtained using an identical protocol as above in slices from mice exposed to a single injection of cocaine 24 hr prior to sacrifice.

(C) Bar graphs of AMPAR/NMDAR EPSC (A/N) ratio at +40 and −70 mV and rectification index in same cells (Rectification index, RI = AMPAR EPSC −70/AMPAR EPSC +40 mV).

(D–F) Corresponding experiments in GluR1^{DATCre} mice: note that in GluR1^{DATCre} mice, A/N ratios and RI after cocaine injection remain unchanged.

(G–I) Corresponding experiments in GluR2^{DATCre} mice: note that in cocaine-treated GluR2^{DATCre} mice, the A/N ratio when measured at −70 and +40 mV, respectively, is higher compared to saline-injected mice.

(J and K) Traces and I-V plots of AMPAR-EPSCs in NR1^{DATCre} mice. The A/N cannot be calculated because NMDARs are absent.

(L) Example of an isolated AMPAR EPSC and corresponding NMDAR EPSC in a non-DA neuron of a NR1^{DATCre} mouse. Note that NMDAR EPSCs are present in this neuron.

(M–O) Basal synaptic transmission is altered in DA neuron of the VTA from NR1^{DATCre} mice. DA neurons in the NR1^{DATCre} mice showed an increased frequency (M and N) but normal amplitudes (M and O) of spontaneous EPSC recorded at −60 mV.

(P) RI in DA cells from NR1^{DATCre} mice. Note that the RI is not increased in NR1^{DATCre} mice after cocaine injection. *p ≤ 0.05, **p ≤ 0.01.

through NMDAR signaling (Dong et al., 2004), we also generated mice lacking GluR2 (GluR2^{DATCre} mice) and NR1 (NR1^{DATCre} mice) in DA neurons. As NR1 is an obligatory subunit, NMDAR function should be abolished in NR1^{DATCre} mice.

We first performed double immunofluorescence labeling for GluR1, GluR2, or NR1 with tyrosine hydroxylase (TH), a marker for DA neurons. In slices from control mice, GluR1, GluR2, and NR1 were expressed in both TH-positive and TH-negative neurons (Figures 1D–1F, 1J–1L, and 1P–1R) but were only expressed in TH-negative neurons in the GluR1^{DATCre}, GluR2^{DATCre}, and NR1^{DATCre} mice, respectively (Figures 1G–1I, 1M–1O, and 1S–1U). This confirms the specific ablation of individual glutamate receptor subunits in the transgenic mouse lines.

Cocaine-Induced Synaptic Strengthening Depends on GluR1 and NR1 in DA Neurons

We next investigated the effects of our specific gene deletions on basal synaptic transmission and cocaine-induced synaptic strengthening by whole-cell patch recordings in acute slices of the VTA. We measured AMPAR and NMDAR mediated excitatory postsynaptic currents (EPSCs) in voltage clamp conditions at −70 mV and +40 mV in slices obtained from control mice injected with a single dose of cocaine or saline (Figures 2A, 2B, and S2). We then used these observations to calculate AMPA/NMDA ratios by dividing AMPAR EPSCs collected at +40 mV and −70 mV by NMDAR EPSCs measured at +40 mV (Figures 2C and S2). We also determined the rectification index RI for AMPAR mediated responses (RI = EPSC −70 mV/EPSC +40 mV). This was done in order to determine the contribution of AMPARs lacking GluR2 since this type of AMPARs are calcium permeable and show strongly rectifying synaptic responses, i.e., currents are smaller at positive potentials compared to currents at symmetrical negative potentials.

In slices from saline-injected mice, we found normal values for all parameters in GluR1^{DATCre} mice (Figures 2D and 2F). In

GluR2^{DATCre} mice the AMPA/NMDA ratio +40 mV/+40 mV was strongly reduced precluding the calculation of RI (Figures 2G–2I), while when the AMPA/NMDA ratio was measured as −70 mV/+40 mV, there was no difference from saline-injected controls (Figures 2I and S2). Taken together, these results reflect a highly rectifying AMPAR population devoid of GluR2. In NR1^{DATCre} mice, the AMPA/NMDA ratio could not be calculated since the NMDAR EPSCs were completely abolished (Figures 2J and 2K) in DA neurons, albeit still present in neighboring GABA neurons (Figure 2L). Strikingly, we found a higher occurrence of spontaneous AMPAR-EPSCs (Figures 2M–2O) in NR1^{DATCre} mice, in line with an adaptive upregulation of AMPARs in the absence of NMDARs (Hall et al., 2007) through a process similar to synaptic scaling observed in neuronal cultures (Turrigiano, 2007). Alternatively, the number of synaptic connections may be higher in the NR1^{DATCre} mice.

When ex vivo recordings were performed in slices from control animals prepared 24 hr after a single exposure to cocaine (15 mg/kg i.p.), we observed significant increases in the AMPAR/NMDAR ratio as well as the RI in all control mouse lines (Figures 2C and S2; $p < 0.01$), in line with previous reports (Bellone and Lüscher, 2006; Ungless et al., 2001). We also examined whether repeated cocaine administration increased the magnitude of the increase in AMPAR-mediated synaptic transmission compared to a single injection in control animals. Mice treated with seven injections of cocaine showed a significantly larger RI (3.19 ± 0.68 versus 1.96 ± 0.18, n = 4, data not shown) 24 hr after the last injection when compared to controls receiving multiple injections of saline. The magnitude of this increase, however, was similar to that elicited by a single injection of cocaine (Figure 2), which is in line with a previous report in rats (Borgland et al. 2004).

Interestingly, in GluR1^{DATCre} mice, the AMPAR/NMDAR ratio and the RI did not differ between cocaine- and saline-injected mice (Figure 2F). In GluR2^{DATCre} the AMPAR/NMDAR ratio was significantly larger in cocaine-treated mice compared to controls (Figure 2I; $p < 0.01$). Both these observations confirm that the expression of the cocaine-evoked plasticity is mediated by the insertion of AMPARs containing GluR1 but not GluR2. Finally, in NR1^{DATCre} mice, no difference in rectification was observed after cocaine treatment (Figure 2P), which supports the idea that cocaine-induced plasticity at this synapse requires functional NMDARs on DA neurons.

Collectively, these results demonstrate that on the functional level the mutagenesis was efficient in DA neurons while not affecting neighboring GABAergic neurons. Further, they show that basal synaptic strength is unaffected in GluR1^{DATCre} and GluR2^{DATCre} mice, while NR1^{DATCre} mice show an increased responsiveness. Finally, the data provide genetic evidence that GluR1, and probably also NMDARs, in DA cells are necessary for the cocaine-induced synaptic strengthening and thus confirm that GluR1^{DATCre} and NR1^{DATCre} mice are valid models for studying the neurochemical and behavioral consequences of such strengthening.

