Behavioral/Systems/Cognitive

In Vivo Metabotropic Glutamate Receptor 5 (mGluR5) Antagonism Prevents Cocaine-Induced Disruption of Postsynaptically Maintained mGluR5-Dependent Long-Term Depression

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Metabotropic glutamate receptor 5 (mGluR5) plays a critical role in psychostimulant-induced behavior, yet it is unclear whether mGluR5 is activated by psychostimulant administration, or whether its role is constitutive. We previously reported that activation of mGluR5 with the group I mGluR agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) can induce a long-term depression (DHPG-LTD) of glutamatergic transmission in the bed nucleus of the stria terminalis (BNST), and that *ex vivo* induction of this LTD is disrupted by repeated *in vivo* administration of cocaine. Here we demonstrate that DHPG-LTD is not maintained by alterations in glutamate release, and that postsynaptic endocytosis is necessary. Furthermore, we find that a single administration of cocaine produces a transient disruption of DHPG-LTD, and the duration of this disruption was increased by repeated days of cocaine administration. The disruption produced by cocaine was not permanent, because DHPG-LTD could be induced 10 d after cocaine administration. To test the role of mGluR5 *in vivo* in the cocaine-induced disruption of DHPG-LTD, we injected mice with the mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine before cocaine. mGluR5 antagonism during *in vivo* cocaine administration rescued subsequent *ex vivo* induction of DHPG-LTD. The effects of *in vivo* cocaine could be mimicked by application of cocaine to BNST-containing slices, suggesting that the actions of cocaine are local. Thus, using a novel strategy of *in vivo* antagonist-induced rescue of *ex vivo* agonist effects for the same receptor, we provide evidence suggesting that mGluR5 activation is actively recruited by *in vivo* cocaine.

Key words: addiction; synaptic plasticity; mGluR5; LTD; cocaine; glutamate

Introduction

Glutamatergic signaling is altered in many pathophysiological states. One candidate glutamatergic receptor thought to play a role in conditions such as schizophrenia, chronic pain, and substance abuse is the metabotropic glutamate receptor 5 (mGluR5) (Spooren et al., 2001; Marino and Conn, 2006). mGluR5 plays a critical role in behavioral responses to multiple substances of abuse. For example, mice harboring a targeted deletion of mGluR5 exhibit marked deficits in responding to the reinforcing and locomotor stimulating effects of cocaine (Chiamulera et al., 2001). Furthermore, acute pharmacological antagonism of

mGluR5 disrupts the reinforcing properties of psychostimulants (Chiamulera et al., 2001) and ethanol (Heilig and Egli, 2006).

Although it is clear that mGluR5 is required for the expression of some reward-related behaviors, the underlying mechanisms are currently unknown. It is also unclear whether a basal constitutive tone of mGluR5 activity is required, or whether mGluR5 is acutely activated in response to these substances. Although the proximate targets of psychostimulants are monoamine transporters, increasing evidence suggests that an important step in the behavioral effects of these compounds is the subsequent modulation of glutamatergic transmission (Kauer and Malenka, 2007). Evidence suggests that mGluR5 regulates glutamatergic transmission in a variety of brain regions, particularly those thought to participate in reward-related behaviors. In one such region, the bed nucleus of the stria terminalis (BNST), we recently found that mGluR5 activation by the group I mGluR agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) can induce long-term depression (DHPG-LTD) of glutamatergic synaptic transmission (Grueter et al., 2006). Moreover, *ex vivo* induction of DHPG-LTD could be disrupted by 10 d of *in vivo* cocaine administration, 24 h before brain slice preparation (Grueter et al., 2006).

A large body of evidence suggests that group I mGluR-

induced LTD plays important roles at synapses throughout the CNS (Grueter et al., 2007). Moreover, induction and maintenance mechanisms for LTD induced by these receptors appear to vary across brain regions. In the present study, we have examined the maintenance mechanisms underlying DHPG-LTD in the BNST. Our data strongly suggest that this LTD is not maintained by alterations in glutamate release and point toward a postsynaptic mechanism. We show that this LTD is transiently disrupted by a single administration of cocaine *in vivo*, and that the duration of this disruption increases with repeated injections. Finally, we have used this disruption as a tool to demonstrate the active participation of mGluR5 *in vivo* in CNS response to cocaine.

Materials and Methods

Animals. Male C57BL/6J mice (5–10 weeks old; The Jackson Laboratory) and mGluR5 (−/− or +/+) males, 5–7 weeks of age on a C57BL/6J mixed background, were used. Mice were housed in cages of two to five animals on a 12 h light/dark cycle with food and water *ad libitum*.

Brain slice preparation. Methods were as described previously (Grueter and Winder, 2005; Grueter et al., 2006). Briefly, mice were decapitated under isoflurane. Brains were quickly removed and placed in an ice-cold, low-sodium/high-sucrose dissecting solution. Hemisected (300 μm) coronal brain slices containing anterior portions of BNST (bregma 0.26–0.02 mm) were prepared on a Leica vibroslicer. Slices were allowed to recover in a submerged holding chamber (25°C) containing artificial cerebrospinal fluid (ACSF) that contained the following (in mM): 124 NaCl, 4.4 KCl, 2.5 CaCl$_2$, 1.3 MgSO$_4$, 1 NaH$_2$PO$_4$, 10 glucose, and 26 NaHCO$_3$ for a minimum recovery period of 120 min. Slices were then removed from the holding chamber and placed in the recording chamber, where they were continuously perfused with oxygenated (95% O$_2$/5% CO$_2$) ACSF at a rate of 2 mL/min at 24–25°C.

Whole-cell voltage-clamp recordings. Whole-cell recordings were performed as previously reported (Grueter and Winder, 2005; Grueter et al., 2006). Briefly, electrodes of 3.0–6.0 MΩ were filled with the following (in mM): 117 Cs gluconate, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl, 4 Na^{2+}ATP, 0.3, Na^{2+}GTP. EPSCs of 100–400 pA were recorded at a frequency of 0.17 Hz while voltage-clamped at −70 mV in the presence of the GABA$_A$ receptor antagonist, picrotoxin (25 μM). After whole-cell configuration was achieved, cells were allowed to equilibrate a minimum of 10 min before baseline recordings were started. Postsynaptic parameters were monitored continuously throughout the duration of the experiments. Data are represented as an average of the peak amplitudes of five sweeps (30 s). In experiments in which *in vivo* manipulations were made, data acquisition and analysis were performed blinded to *in vivo* treatment.