Normal Extracellular DA Release and Locomotor Behavior in the Mutant Mice

The observed alterations in excitatory afferent transmission in DA neurons in GluR1^{DATCre} and NR1^{DATCre} mice prompted us to study the release properties of DA neurons in these mutants. To this end, we used microdialysis in freely moving mice. Guide cannulas were implanted in the nucleus accumbens, and sampling was performed 1 week later in the home cage. In controls, the basal DA levels were 576 ± 148 pM. No significant differences were seen in comparison to GluR1^{DATCre} (553 ± 152 pM) and NR1^{DATCre} mice (808 ± 365 pM), although a trend toward higher values and more variability was seen in NR1^{DATCre} mice (Figure 3A). Since DA release in the nucleus accumbens strongly influences locomotor activity, we also monitored home cage activity for 48 hr before the microdialysis experiment started (Figures 3C and 3F). In addition, we measured the exploration behavior of these mice in an open-field arena (Figures 3D and 3G). Finally, further activity was monitored during the microdialysis experiment (Figures 3E and 3H). Under all tested conditions the mutant mice did not differ significantly from the control mice, although NR1^{DATCre} mice had a tendency to show enhanced basal locomotor activity (Figures 3F and 3H).

An important feature of addictive drugs such as cocaine is the increase of extracellular DA levels within the nucleus accumbens following acute application (Di Chiara and Imperato, 1988; Spanagel and Weiss, 1999; Lüscher and Ungless, 2006). After an i.p. administration of a 10 mg/kg dose of cocaine, all genotypes responded with an approximately 5-fold increase in DA levels (Figure 3B). Three hours later, all animals were injected with a 20 mg/kg dose of cocaine leading to a 10-fold increase in DA release. The changes in extracellular DA after cocaine treatment were significant in all genotypes ($p < 0.0001$), whereas there were no significant differences between the genotypes for both doses ($p = 0.40$). In parallel, on-line monitoring of cocaine-induced locomotor activity during the microdialysis experiment (Figures 3E and 3H) did not reveal any differences between the genotypes in response to the 10 mg/kg i.p. dose of cocaine. At the higher dose, activity measurement was confounded by the occurrence of stereotypic behavior in some animals.

Normal Cocaine-Induced Sensitization and Conditioned Place Preference (CPP) in the Mutant Mice

To assess the short-term and long-term behavioral consequences of GluR1, GluR2, and NR1 gene inactivation in DA neurons, we used the mutant mice in various behavioral tests that serve a models of core components of drug addiction (Sanchis-Segura and Spanagel, 2006). First, we tested locomotor activity following saline injection, which did not differ between genotypes. Moreover, all animals similarly increased their locomotor activity in response to an acute cocaine challenge (10 mg/kg; i.p.; Figures 4A–4C). These results are in line with the normal DA levels seen in the mutant mice. Then we tested the development of cocaine-induced behavioral sensitization, a progressively escalating locomotor response to a fixed drug dose (Sanchis-Segura and Spanagel, 2006). Mice of all genotypes showed a progressive increase in cocaine-induced activity, and no differences between genotypes were observed (Figures 4A–4C). Our data suggest that GluR1-driven synaptic strengthening in DA neuron and other cocaine-evoked adaptations dependent on GluR1, GluR2, and/or NR1

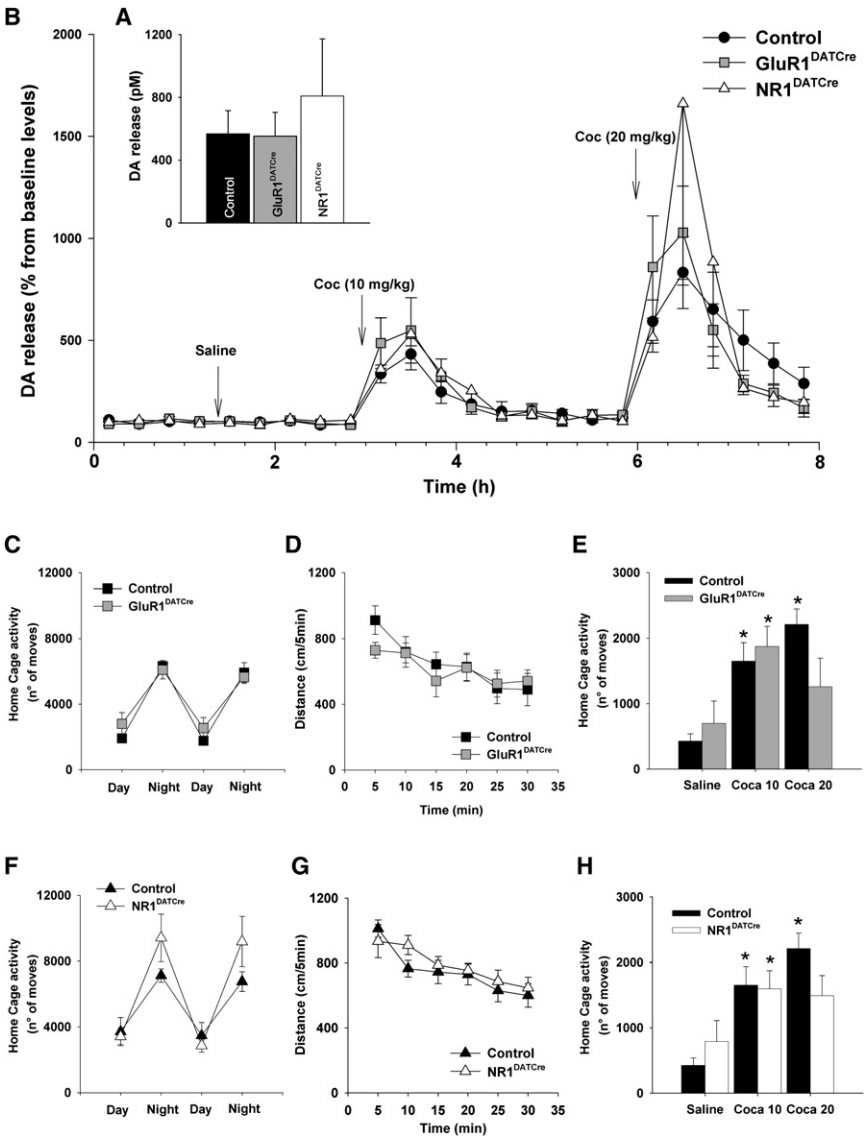

Figure 3. Basal and Cocaine-Induced Extracellular Dopamine Levels and Locomotor Activity in GluR1^{DATCre} and NR1^{DATCre} Mice

(A and B) Measurement of basal and cocaine-induced extracellular dopamine levels in GluR1^{DATCre}, NR1^{DATCre} mice and their respective controls. (A) Basal levels of extracellular DA release in pM measured in the nucleus accumbens of GluR1^{DATCre} (n = 6), NR1^{DATCre} (n = 4), and control mice (n = 8; since no differences occurred between both control groups, all control mice were pooled together). These values correspond to the averaged value of the first four samples collected. (B) Percentage of increase of DA release compared to baseline levels during 8 hr and following saline injection and successive cocaine challenges (10 mg/kg and 20 mg/kg i.p.). The changes after cocaine treatment were significant in all genotypes (ANOVA for repeated-measures; *Treatment* $F_{23,\ 345} = 18.28$, $p < 0.0001$) whereas there were no statistically significant differences between the genotypes for both doses (ANOVA for repeated-measures; *Treatment* Genotype* $F_{46,345} = 1.04$, $p = 0.40$).