A paired-pulse ratio (PPR) was acquired by applying a second stimulus of equal intensity 50 ms after the first stimulus, where PPR = EPSC$_1$/EPSC$_2$. Miniature EPSCs were collected at a holding potential of −90 mV in the presence of 1 μM TTX. Two minute blocks of events were acquired via a Multiclamp 700A, digitized at 10 kHz, and analyzed using Mini-analysis software (Synaptosoft) with detection parameters set at >5 pA amplitude and <3 ms rise time. All events were verified by eye.

Immunohistochemistry. Immunohistochemistry of mouse brain slices was performed as previously described (Egli et al., 2005). Briefly, 6- to 9-week-old male C57BL/6 mice were pericardially perfused with 4% paraformaldehyde, brains were excised and suffused in 20% sucrose, and 30–50 μm coronal slices were cut on a Leica CM3050S cryomicrotome. Slices were washed in PBS and blocked in 4% normal donkey serum (NDS) and 0.2% Triton X-100 in PBS overnight at 4°C. Slices were exposed overnight at 4°C to primary (rabbit polyclonal anti-mGluR5 (Millipore) and mouse anti-MAP2 (microtubule-associated protein 2) (Millipore)), and secondary antibodies (6 h at 4°C) in 2% NDS and 0.1% Triton X-100 in PBS, exposed to 20 μM DRAQ (Biostatus) in PBS (10 min at room temperature), and were washed extensively in PBS between each step. Slices were mounted in AquaPolyMount (Polysciences) and visualized on a Zeiss Inverted LSM510 confocal microscope in the Vanderbilt Cell Imaging Resource Center.

Intraperitoneal cocaine administration. C57BL/6J mice at 5–6 weeks of age were given intraperitoneal injections of either saline or cocaine (20 mg/kg) based on weight. Injections were given in a home cage environment. All mice received a minimum of five daily injections before *ex vivo* studies to allow animals to acclimate to handling and injections. Mice were killed and recordings were made 24 h after the last injection except in the acutely treated mice. To examine the acute effects of *in vivo* cocaine in BNST slices, mice were killed 30 min or 4 h after injections. To examine the role of mGluR5 in cocaine-induced changes in DHPG-LTD, the mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) was dissolved in DMSO and diluted to a 10% DMSO/0.9% saline solution. Intraperitoneal injections of MPEP (10 mg/kg) or vehicle were given 30 min before cocaine/saline treatment.

Reagents. The following drugs were purchased from Sigma-Aldrich: TTX (1 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX, 10 μM). The following reagents were purchased from Tocris: DHPG, MPEP, γ-D-glutamylglycine (γ-DGG), and dynamin inhibitory peptide (Gln-Val-Pro-Ser-Arg-Pro-Asn-Arg-Ala-Pro). Jasplakinolide was purchased from Invitrogen. (1*S*,2*S*,5*R*,6*S*)-2-Aminobicyclo [3.1.0]hexane-2,6-dicarboxylic acid (LY 354740) was a gift from Dr. Darryle Schoepp (Eli Lilly). Dimethyl sulfoxide was the solvent used for DHPG, MPEP, and jasplakinolide.

Results

DHPG-LTD in BNST is mGluR5 dependent

As reported previously (Grueter et al. 2006), we find that application of the group I mGluR agonist DHPG elicits LTD at glutamatergic synapses in the adult mouse BNST (Fig. 1*A*). DHPG has a similar affinity for both mGluR1 and mGluR5 (Conn and Pin, 1997). As shown in Figure 1, *B* and *C*, immunohistochemical studies demonstrate heavy expression of mGluR5 throughout the anterolateral BNST. We previously demonstrated that infusion of the postsynaptic neuron in the BNST with GDPβS to block postsynaptic G-protein function disrupted DHPG-LTD (Grueter et al., 2006). Furthermore, consistent with the involvement of a postsynaptic mGluR5 in this process, we find that mGluR5 immunoreactivity colocalizes with MAP2 immunoreactivity in the BNST (Fig. 1*D–F*).

Consistent with the expression of this receptor in BNST, we previously reported that DHPG-LTD in the BNST is blocked by a noncompetitive antagonist of mGluR5, MPEP, whereas additional antagonism of mGluR1 was required to reduce the early component of the DHPG-induced synaptic depression (Grueter et al., 2006). Furthermore, consistent with the involvement of mGluR5 in this LTD, we found that DHPG-LTD is absent in slices prepared from mGluR5 knock-out mice (Fig. 1*A*) (wild-type, 24.3 ± 5.0% depression; $n = 7$; mGluR5 knock-out, 4.5 ± 4.9% depression; $n = 6$; $p < 0.05$, comparing mGluR5 wild-type with mGluR5 knock-out).

DHPG-LTD is not maintained by reductions in glutamate release probability

We previously reported that DHPG-LTD was associated with transient, but not persistent, alterations in PPRs of evoked glutamatergic responses, suggesting that this LTD is not mediated by an alteration in release probability (Grueter et al., 2006). To further test whether DHPG-LTD is maintained by a reduction in glutamate release probability, we performed experiments in a low Ca^{2+}/high Mg^{2+} ACSF (1 mM Ca^{2+}/2.8 mM Mg^{2+}) to artificially lower basal release probability. Average basal PPRs in our "normal" extracellular solution were 1.02 ± 0.02, and values were significantly elevated to 1.23 ± 0.08 in 1 mM Ca^{2+}/2.8 mM Mg^{2+} ($n = 6$) (Fig. 2*C*), consistent with a lowered basal release probability. In a previous study it was reported that this manipulation of extracellular divalent cations blocked DHPG-LTD in
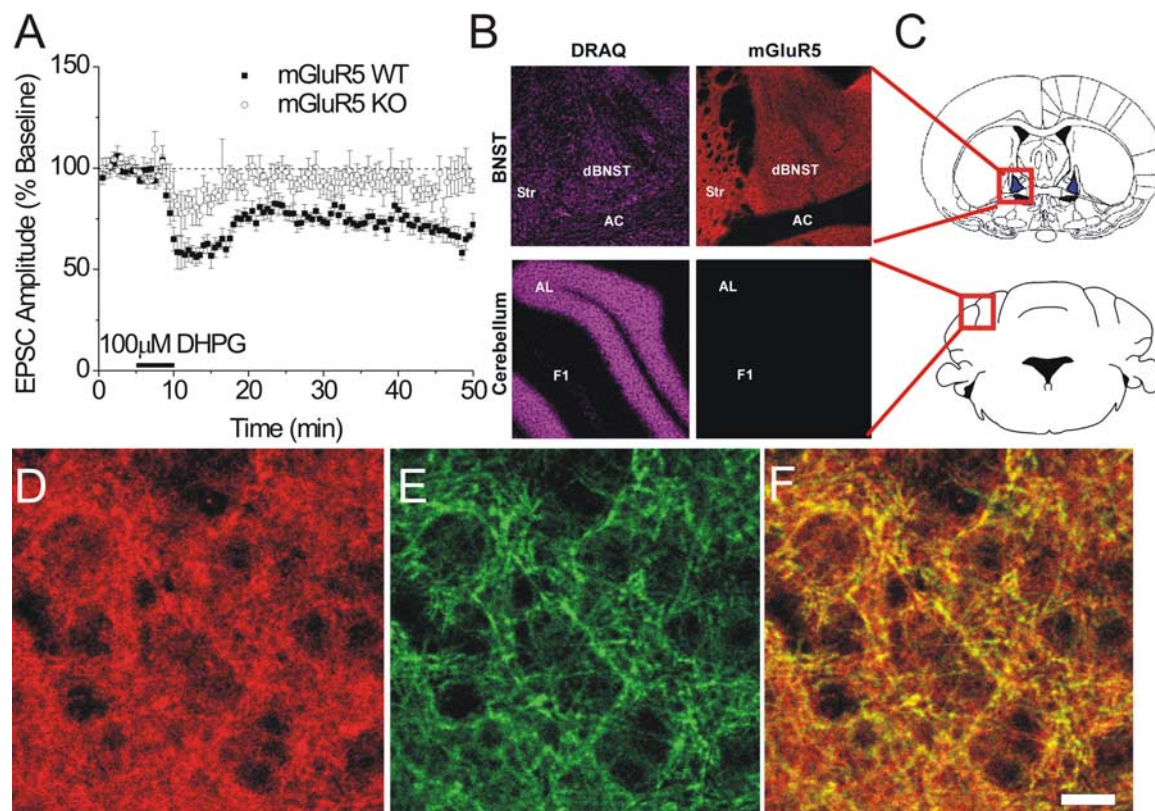
Figure 1. mGluR5-LTD in the BNST. *A*, Group I mGluR LTD in the BNST is dependent on mGluR5 function. Average time courses of normalized EPSC amplitudes evoked by local stimulation in the BNST in acute slice preparations are shown. DHPG (100 μM) was applied for 5 min as indicated. $\bigcirc$, mGluR5 knock-out; $\blacksquare$, wild-type littermates. *B*, Mouse brain coronal slices containing the dorsal BNST (top) or a portion of the cerebellum (bottom) stained with DRAQ nuclear stain (left) or anti-mGluR5 (right) show a specific mGluR5 signal in the BNST. AC, Anterior commissure; Str, striatum; AL, ansiform lobule; F1, fissure 1. *C*, Schematic depicting the region contained in the micrographs (red squares). Dorsal BNST in blue (top), cerebellum (bottom). *D–F*, Mouse brain coronal slices were stained with anti-mGluR5 (*D*; red) or the dendritic marker anti-MAP2 (*E*; green) (*F*; merge). Scale bar, 20 μm.

area CA1 of hippocampus (Watabe et al., 2002). In the BNST, bath application of DHPG in 1 mM Ca^{2+}/2.8 mM Mg^{2+} ACSF produced reductions in evoked EPSC amplitude similar to those observed in 2.5 mM Ca^{2+}/1.3 mM Mg^{2+} ACSF (29.9 $\pm$ 9% vs 27 $\pm$ 4% depression, 1 mM Ca^{2+}/2.8 mM Mg^{2+}, and 2.5 mM Ca^{2+}/1.3 mM Mg^{2+}, respectively; $n = 6$) (Fig. 2*A*) (Grueter et al., 2006). To further assess whether DHPG-LTD is associated with alterations in basal or DHPG-stimulated glutamate release probability, we tested the effects of group I mGluR activation on PPR of evoked EPSCs in BNST neurons under low extracellular Ca^{2+}/high Mg^{2+} conditions. We found no effect of DHPG on PPR 30 min after DHPG washout in 1 mM Ca^{2+}/2.8 mM Mg^{2+} (95.5 $\pm$ 4% of basal PPR).

Although the roles of Ca^{2+} in regulating the probability of transmitter release and inducing synaptic plasticity in the postsynaptic cell are well established, more recent data suggest that extracellular divalent ion concentrations can additionally influence synaptic function by acutely regulating quantal size (Hardingham et al., 2006). Interestingly, Ca^{2+}, as well as Mg^{2+}, has been shown to activate group I mGluRs (Francesconi and Duvoisin, 2004). Recently, it has been suggested that extracellular Ca^{2+} and Mg^{2+} can influence quantal size via group I mGluRs (Hardingham et al., 2006). To address the general role of extracellular divalent cations in DHPG-LTD, we lowered the extracellular concentrations of both Ca^{2+} and Mg^{2+} to 1 mM. This also had no effect on DHPG-LTD (22.6 $\pm$ 4% depression; $n = 6$) (Fig. 2*B*) or on the effects of DHPG on PPR 30 min after DHPG (104.4 $\pm$ 12% of baseline). In total, these data suggest that DHPG-LTD is not maintained by alterations in probability of

glutamate release or by changes in quantal size produced by altered extracellular ion concentrations.

Synaptic cleft glutamate concentrations are unaltered during group I mGluR LTD in BNST

Although data suggest that DHPG-LTD is not maintained by constitutive alterations in glutamate release probability (Fig. 2) (Grueter et al., 2006), presynaptic mechanisms that altered vesicular glutamate concentration, fusion pore release dynamics, or synaptic glutamate buffering could still account for DHPG-induced LTD. To begin to address these possibilities, we performed experiments comparing the effects of bath application of a low-affinity AMPA receptor (AMPAR) antagonist, γ-DGG (Chen and Diamond, 2002), before and after DHPG application (Fig. 2*D,E*). Alterations in synaptic cleft glutamate concentration by DHPG would be predicted to alter the antagonism of the EPSC by γ-DGG. We found that brief application of 500 μM γ-DGG produced a 40.3 $\pm$ 4% inhibition of the EPSC, which nearly completely reversed on washout. The percentage inhibition by this application was unchanged when γ-DGG was again applied 30 min after DHPG application (42.6 $\pm$ 6% depression; $n = 5$; $p > 0.05$ before and after DHPG) (Fig. 2*E*). These data suggest that DHPG-induced LTD is not likely due to alterations in glutamate concentrations at the synaptic cleft.