(C–H) Measurement of basal and cocaine-induced locomotor activity in GluR1^{DATCre}, NR1^{DATCre} mice, and their respective controls. Home cage locomotor activity was monitored over 48 hr for both genotypes and appropriate control animals (n = 10–11 mice per genotype). Neither GluR1^{DATCre} nor NR1^{DATCre} mice differed significantly from their respective control mice. (D and G) Furthermore, open field locomotor activity was assessed by the distance traveled every 5 min for 30 min (n = 10–12 mice per genotype). Again, no significant difference could be measured in GluR1^{DATCre} and in NR1^{DATCre} mice compared to their respective controls. (E and H) Finally, locomotor activity was assessed during the microdialysis experiments. Both doses of cocaine enhanced activity in controls and both genotypes (Newman-Keuls post hoc for all genotypes, *$p < 0.01$ compared to saline injection). However, at the 20 mg/kg dose augmented stereotypic behavior was observed in some animals.

All data presented are mean ± SEM.

in these cells do not play a role in cocaine-induced locomotor sensitization.

Next, we tested the ability of these mice to acquire a CPP for cocaine. In this test, which is used to measure reinforcement and drug-seeking behavior (Tzschentke, 2007), mice are trained to associate a given context with the drug, resulting in preference for this environment compared to a saline-paired context. All genotypes showed a robust CPP (Figures 5A–5C) for the cocaine-paired compartment, identical to that of control mice. We found no differences between genotypes in locomotor activity during the CPP (Figure S3), and a pairwise correlation (Pearson's index) between the percental increase in locomotion and the CPP did not covariate ($r = -0.07$, $p = 0.60$). In summary, GluR1^{DATCre}, GluR2^{DATCre}, and NR1^{DATCre} mice had CPP scores that were indistinguishable from that exhibited by the appropriate control mice.

Reduced Extinction of CPP in GluR1^{DATCre} Mice

Drug addiction is characterized by persistent drug-seeking behavior (Sanchis-Segura and Spanagel, 2006). In order to assess the persistence of cocaine-seeking behavior in our mutant mice, we next studied the extinction of the CPP response. This was done by saline injections in the previously drug-paired environment. As expected, control mice showed a robust, almost complete, extinction of CPP after 16 extinction sessions (Figure 5A–5C). Robust extinction was also observed in the GluR2^{DATCre} and NR1^{DATCre} mice to a level identical to that of control mice. Interestingly, GluR1^{DATCre} mice displayed no extinction of the CPP (Figure 5A). Thus, there was a significant difference between genotypes (control versus GluR1^{DATCre}) in extinction of the CPP response ($p < 0.01$), showing that GluR1 in DA neurons are essential for extinction of cocaine-induced CPP. Locomotor activity during extinction testing did not differ between genotypes (Figure S3).

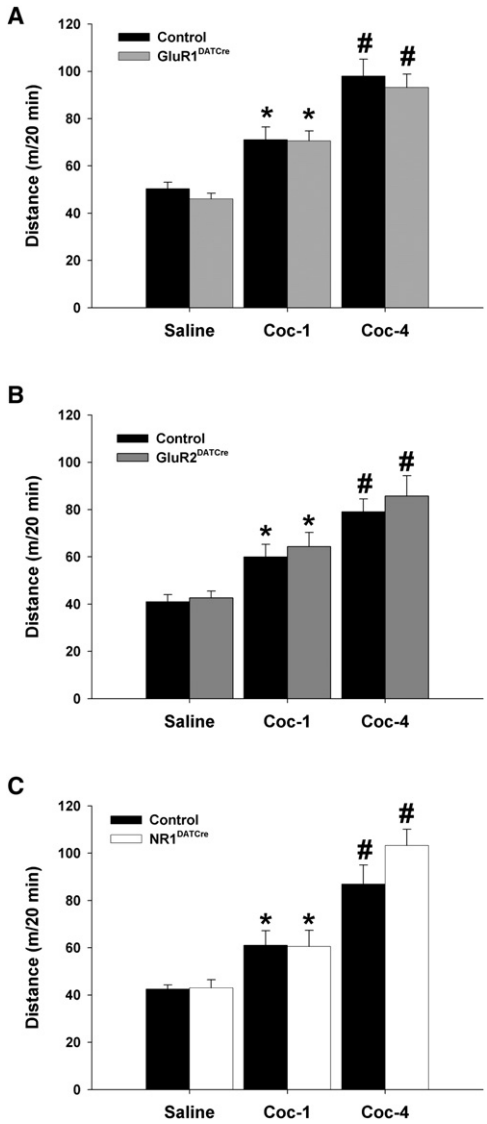

Figure 4. Acute Cocaine-Induced Locomotion and Cocaine-Induced Behavioral Sensitization

Behavioral effects of cocaine in GluR1^{DATCre} (n = 14), GluR2^{DATCre} (n = 15), NR1^{DATCre} (n = 18), and control mice for each genotype (n = 14–19).

(A–C) Effects of acute and repeated cocaine injections on locomotor activity. A single cocaine injection (10 mg/kg; i.p.) increased locomotion regardless of the genotype (Coc-1) The development of sensitization was also independent of the genotype, and it was expressed as a progressive increase of the locomotion elicited by a fixed (10 mg/kg, i.p.) cocaine dose (Coc-4) (ANOVA for repeated-measures, *Treatment*, GluR1^{DATCre}: $F_{2,50} = 97.90$, $p < 0.001$; GluR2^{DATCre}: $F_{2,46} = 33.52$, $p < 0.001$; NR1^{DATCre}: $F_{2,66} = 66.57$, $p < 0.001$) . All data presented are mean levels ± SEM. Newman-Keuls post hoc for all genotypes: *$p < 0.01$ compared to saline injection; #$p < 0.01$, compared to day 1.