Group I mGluR activation results in a decrease in miniature EPSC frequency but not amplitude

Spontaneous, TTX-insensitive, CNQX-sensitive (data not shown) miniature EPSCs (mEPSCs) were also analyzed in the

BNST before and after DHPG application to further assess mechanisms by which transmission is modulated. Mean basal mEPSC amplitude and frequency were 21.0 ± 0.9 pA and 3.9 ± 0.7 Hz, respectively. Representative traces of mEPSCs measured at -90 mV before and after DHPG application are shown in Figure 3A. Application of 100 μM DHPG significantly decreased the frequency of mEPSCs 30 min after DHPG washout ($64.4 \pm 9\%$ of baseline, $n = 11$; $p < 0.05$) (Fig. 3B). However, there was no significant effect on mEPSC amplitude ($97.6 \pm 7\%$ of baseline, $n = 11$; $p > 0.05$) (Fig. 3C). Additionally, no gross changes in the kinetics of mEPSCs were observed after DHPG application (Fig. 3C, inset). These data are consistent with the γ-DGG data suggesting a lack of change of quantal size.

Interfering with postsynaptic endocytic machinery prevents group I mGluR-LTD in the BNST

The observed alteration in mEPSC frequency could be considered to be in conflict with the lack of effect observed on PPR of evoked EPSCs. However, alterations in quantal content occur through a change in either p (unitary release probability) or n (number of releases per response site), either of which would be expected to result in a decrease in mEPSC frequency. Thus, a change in n through either presynaptic or postsynaptic mechanisms could account for the data. The postsynaptic silencing/unsilencing of synapses through the addition/deletion of AMPARs is thought to be a major mechanism underlying forms of NMDA receptor–long-term potentiation (LTP)/LTD as well as some forms of mGluR LTD (Oliet et al., 1997; Snyder et al., 2001; Xiao et al., 2001; Derkach et al., 2007).

Endocytosis of AMPARs has been identified as a major mechanism mediating both group I mGluR-dependent and NMDA receptor-dependent LTD in other brain regions (Carroll et al., 1999; Lüscher et al., 1999; Morishita et al., 2005; Derkach et al., 2007; Grueter et al., 2007). To test whether processes involving endocytosis are involved in DHPG-LTD in the BNST, through the patch pipette we perfused the postsynaptic BNST neurons with a dynamin-inhibitory peptide (2 mM), a peptide designed to prevent interaction of dynamin with amphiphysin (Xiao et al., 2001). On allowing the peptide to perfuse into the cell for at least 30 min, DHPG induced an acute depression in EPSC amplitude ($42.2 \pm 5\%$ of baseline) that reversed to $1.3 \pm 11\%$ depression 30 min
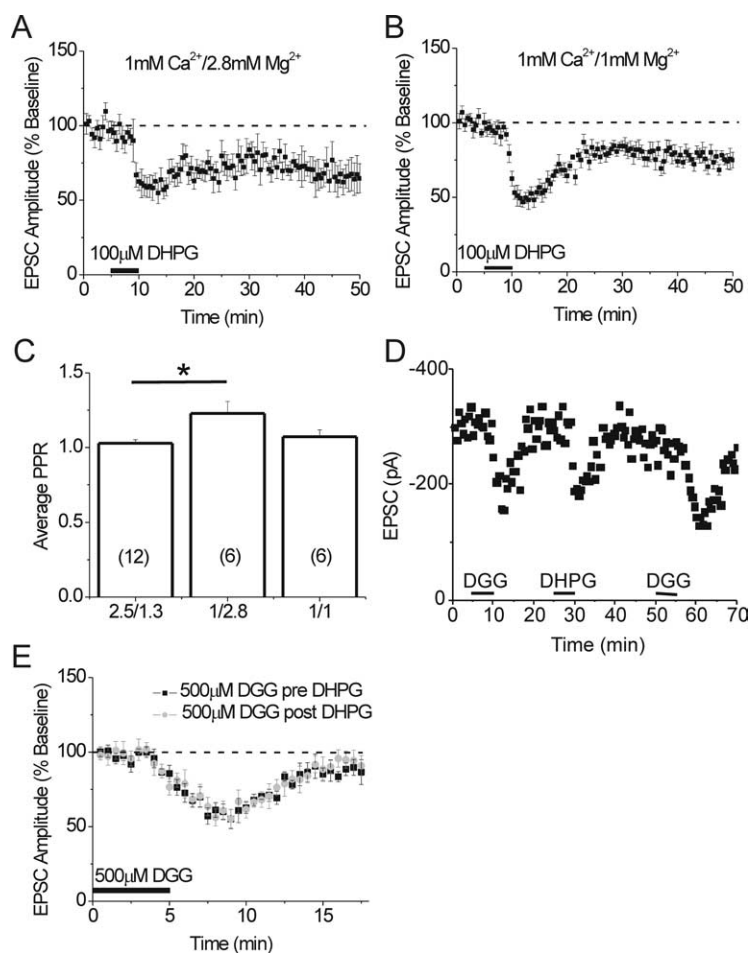

Figure 2. **A**, **B**, Normalized time course of effects of DHPG on EPSC amplitude in external ACSF containing 1 mM Ca^{2+}/2.8 mM Mg^{2+} (**A**) or 1 mM Ca^{2+}/1 mM Mg^{2+} (**B**). **C**, Summary graph of effects of varying extracellular cations on average basal PPR ($EPSC_2/EPSC_1$). *$p < 0.05$. **D**, Varying extracellular cations did not affect PPR normalized to baseline 30 min after DHPG washout. **E**, Representative experiment depicting time course of γ-DGG-induced depression of EPSCs before and after DHPG application. **F**, Percentage of inhibition by γ-DGG is unchanged before and after DHPG application. Error bars indicate SEM.
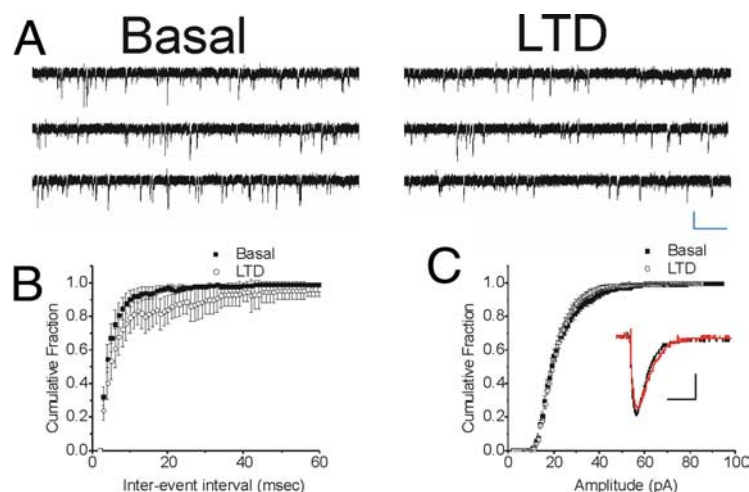