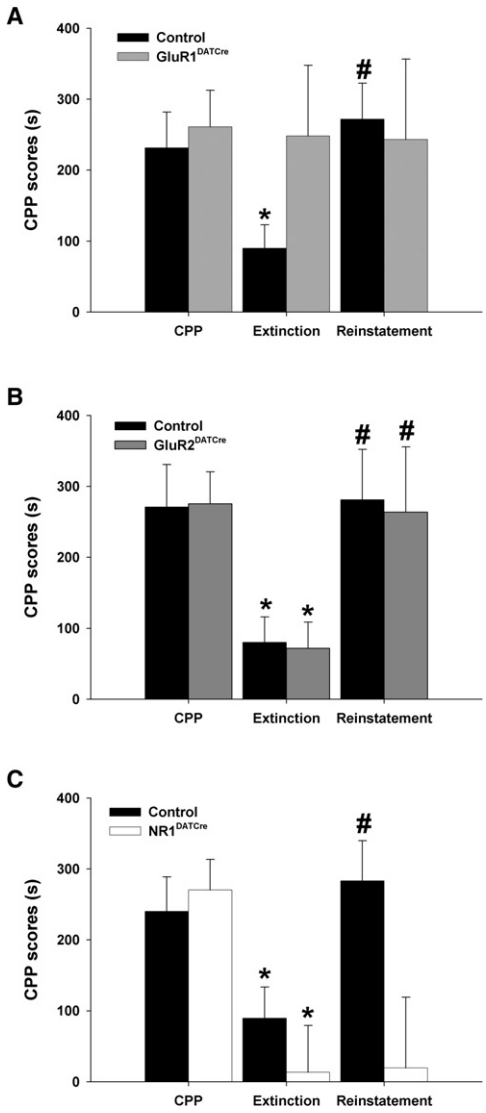

Figure 5. Cocaine-Induced CPP, Extinction, and Reinstatement

The CPP score represents the time spent (seconds) in the cocaine-paired floor minus the time spent in the saline-paired floor during the test day (test duration, 900 s). All animals exhibited a significant cocaine-induced CPP, and no genotype effect was observed (ANOVA for repeated-measures, GluR1^{DATCre}: $F_{1,50} = 0.3$, $p = 0.5$; GluR2^{DATCre}: $F_{1,18} = 0.01$, $p < 0.9$; NR1^{DATCre}: $F_{1,31} = 2.49$, $p < 0.1$). Control, GluR2^{DATCre} (A) and NR1^{DATCre} (B) mice, but not GluR1^{DATCre} (C) mice displayed a significant reduction of the time spent on the cocaine-paired floor after extinction training (Newman-Keuls post hoc, CPP response compared to extinction in GluR1^{DATCre} $p = 0.8$; GluR2^{DATCre} $p = 0.04$; NR1^{DATCre} $p = 0.003$). Reinstatement of cocaine-seeking behavior was induced by a priming injection (7.5 mg/kg, i.p.) of cocaine. Whereas control mice and GluR2^{DATCre} showed a significant reinstatement of cocaine-seeking behavior, NR1^{DATCre} mice showed no reinstatement. All data are depicted as mean ± SEM. *$p < 0.01$ compared to CPP; #$p < 0.01$ compared to extinction levels.

Reinstatement of CPP Depends on NMDARs in DA Neurons

Another key feature of drug addiction is relapse (Sanchis-Segura and Spanagel, 2006). Reinstatement of cocaine-seeking can be triggered by drug-associated cues, stress, or drug re-exposure (Shaham et al., 2003). Since control, GluR2^{DATCre}, and NR1^{DATCre} mice showed a normal extinction, we tested for reinstatement of CPP in these lines, a test modeling relapse-like behavior (Sanchis-Segura and Spanagel, 2006; Shaham et al., 2003). As expected, a priming dose of cocaine completely reinstated CPP

in control mice (Figures 5A–5C). In stark contrast, mice lacking NMDARs in DA neurons did not display reinstatement of cocaine-seeking behavior (Figure 5C; $p < 0.01$). Again, GluR2^{DATCre} mice did not exhibit any obvious phenotype in this specific test (Figure 5B). Since GluR1 mice did not show extinction, it was not possible to test reinstatement. They maintained their preference for the cocaine-paired compartment as expected. Notably, locomotor behavior during reinstatement testing did not differ between genotypes (Figure S3), showing that the mechanism triggering reinstatement is distinct from the mechanisms maintaining elevated locomotor activity in response to the drug. These results show that NMDAR signaling in DA neurons is critical for relapse-like behavior.

Inducible Mutation of NR1 in DA Neurons

As described above, we observed an upregulation of AMPAR-responses in the NR1^{DATCre} line. We can therefore not rule out that the absence of cocaine-evoked plasticity was due to occlusion and that the basal enhanced AMPAR transmission could also influence the behavioral phenotype. To circumvent these adaptive phenomena, we used a mouse line in which recombination specific to DA neurons can be induced on demand. In this DATCreERT2 mouse line, a Cre recombinase fused to a modified ligand-binding domain of the estrogen receptor is expressed under control of the DAT promoter (Figure 6A). Thus recombination selectively in DA neurons can be triggered by repeated tamoxifen administration (Feil et al., 1997; Erdmann et al., 2007). We generated NR1^{DATCreERT2} mice and injected them twice per day for 5 days with 1 mg tamoxifen starting at P15 to switch off NR1 synthesis. We then allowed 8 days for the downregulation of endogenous NMDARs, injected cocaine at day 9 and sacrificed the mice 24 hr later. In slices from these mice, synaptic NMDAR responses were absent in DA neurons but still present in neighboring non-DA neurons, demonstrating the specificity of the deletion (Figures 6B–6E). In tamoxifen-treated NR1^{DATCreERT2} mice, exposure to cocaine did not affect the RI, and the current-voltage relationships of the recorded neurons were linear reflecting transmission mediated by GluR2-containing AMPARs (Figures 6B–6D). Taken together, our results confirm that functional NMDARs are necessary for the induction of the cocaine-evoked plasticity of glutamatergic synapses onto DA neurons.

We next measured the CPP response to cocaine in NR1^{DATCreERT2} mice. For this experiment, the mutation was induced in 8-week-old male mice and CPP training was started 8 days later. Similar to NR1^{DATCre} mice, tamoxifen-treated NR1^{DATCreERT2} mutants showed a normal CPP response to cocaine (Figure 7A). It is unlikely that the tamoxifen treatment schedule applied here affects general behaviors such as locomotion, learning, and memory (Vogt et al., 2008). Nevertheless, we also quantified the locomotor activity in the home cage and in an open-field arena and did not observe any differences compared to control animals that received vehicle injections (Figures 7B and 7C), showing that tamoxifen treatment per se had no impact on the behavior in the CPP test. Finally, we tested extinction and reinstatement of the CPP response in NR1^{DATCreERT2} and control mice. Both genotypes showed normal extinction (Figure 7A). A priming dose of cocaine completely reinstated

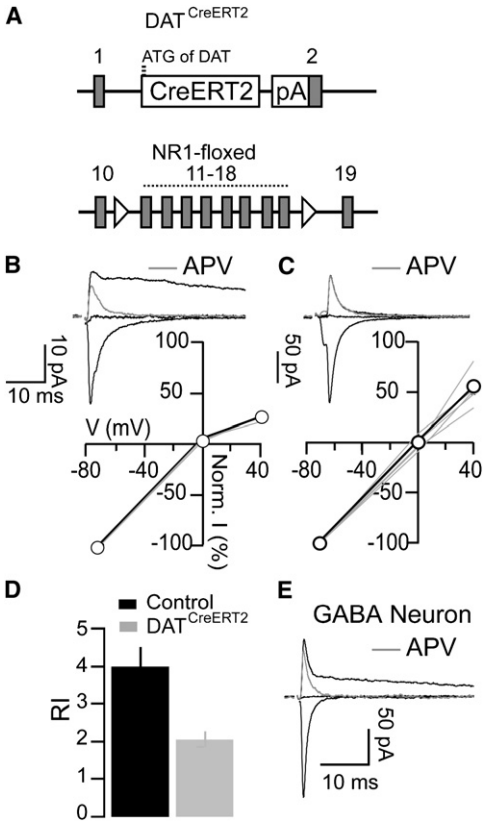

Figure 6. Generation of NR1^{DATCre} Inducible Mice and Absence of Cocaine-Evoked Plasticity in NR1^{DATCreERT2} Mice

(A) Schematic representation of the construct for the DATCreERT2 mouse line.