Figure 3. DHPG decreased the frequency but not the amplitude of BNST mEPSCs. **A**, Sample current traces of mEPSCs in a BNST neuron before DHPG application (basal) and at 30 min after (late; LTD) DHPG. Calibration: 20 pA, 512 ms. **B**, Cumulative probability plot of DHPG induced effects on mEPSC inter-event interval within the basal (3–5 min, open squares) and late (LTD; min 35–37, closed circles) time periods ($n = 11$; $p < 0.05$, Kolmogorov–Smirnov test). **C**, Cumulative probability of effects of DHPG on mEPSC amplitude within the same current recordings as shown in **B**. Inset, Representative traces before DHPG application and during DHPG-mediated LTD. Inset calibration, 5 pA. Error bars indicate SEM.
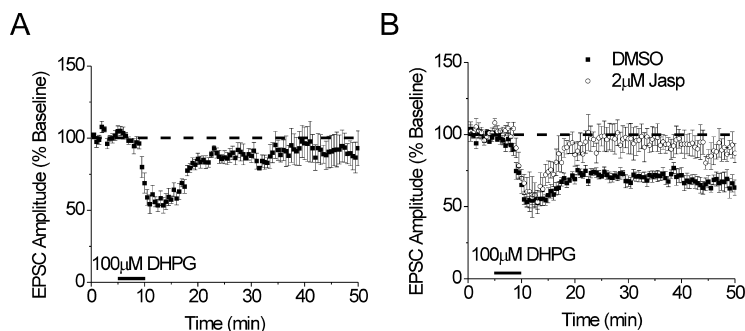
Figure 4. Maintenance of mGluR5 LTD is dependent on postsynaptic endocytosis machinery. **A**, Average time course of effects of 100 μM DHPG on normalized EPSC amplitudes from BNST neurons perfused with 2 mM dynamin-inhibitory peptide. **B**, Time course of effects of 100 μM DHPG on EPSCs from BNST neurons postsynaptically perfused with either jasplakinolide (2 μM) (○) or vehicle (DMSO) (■). $n = 7$ (1 cell was excluded from this analysis because DHPG produced a depression of the EPSC twice the normal observed amplitude in naive neurons). Error bars indicate SEM.
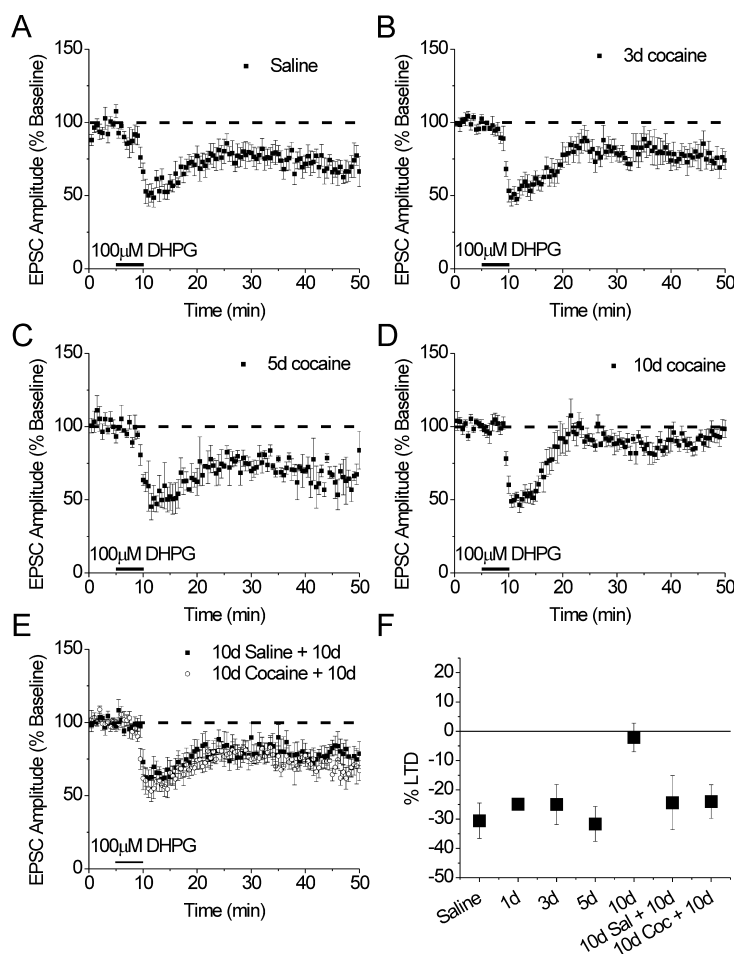

Figure 5. Repeated exposure to cocaine attenuates group I mGluR LTD in the BNST. **A–E**, Average time courses of normalized EPSC amplitudes evoked by local stimulation in the BNST in slices taken from saline and cocaine (20 mg/kg)-treated mice. DHPG (100 μM) was applied for 5 min as indicated. Specific mouse treatments were as follows: **A**, mice killed and slices prepared 24 h after 10 d of intraperitoneal saline injections; **B–D**, mice killed and slices prepared 24 h after 3, 5, and 10 d of intraperitoneal cocaine injections, respectively; **E**, mice killed and slices prepared 10 d after 10 d of intraperitoneal cocaine or saline injections. **F**, Summary graph in which values represent percentage change in EPSC ± SEM, 30 min after DHPG washout. *$p < 0.05$ (Bonferroni and Tukey test after one-way ANOVA). Error bars indicate SEM.

the group II mGluR agonist, LY 354740 (1 μM), on EPSCs from cells filled with the dynamin-inhibitory peptide. Previous studies indicate that group II mGluRs depress excitatory transmission at these synapses through a presynaptic mechanism (Grueter and Winder, 2005; Muly et al., 2007). Similar to previously published results (Grueter and Winder, 2005), LY 354740 caused a decrease in EPSC amplitudes (54.4 ± 7% depression; $n = 3$; $p < 0.05$) (data not shown) in dynamin-inhibitory peptide-filled BNST neurons.