(B and C) EPSCs recorded at −70, 0, and +40 mV before and after APV application and corresponding I-V plots of AMPAR-EPSCs from control and NR1^{DATCreERT2} mice.

(D) Corresponding RI. Note that while increased in control mice, the RI is not increased NR1^{DATCreERT2} mice 24 hr after cocaine injection. The A/N cannot be calculated because NMDARs are absent.

(E) Example of an isolated AMPAR EPSC and corresponding NMDAR EPSC in a nondopaminergic neuron of an NR1^{DATCreERT2} mouse. As expected, NMDAR EPSCs are present in this neuron.

Error bars indicate SEM.

CPP in control mice (Figure 7A), while the mutants did not reinstate cocaine-seeking behavior (Figure 7A; $p < 0.01$).

To determine whether NR1^{DATCreERT2} mice still showed the scaling of the AMPAR-mediated responses, we performed electrophysiological recordings in the same mice that were previously used for the behavioral test, which at that time were 80 days old (Figures 7D–7I). We found no difference in the frequency nor in the amplitude of spontaneous EPSCs between the two genotypes. We also determined the RI in DA neurons 48–72 hr after the last cocaine injection and found linear current-voltage relationships in the NR1^{DATCreERT2} mice and significant rectification in control mice (Figures 7D–7I).

Taken together, we conclude that NMDARs expressed on DA neurons are required for the induction of the cocaine-induced plasticity as well as reinstatement.

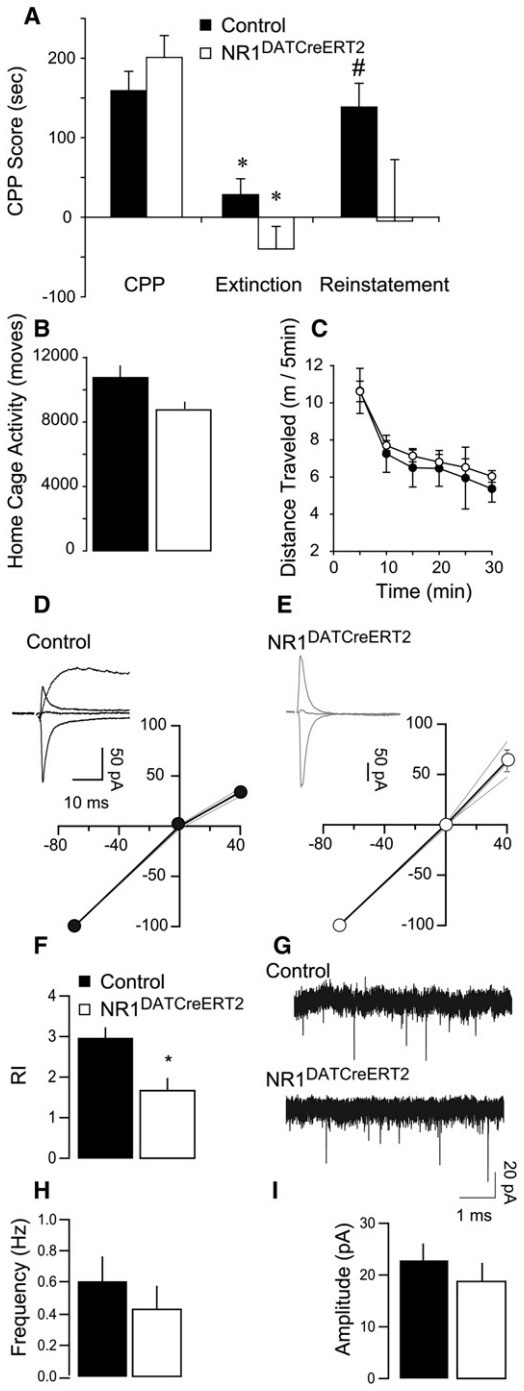

Figure 7. Behavioral Testing followed by Ex Vivo Electrophysiological Characterization of Adult NR1^{DATCreERT2} Mice

(A–C) Cocaine-induced CPP, extinction, reinstatement, and locomotor activity in NR1^{DATCreERT2} mice. (A) The CPP score represents the time spent (s) in the cocaine-paired floor minus the time spent in the saline-paired floor during the test day (test duration, 900s). All control (n = 6) and NR1^{DATCreERT2} (n = 7) mice exhibited a significant cocaine-induced CPP and displayed a significant reduction of the time spent on the cocaine-paired chamber after extinction training (compared to CPP). When the reinstatement of the CPP was induced by a priming injection (7.5 mg/kg) of cocaine, only control but not NR1^{DATCreERT2} mice showed reinstatement of cocaine-seeking behavior (compared to extinction). NR1^{DATCreERT2} and control mice were also monitored for behavior in their home cage (B) and when they explored an open field arena for 30 min (C). Home cage activity measured during 24 hours or the distance traveled in the open field each 5 min intervals did not differ between genotypes.

(D–I) NMDAR-mediated EPSPs in NR1^{DATCreERT2}. (D and E) Traces recorded at −70, 0, and +40 mV and the mathematically subtracted NMDAR-mediated EPSC recorded at +40 mV and I-V plots of AMPAR-EPSCs obtained from control and NR1^{DATCreERT2} mice which underwent behavioral experiments. Mice were sacrificed 48 hr after they were injected with cocaine during reinstatement experiment. (F) Corresponding RI. Note that RI is increased in DA neurons from control mice. (G) Sample traces showing spontaneous AMPAR EPSCs from control and NR1^{DATCreERT2} mice. (H and I) Bar graph of spontaneous AMPAR EPSCs frequency and amplitude.

Error bars indicate SEM. #, *p $\leq$ 0.05.

DISCUSSION

A major hypothesis in the etiology of addictive behavior points at drug-induced plasticity within the mesocorticolimbic DA system as one of the major causes of compulsive cocaine-seeking and relapse behavior (Kauer and Malenka, 2007; Thomas et al., 2008). In particular, it is suggested that cocaine strengthens excitatory synapses on midbrain DA neurons, presumably by NMDAR-dependent synaptic incorporation of GluR1-containing AMPARs (Kauer and Malenka, 2007; Lüscher and Bellone, 2008). The neurochemical and behavioral consequences of these drug-induced molecular alterations are still unclear. Using conditional knockout mice—lacking either GluR1, GluR2, or NR1 receptor subunits selectively in DA neurons—we measured DA release properties and cocaine-induced behaviors and correlated our findings with the electrophysiological characterization of excitatory synaptic transmission onto DA neurons of the VTA. In summary, we show that mice lacking cocaine-induced synaptic strengthening in DA neurons due to perturbed AMPAR plasticity or NMDAR signaling exhibit normal basal and cocaine-induced DA release properties. These mice also exhibit normal reinforcement and behavioral sensitization to cocaine. However, we found two alterations in the persistence of drug-seeking behavior. First, a genetic deletion of the AMPAR GluR1 subunit within DA neurons resulted in a specific deficit of extinction of cocaine-induced reinforcement. Second, blocking NMDAR signaling in DA neurons abolished reinstatement of cocaine-seeking behavior in the CPP test, suggesting a critical role of this molecular mechanism in relapse behavior. Reinstatement was absent both in mice with a constitutive deletion of the NR1 subunit selective to DA neurons, as well as in mice where NR1 was removed in these neurons in adulthood.

The mouse models presented here have some advantages compared to other approaches for gene inactivation. Most importantly, we achieved highly specific targeting of glutamate receptor subunits in DA neurons. Such specificity is not achieved by typical viral-mediated gene suppression methods, due to infection of non-DA neurons. Our BAC-based Cre transgenesis approach also has a clear advantage compared to previously reported DATCre mice (Zhuang et al., 2005), since those mice have only one allele for DAT, and it is well known that this causes phenotypic effects that interfere with several DA-mediated behaviors (Giros et al., 1996; Spielewoy et al., 2000). In our DATCre

line, the DAT gene is not affected, allowing us to draw more precise conclusions regarding the gene of interest. Finally, the tamoxifen-inducible system for selective mutagenesis in DA neurons in the adult brain circumvents developmental adaptations, such as the scaling of spontaneous AMPAR-mediated transmission.

Our electrophysiological data confirm the immunohistochemical analysis showing that the mutagenesis was efficient in DA neurons and that the AMPA/NMDA ratio is unaffected in GluR1^{DATCre} and GluR2^{DATCre} mice, while NR1^{DATCre} mice show a scaling of spontaneous AMPAR transmission which reflects an adaptation during synaptogenesis (Adesnik et al., 2008). Finally, the electrophysiological data show that GluR1 and NMDARs in DA cells are necessary for the cocaine-induced synaptic strengthening and thus confirm that GluR1^{DATCre} and NR1^{DATCre} mice may be used to study the behavioral consequences of such strengthening.

The behavioral characterization of mice lacking GluR1 in DA neurons adds an important piece of information to the long-standing debate on the functional role of GluR1-upregulation and synaptic potentiation in these cells. It is known that GluR1 is upregulated in DA neurons in response to cocaine administration (Fitzgerald et al., 1996; Churchill et al., 1999; Grignaschi et al., 2004; but see also Lu et al., 2002) and blockade of AMPARs by intra-VTA administered antagonists reduces locomotor sensitization (Carlezon and Nestler, 2002). In an attempt to link cocaine-evoked plasticity with behavior Dong et al. (2004) tested knockout mice lacking GluR1 and found that locomotor sensitization did not differ from wild-type mice. However, these mice show an increased basal locomotor activity and a basal AMPA/NMDA ratio as high as after cocaine administration in wild-type mice (Dong et al., 2004). Moreover, since GluR1 is absent in the entire brain, brain regions other than the VTA may mediate the behavioral effects. The use of GluR1^{DATCre} mutants in the present study now demonstrates a clear dissociation between synaptic potentiation in DA neurons and locomotor sensitization.

The link between CPP and GluR1 is even more controversial. In a study using GluR1 knockout mice, cocaine-induced CPP was normal (Mead et al., 2005) while another group reported a complete absence of CPP (Dong et al., 2004). An obvious difference between these studies is that Dong et al. (2004) used a biased CPP protocol, whereas in Mead et al. (2005) and our present study in GluR1^{DATCre} mutants a nonbiased CPP protocol was used, and different outcomes have been reported in several other CPP studies when using biased versus nonbiased protocols (Tzschentke, 2007). In order to further resolve this discrepancy in the literature, we also tested if mice lacking GluR1 in the whole brain can exhibit a normal CPP response to cocaine administration in our nonbiased procedure. We found that GluR1 knockouts showed in fact a CPP response comparable to control mice (Figure S4), indicating that GluR1 is dispensable for CPP under the conditions used in this study. This conclusion is further supported by the results in our GluR1^{DATCre} mice. They have normal locomotor activity, normal levels of DA, and normal AMPA/NMDA ratio under basal conditions but show no synaptic potentiation in DA neurons in response to cocaine. The normal CPP response in these mice shows that GluR1-upregulation and synaptic potentiation in DA neurons is not related to the development of cocaine reinforcement.

NR1^{DATCre} mutants also showed a normal CPP response to cocaine and developed behavioral sensitization to the same degree as control animals. At first sight, these findings might be surprising in the light of previous studies using local VTA injections of NMDAR antagonists (Kalivas and Alesdatter, 1993; Harris and Aston-Jones, 2003). Furthermore, in a very recent report it was shown that cocaine-induced sensitization and CPP are attenuated in NMDAR-deficient mice (Ramsey et al., 2008). However, similar to the case with GluR1, our present study differs fundamentally from these previous reports since we targeted only DA neurons. These observations suggest that NMDAR-dependent mechanisms in cells other than DA neurons mediate sensitization and reinforcement. For example, in the VTA, GABA and glutamate neurons are also present (Yamaguchi et al., 2007). Moreover, expression of mutant NMDARs in D1 receptor-expressing neurons (i.e., non-DA neurons), prevents cocaine sensitization and decreases cocaine preference (Heusner and Palmiter, 2005).

One of our key findings is that a GluR1-dependent mechanism in DA neurons is important for extinction of cocaine-seeking behavior. In line with this, a previous report has observed extinction deficits in nonconditional GluR1 knockout mice following self-administration of cocaine (Stephens and Mead, 2003).

Our second key finding is that NMDARs in DA neurons are necessary for reinstatement of cocaine-seeking behavior. This is in line with previous studies indicating that DA neurons projecting to the medial prefrontal cortex are important for reinstatement of cocaine self-administration by activating glutamatergic neurons projecting to the nucleus accumbens (reviewed in Schmidt et al., 2005). However, while the NR1^{DATCre} mouse line achieves selectivity for DA neurons in the midbrain, they have altered baseline transmission, which may influence the behavior. To circumvent these limitations, we generated the NR1^{DATCreERT2} mice where the NR1 subunit can be removed once development is completed. Since our NR1^{DATCre} and NR1^{DATCreERT2} mice exhibit a normal CPP, we suggest that postlearning reactivation of NMDARs is crucial for reinstatement of drug-seeking behavior. In this respect, it has been shown previously that postlearning reactivation of NMDARs plays a more general role in consolidation of memories (Wang et al., 2006).