We also tested the effects of the actin-stabilizing drug jasplakinolide, on DHPG-induced LTD in the BNST. Jasplakinolide has previously been demonstrated to disrupt AMPA receptor endocytosis, as well as DHPG-LTD, in hippocampal neurons (Xiao et al., 2001). Jasplakinolide (2 μM) perfused into the postsynaptic cell prevented the lasting depression induced by DHPG in seven of eight cells recorded from (8.4 ± 6% depression in an average of 7 of 8 and 73.7% depression in 1 of 8 cells), whereas the vehicle had no effect (29.4 ± 5% depression; $n = 9$; $p < 0.05$) 30 min after DHPG washout (Fig. 4B). In total, these data suggest that postsynaptic endocytosis is required for DHPG-LTD in the BNST.

Chronic cocaine administration is required to disrupt *ex vivo* DHPG-LTD 24 h after the last cocaine dose

Previously, we reported that 10 d of either contingent or non-contingent administration of cocaine resulted in disruption of the ability of DHPG (100 μM, 5 min) to induce LTD in the BNST in brain slices prepared 24 h after the last cocaine exposure (Grueter et al., 2006). This disruption of DHPG-LTD 24 h after a 10 d cocaine administration regimen was not mimicked by a single injection of cocaine administered 24 h before LTD induction, demonstrating that repeated administration was necessary (Grueter et al., 2006). To further define the relationship between *in vivo* cocaine administration and subsequent disruption of *ex vivo* DHPG-LTD, we prepared BNST slices 24 h after 3, 5, or 10 d of intraperitoneal cocaine (20 mg/kg) or saline administration and assessed DHPG-LTD. We found that DHPG-LTD measured 24 h after 1, 3, or 5 d of exposure to cocaine was normal [saline, 32.8 ± 4.0% depression (Fig. 5A,F); 1 d, 24.9 ± 1.8% depression; $n = 6$ (Grueter et al., 2006); 3 d, 25.0 ± 6.8% depression; $n = 7$ (Fig. 5B,F); 5 d, 31.6 ± 6.0% depression; $n = 4$) (Fig. 5C,F)], but that it was dramatically disrupted after 10 d of intraperitoneal cocaine injections (6.4 ± 7.7% depression) (Fig. 5D,F).

after washout ($n = 7$; $p > 0.05$) (Fig. 4A). No difference was observed in time-matched recordings of AMPAR EPSC amplitudes in cells filled with the dynamin-inhibitory peptide (98% of baseline, $n = 2$). We also tested the effects of bath application of
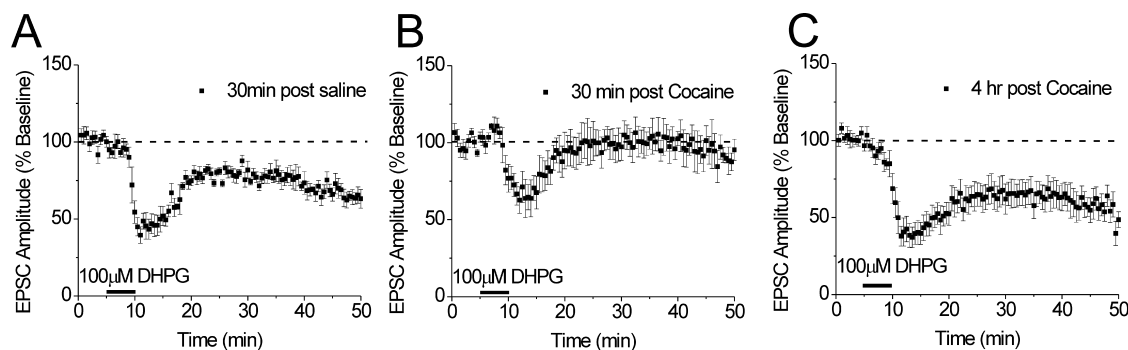
Figure 6. A single cocaine exposure transiently attenuates group I mGluR LTD in the BNST. ***A–C***, Average time courses of normalized EPSC amplitudes evoked by local stimulation in the BNST in slices taken from saline and cocaine (20 mg/kg)-treated mice 30 min (***A***, ***B***) or 4 h (***C***) after injection. DHPG (100 μM) was applied for 5 min as indicated.

As can be seen in Figure 5*D*, although the LTD induced by DHPG is disrupted by 10 d of cocaine administration, DHPG still produces a transient depression of the EPSC. We previously demonstrated that the acute depression produced by DHPG was distinct from the LTD in that it was dependent on mGluR1 as well as mGluR5, was blocked by CB1 receptor antagonists and absent in CB1 receptor knock-out mice, and was associated with a robust enhancement of PPR (Grueter et al., 2006). The transient DHPG-induced depression observed in slices obtained from cocaine-treated mice was associated with an increase in PPR (1.39 ± 0.10, $p < 0.05$), suggesting that the depression produced by mGluR1 and CB1-dependent actions remains intact.

Having established that multiple days of cocaine administration were necessary to disrupt DHPG-LTD 24 h after the last exposure, we assessed the duration of DHPG-LTD disruption by assessing DHPG-LTD *ex vivo* 10 d after the 10 d intraperitoneal cocaine administration. At this time point after cocaine administration, DHPG-LTD was again normal (Fig. 5*E*,*F*) (saline, 24.4 ± 9.2%, $n = 6$; cocaine, 24.0 ± 5.7%, $n = 8$). Thus, the duration of the cocaine-induced disruption of DHPG-LTD in the BNST is on a time scale similar to that observed with cocaine regulation of NMDA receptor-dependent LTP in the ventral tegmental area (VTA) (Ungless et al., 2001).

A single injection of cocaine disrupts *ex vivo* DHPG-LTD induction from slices prepared 30 min, rather than 24 h, after injection
In addition to examining the number of daily injections required to produce a disruption of DHPG-LTD 24 h after the last exposure, we also considered whether fewer exposures might also produce disruptions of a more transient nature. We tested this idea by preparing brain slices 30 min or 4 h, rather than 24 h, after a single injection of cocaine and measuring DHPG-LTD. We found that DHPG-LTD was absent in BNST slices prepared 30 min after a single cocaine exposure (Fig. 6*A*,*B*) ($n = 5$; −0.9 ± 5.0% vs 28.8 ± 5% depression in cocaine- and saline-treated groups, respectively). If slices were prepared 4 h rather than 30 min after a single injection, DHPG-LTD was indistinguishable from control (64 ± 8% of baseline, $n = 5$) (Fig. 6*C*). Thus, these data suggest that cocaine produces transient disruptions of DHPG-LTD in the BNST, and the duration of this period of induction increases with multiple days of exposure.