It may be surprising that the behavioral phenotype of GluR1^{DATCre} mice differs from NR1^{DATCre} mice—despite the absence of cocaine-evoked plasticity in both mouse lines. Since the inducible NR1^{DATCreERT2} and the constitutive NR1^{DATCre} line showed similar behavioral alterations, we do not believe that the scaling of AMPAR EPSC neurons observed in the NR1^{DATCre} line could explain this difference. Our data rather suggest that extinction relies on a mechanism that requires GluR1 but is distinct from the NMDAR-dependent cocaine-evoked plasticity. Since testing reinstatement is contingent to normal extinction, it cannot be reliably done in the GluR1^{DATCre} mice, but our model would predict reinstatement to be absent in these mice. Alternatively, since in the GluR1^{DATCre} line GluR1 is constitutively absent in all DA neurons, we cannot exclude that developmental adaptations or changes in DA neurons outside the VTA (e.g., substantia nigra) influence this behavior.

In conclusion, we show that AMPAR and NMDAR dependent cocaine-induced synaptic strengthening in DA neurons seems not to be related to cocaine-induced behavioral sensitization and CPP. However, our data cannot exclude the possibility that GluR1 and/or NR1 subunits in DA cells are important for sensitization and reinforcement when using other experimental paradigms e.g., self-administration procedures or other addictive drugs.

We further show that a genetic deletion of the AMPAR GluR1 subunit within DA neurons results in a specific deficit of extinction of cocaine-induced reinforcement. This finding provides a new rationale in the treatment of cocaine addiction: the selective activation of the GluR1 subunit could potentially improve the outcome of any given exposure therapy. In fact, it has recently been shown that an AMPAR potentiator can facilitate extinction learning for contextual fear memory (Zushida et al., 2007). Therefore, the use of AMPAR potentiators may also facilitate extinction processes related to cocaine taking behavior. Finally, we provide evidence for a critical role of NMDAR signaling in DA neurons for relapse behavior. Because of the temporal difference, the cocaine-evoked plasticity can neither be the cellular correlate of extinction nor reinstatement. However, synaptic plasticity in the VTA could trigger adaptations in other parts of the brain. Thus cocaine-evoked plasticity in the VTA could represent a first step in a cascade of adaptive pathways that develop in parallel and eventually lead to the persistent changes associated with addiction (Lüscher and Bellone, 2008).

EXPERIMENTAL PROCEDURES

Mice

GluR1^{DATCre} and GluR2^{DATCre} mice were generated by crossing mice carrying the DATCre transgene with GluR1$^{fl/fl}$ and GluR2$^{fl/fl}$ mice (Shimshek et al., 2006) having loxP-flanked exons 11 of the *Gria1* or *Gria2(586R)* alleles, respectively. GluR1$^{fl/fl}$ mice were generated as described in Zamanillo et al. (1999). NR1^{DATCre} mice were generated by crossing mice carrying the DATCre transgene with NR1$^{fl/fl}$ mice having exon 11–18 of *Grin1* flanked by loxP sites (Niewoehner et al., 2007). The DATCre construct consists of an improved Cre inserted at the transcriptional start of the DAT gene (Parlato et al., 2006). DATCre mice have been characterized previously (Parlato et al., 2006). In order to monitor the recombination, DATCre mice were also crossed with mice in which the gene for LacZ has been introduced under control of the ROSA26 promoter but is interrupted by a loxP-flanked stop-cassette (Gt(ROSA)26-Sor^{tm1Sor}), generating ROSA26^{DATCre} mice. In addition, we crossed DATCre mice with a second reporter line (Z/EG; Cg-Tg(CAG-Bgeo/GFP)21Lbe/J), in which the expression of EGFP is triggered by Cre-mediated recombination. All mice were backcrossed in C57BL6/N for at least six generations. For behavioral and electrophysiological experiments, GluR1^{DATCre} (GluR1$^{fl/fl}$; DAT-Cre), GluR2^{DATCre}, and NR1^{DATCre} were used. As controls, fl/fl littermates not carrying the Cre were used.

NR1^{DATCreERT2} mice were generated by crossing mice with an inducible Cre-recombinase under the DAT-promoter with mice carrying floxed alleles for NR1. The DATCreERT2 mice were generated by recombining a construct containing an improved Cre-recombinase fused to a modified ligand binding domain of the estrogen receptor (CreERT2) into a BAC containing the gene encoding DAT (the same BAC as used for the DATCre line), using BAC recombineering. To induce the mutation, 1 mg of tamoxifen was administered i.p. twice a day for 5 days, according to previously published protocols (Erdmann et al., 2007).

Mice were housed individually, kept under a 12 hr light/12 hr dark conditions (lights on 07–19) and fed ad libitum. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe, License number 35-9185.81/G-153/05) and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Histochemistry

Immunohistochemistry detecting the Cre-recombinase was performed using a custom-made antiserum against Cre. The immunoreactivity was visualized using an ABC, peroxidase, and DAB-based protocol (Parlato et al., 2006). EGFP was detected with a polyclonal antibody (1:10000, Invitrogen, A11122). X-gal histochemistry in combination with immunohistochemistry for tyrosine hydroxylase was performed as previously described (Parlato et al., 2006), with minor modifications. Thus mice were perfused with 4% PFA, and brains were immediately transferred to 30% sucrose in PBS and incubated overnight. Coronal sections were cut on a cryostat and incubated, free-floating, in X-gal substrate overnight. Subsequently, immunohistochemistry was performed using an anti-tyrosine hydroxylase antibody (1:2000; Chemicon). For dual-labeling immunohistochemistry against glutamate receptors and TH, affinity-purified polyclonal antibodies against GluR1 (AB1504), GluR2 (AB1768-25UG), and NR1 (AB9864) from Chemicon and a monoclonal antibody against TH (Calbiochem) were used. Animals were deeply anaesthetized and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Tissue blocks containing the area of DA neurons were embedded in 4% agarose and sectioned at 60 μm with a microtome. Sections were blocked in 10% normal goat serum (NGS) and subsequently incubated in a mixture of primary antibodies for (1) GluR1 and TH, (2) GluR2 and TH, or (3) NR1 and TH, in Tris-buffered saline (TBS) containing 2% NGS overnight at 4°C. For NR1 labeling, the sections were pretreated with 2 μg/ml Pepsin in order to improve accessibility to the epitopes. After washes, sections were further incubated in a mixture of secondary antibodies (anti-rabbit Alexa 488 for GluR1, GluR2, or NR1 and anti-mouse cyanine-derived fluorochrome Cy3 for TH) made up in TBS for 2 hr at room temperature. Subsequently, sections were washed, mounted, and coverslipped. Fluorescent signals were examined using an epifluorescence microscope (Nikon).