DHPG-induced reductions of mEPSC frequency are blocked by both *in vivo* and *in vitro* administration of cocaine
We hypothesized that the disruption of LTD by cocaine was dependent on cocaine-mediated disruptions of glutamatergic
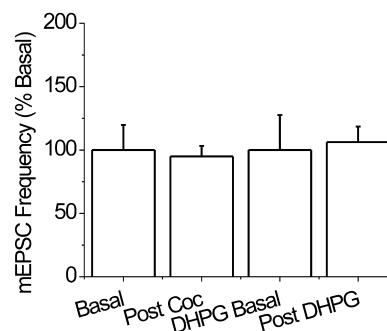

Figure 7. Cocaine has local effects on mGluR5 signaling in BNST. Summary graph representing percentage change in mEPSC frequency after bath application of 30 μM cocaine (Coc) and 30 min after subsequent DHPG (100 μM) application.

transmission resulting in the recruitment of mGluR5. Because *in vivo* cocaine was administered intraperitoneally, it is possible that such alterations in glutamatergic transmission could occur anywhere in the CNS. To test this idea, we compared the effects of both *in vivo* and *in vitro* administration of cocaine. Because cocaine can work indirectly via inhibition of voltage-gated sodium channels, we took advantage of our finding that DHPG produces a long-term decrease in mEPSCs in the presence of TTX to allow us to bypass this indirect action.

Consistent with the effects we observed of *in vivo* cocaine on DHPG-LTD, we found that, in slices prepared 24 h after 10 daily injections of cocaine, DHPG failed to produce a persistent decrease in mEPSC frequency (mEPSC frequency 98.7 ± 14% of baseline 30 min after DHPG application; $n = 5$). Next, we prepared slices from naive mice. After establishing a stable baseline of mEPSC frequency, we applied cocaine (30 μM) for 30 min. This administration of cocaine had no effect on mEPSC frequency (Fig. 7) (94.9 ± 8% of baseline; $n = 7$). After cocaine application, we applied DHPG. As in the case of the *in vivo* cocaine application experiments, we again found that DHPG did not reduce mEPSC frequency after cocaine bath application (Fig. 7) (106 ± 12% of baseline; $n = 7$). Thus, these data suggest that cocaine acts locally within the BNST to regulate BNST mGluR5 LTD.

In vivo MPEP prevents cocaine-induced disruption of *ex vivo* mGluR5-dependent LTD
Given that DHPG-LTD in BNST was blocked by bath application of MPEP (Grueter et al., 2006) as well as absent in mGluR5 knock-out mice, we tested the hypothesis that cocaine disrupts *ex vivo* DHPG-LTD by *in vivo* recruitment of mGluR5. To test this
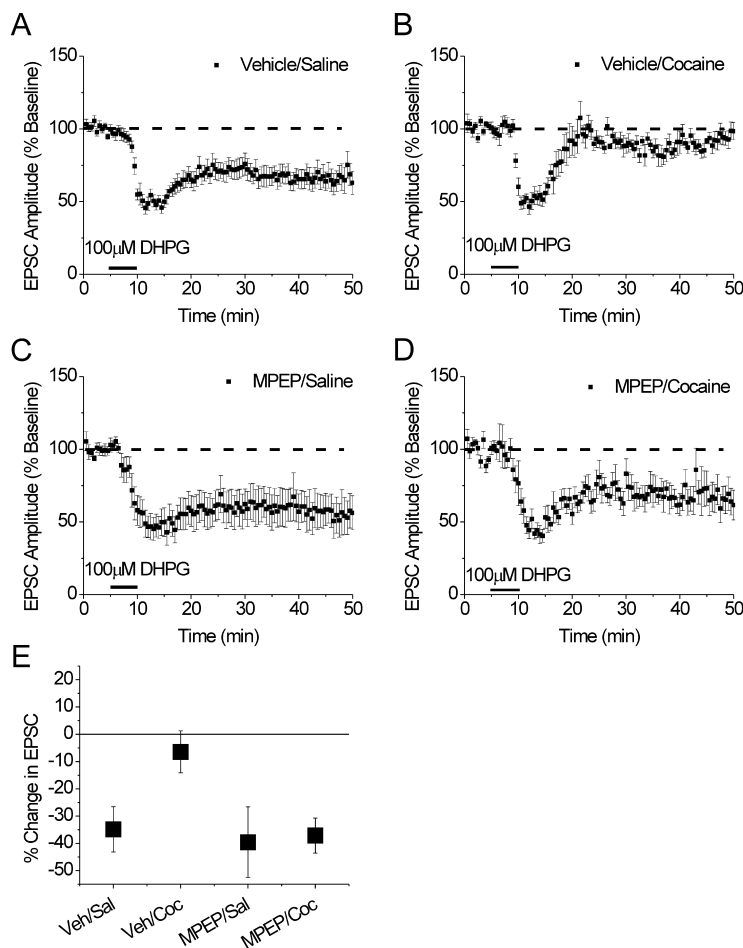
Figure 8. Repeated cocaine-induced changes in group I mGluR LTD are dependent on mGluR5 function *in vivo*. *A–D*, Average time courses of normalized EPSC amplitudes evoked by local stimulation in the BNST in acute slice preparations. DHPG (100 μM) was applied for 5 min as indicated. In each case, mice underwent 10 d of intraperitoneal injections and were killed 24 h after the last injection. Specific daily mouse treatments were as follows: *A*, intraperitoneal vehicle followed 30 min later by intraperitoneal saline; *B*, intraperitoneal vehicle followed 30 min later by intraperitoneal cocaine (20 mg/kg); *C*, intraperitoneal MPEP (10 mg/kg) followed 30 min later by intraperitoneal saline; *D*, intraperitoneal MPEP (10 mg/kg) followed 30 min later by intraperitoneal cocaine (20 mg/kg). *E*, Summary graph in which values represent percentage change in EPSC $\pm$ SEM, 30 min after DHPG washout. *$p < 0.05$ (Bonferroni and Tukey test after one-way ANOVA). Error bars indicate SEM.

fore the acutely administered cocaine again rescued *ex vivo* LTD induction [(Fig. 6*A*) saline, 28.8 $\pm$ 5.5%; (Fig. 6*B*) cocaine, $-0.9 \pm 5.0\%$; (Fig. 9*A,C*) MPEP/cocaine, 30.5 $\pm$ 5.6% depression; $n = 5$]. Furthermore, we verified that the rescued DHPG-LTD retained MPEP sensitivity in these experiments, demonstrating that *in vivo* MPEP administration rescues MPEP-sensitive DHPG LTD *ex vivo* [(Fig. 9*B,C*) MPEP/cocaine + MPEP, 7.7 $\pm$ 6% depression; $n = 6$]. These data suggest that cocaine administration promotes the activation of the mGluR5 signaling pathway in the BNST, resulting in either the occlusion or the subsequent desensitization of mGluR5.