Electrophysiology in Acute Slices

For the electrophysiological experiments, mice at postnatal days 18–80, 7–30 g bodyweight were used. Horizontal slices (250 μm thick) of the midbrain were prepared in cooled 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, and bubbled with 95% O_2 and 5% CO_2. Whole-cell voltage-clamp recording techniques were used (32°C–34°C, 2–3 ml min^{-1}, submerged slices) to measure the holding currents and synaptic responses of DA neurons of the VTA, identified as the region medially to the medial terminal nucleus of the accessory optical tract. DA neurons were identified by the presence of a large hyperpolarization-activated (I_h) current immediately after obtaining a whole-cell configuration. The internal solution contained (mM):130 CsCl, 4 NaCl, 2 $MgCl_2$, 1.1 EGTA, 5 HEPES, 2 Na_2ATP, 5 sodium creatine-phosphate, 0.6 Na_3GTP, and 0.1 spermine. Currents were amplified, filtered at 5 kHz, and digitized at 20 kHz. The liquid junction potential was small (−3 mV), and therefore traces were not corrected. All experiments except where noted were carried out in the presence of picrotoxin (100 μM) and D,L-APV (100 μM). The holding potential was −50 mV, and the access resistance was monitored by a hyperpolarizing step to −60 mV with each sweep, every 10 s. Experiments were terminated 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 positioned rostral to the VTA. The NMDAR component was calculated as the difference between the EPSCs measured in the absence and presence of D,L-APV (100 μM). 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.

Microdialysis

Eight-month-old male GluR1^{DATCre}, NR1^{DATCre} mice and their respective controls were used. Mice were mounted in a stereotactic device (Stoelting) under isoflurane anesthesia and implanted unilaterally with a CMA7 guide cannula (CMA Microdialysis AB) aiming at the nucleus accumbens (stereotaxic coordinates, AP + 1.5 mm, L ± 0.8 mm, V −3.5 mm from bregma and dura surface).

Guide cannulas were fixed to the brain using two anchor screws and dental cement. After surgery, mice were placed back in their home cage equipped with a Plexiglas cage extension (height, 20cm). Seven days after surgery, CMA7/1 microdialysis probes of 1 mm membrane length (CMA Microdialysis AB) were slowly inserted and mice were connected to a single-channel liquid swivel and a counterbalancing system (Instech Laboratories). Microdialysis probes were perfused with sterile Ringer solution (Fresenius Kabi GmbH) at a flow rate of 1 μl/min using a PHD2000 microinfusion pump (Harvard Apparatus). After overnight stabilization, the sampling period started from 8 hr to 16 hr. Microdialysis samples were collected every 20 min in tubes containing 4 μl of 100 mM $HClO_4$ for stabilization and stored at −80°C until HPLC analysis. After the first four samples (baseline), the mice were injected with a saline solution. Subsequently, 10 mg/kg and 20 mg/kg cocaine was administered (i.p.) to the mice at 11 a.m. and 2 p.m., respectively. At the end of the experiment, the mice were killed and the probe placements were checked histologically in 50 μm brain sections. DA content in the dialysate samples was determined by high-pressure liquid chromatography. Electrochemical detection was acquired with the ALEXIS 100 cooled-micro LC-EC system (Antec Leyden bv) equipped with a microbore VT-03 flow cell. The working potential of the cell was set at 400 mV and the oven temperature of the DECADE II at 35°C. The mobile phase of pH 6 contained 50 mM phosphoric acid, 400 mg/l OSA, 0.1 mM EDTA, 8 mM KCl, and 15% methanol and was perfused with a flow rate of 200 μl/min. Duplicates of 4 μl aliquots of each sample were injected onto a reversed phase column (C18, ALF-205 column, 50 × 2.1 mm ID, 3 μm; Axel Semrau GmbH & Co. KG), and the DA content was determined using the area under the peak with an external standard curve as a reference. Detection limits for DA was 50 pM with a signal-to-noise ratio of 2. All data were analyzed with two-way ANOVAs for repeated-measures. Data presented are mean ± SEM.

Behavioral Procedures

Animals (12–24 weeks) were treated i.p. with a standard dose of 10 mg/kg of cocaine (Sigma), except for the reinstatement, where 7.5 mg/kg was used. This dose of cocaine dose was chosen because it proved to be most reliable in a previous study involving cocaine CPP in complete GluR1 and GluR2 knockout mice (Mead et al., 2005). Behavioral experiments started 1 week after the arrival of the animals in the laboratory environment and were always conducted between ZT2-6.

The procedure of acquisition, extinction, and reinstatement of cocaine-induced CPP was adapted from the original description (Itzhak and Martin, 2002), and other effects of cocaine (acute locomotion and locomotor sensitization) were also measured. CPP was conducted in six gray acrylic chambers (32 cm long × 16 cm wide × 22 cm high) as described (Abarca et al., 2002). The whole procedure consisted of six phases: preconditioning (1 session), conditioning (8 sessions), preference test (1 session), extinction pairings (16 sessions), extinction tests, and the reinstatement test (1 session). All sessions were monitored by a video-tracking system (Ethovision 2.0, Noldus), which enabled us to determine locomotion and spatial placement of each mouse each 0.2 s across the whole session.

At day 1 (preconditioning session; duration, 30 min) all subjects were placed inside the conditioning chamber with a distinctive floor (a smooth plastic surface covered with soft tissue paper). The conditioning phase started the next day and followed an unbiased Pavlovian conditioning procedure in which mice belonging to the different genotype-based groups were randomly assigned to one of two experimental conditions (CS^+, Rod^+ or $Hole^+$; or CS^-, Rod^- or $Hole^-$). During the conditioning phase (session length: 30 min) subjects had access to the entire apparatus but only one floor type (Rod or Hole) was presented. On CS^+ trials, each mouse received an injection of cocaine (10 mg/kg; i.p.), whereas in the CS^- trials, animals were injected with equal volumes of saline. Immediately after injection, mice were placed in the conditioning apparatus containing the corresponding floor. The order of the CS^+ and CS^- trials was counterbalanced among animals, and each floor were designed as CS^+ or CS^- for half of the animals of each genotype-based group. The effects of an acute cocaine challenge on locomotion were assessed by comparing the distance traveled (cm/30 min) during the first CS^+ and the first CS^- trials, and cocaine-induced behavioral sensitization was assessed by analyzing the changes in locomotion across the four CS^+ trials. The preference test took place 24 hr after the last conditioning trial. To conduct this test, the floor was divided with half Rod and half Hole, and mice were placed in the center of the chamber without any previous injection. The relative position (left versus right) of each floor was counterbalanced. Preference test duration was 15 min. The primary dependent variable of this test was the amount of time spent on CS^+ and CS− floor during the whole 15 min test session, although locomotion was also measured. Extinction consisted of a total of 16 daily sessions in which both floors were paired with saline. The extinction test on day 16 was identical to the preference test described above. On the following day, a priming injection (7.5 mg/kg) was used to reinstate the extinguished CPP (Itzhak and Martin, 2002). CPP reinstatement was assessed in a test identical to the first preference test described above. All data are presented as means ± the standard error of the mean (SEM), and a significance level of $p \leq 0.01$ was used. Most of the statistical treatment of the data was conducted using one- or two-way ANOVA, with a repeated-measures factor when necessary, followed by Newman-Keuls post hoc tests, when appropriate.

SUPPLEMENTAL DATA

The Supplemental Data include four figures and can be found with this article online at http://www.neuron.org/cgi/content/full/59/3/497/DC1/.

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