Discussion

In total, our data suggest that mGluR5-dependent LTD in the BNST is transiently disrupted by *in vivo* cocaine administration, and that the duration of this disruption increases with repeated cocaine administration, such that after 10 d of administration, the disruption persists at least 24 h after the last dose of cocaine. Even after chronic cocaine administration, however, this disruption is not permanent, because normal DHPG-LTD can be induced 10 d after the last cocaine dose. *In vivo* administration of cocaine has previously been reported to disrupt *ex vivo* NMDA receptor-dependent LTP induction in the VTA (Ungless et al., 2001; Borgland et al., 2004), and *ex vivo* NMDA receptor- as well as mGluR5/CB1 receptor-dependent LTD induction in the nucleus accumbens (Thomas et al., 2001; Fourgeaud et al., 2004). Thus, these data add to a growing literature suggesting that cocaine administration can transiently regulate synaptic plasticity at glutamatergic synapses. These data emphasize that these alterations in glutamatergic transmission in distinct brain regions (i.e., the VTA, nucleus accumbens, and BNST) occur with subtly distinct time courses which may be of behavioral relevance. For example, disruption of VTA LTP is observable 24 h after a single administration of cocaine, whereas disruption of mGluR5-LTD in the BNST is not. Brain regions implicated in mediating behavioral responses to drugs of abuse are known to have rich interconnections, with the VTA projecting to the nucleus accumbens and BNST, and strong excitatory projections returning from the BNST to the VTA (Georges and Aston-Jones, 2001, 2002; Kash et al., 2008). Therefore, one possibility is that cocaine-induced alterations in one brain region may result in the alterations of transmission in other brain regions.

It is important to note that the proximate molecular targets of cocaine in the brain are the monoamine transporters. The BNST receives rich innervation from dopaminergic, serotonergic, and noradrenergic brain centers. Thus, at present, it is unclear whether the effects of cocaine are mediated by one or a combination of monoamine systems in the brain.

hypothesis, we again administered cocaine once daily for 10 d, preceding each injection by 30 min with either an injection of MPEP (10 mg/kg) or vehicle. For comparison, we also examined the effects of MPEP and vehicle injections on mice that received daily saline rather than cocaine injections. As previously demonstrated, we found that this regimen of cocaine administration resulted in disruption of DHPG-LTD, and this effect on DHPG-LTD was unaffected by pretreatment with vehicle (Fig. 8*B,E*) (vehicle/cocaine, 6.4 $\pm$ 7.7% depression; $n = 7$). In contrast, we found that daily *in vivo* administration of MPEP before cocaine prevented the disruption of DHPG-LTD *ex vivo* (Fig. 8*D,E*) (MPEP/cocaine, 37.2 $\pm$ 6.4% depression; $n = 7$). Importantly, neither MPEP nor vehicle alone had a significant effect on DHPG-LTD when administered with saline [(Fig. 8*A,E*) vehicle/saline, 34.9 $\pm$ 8.3% depression; $n = 10$; (Fig. 8*C,E*) MPEP/saline, 39.6 $\pm$ 13.0% depression; $n = 8$].

We next assessed whether the disruption of LTD shortly (30 min) after cocaine administration was similarly mediated by *in vivo* mGluR5-dependent processes. As with the 10 d cocaine treatment regimen, we found that pretreatment with MPEP be-

DHPG-LTD is not mediated by alterations in glutamate release

In this study, we extensively analyzed the potential for DHPG-LTD to be mediated by alterations in glutamate release properties. There are two primary means by which this LTD could be maintained presynaptically. First, it is possible that this LTD is mediated by a reduction in the probability of glutamate release. Indeed, this has been suggested for group I mGluR LTD in the striatum (Choi and Lovinger, 1997; Robbe et al., 2002; Kreitzer and Malenka, 2005) and, in certain cases, in the hippocampus (Nosyreva and Huber, 2005). In these cases, LTD (1) was associated with an alteration in PPR, (2) was disrupted by lowering the extracellular Ca^{2+}/Mg^{2+} ratio, and (3) in some cases is dependent on endocannabinoid activity as a retrograde messenger. Across a variety of sampling conditions, we find that DHPG-LTD in the BNST is not associated with persistent alterations in PPR, nor is the degree of LTD altered by changes in the divalent cation ratio. Furthermore, we previously demonstrated that the LTD induced by DHPG persists in the presence of a CB1 receptor antagonist and in slices made from a CB1 receptor knock-out mouse (Grueter et al., 2006).

A second possibility is that, rather than an alteration of transmitter release probability, DHPG could induce an alteration in quantal amplitude through, for example, a change in vesicular glutamate loading or mechanism of glutamate release to alter the average cleft glutamate concentration. Indeed, recent studies have suggested that in the hippocampus, group I mGluRs control quantal amplitude in an extracellular divalent cation concentration-dependent manner (Hardingham et al., 2006). However, we find that (1) manipulation of extracellular divalent ion concentrations does not impact DHPG-LTD, and (2) the effectiveness of a low-affinity AMPA receptor antagonist at reducing glutamatergic transmission is unaltered by DHPG administration. Thus, in total, these data are strongly suggestive that DHPG-LTD is not mediated by alterations in glutamate release.

DHPG-LTD is diminished by postsynaptic disruption of endocytosis

Given the strong data described above, indicating that DHPG-LTD is not mediated by alterations in transmitter release, it was surprising to observe that DHPG produced a persistent reduction of mEPSC frequency without significantly affecting mEPSC amplitude. It is important to point out that although monitoring of mEPSC frequency is frequently used as a means to monitor glutamate release probability, mEPSC frequency depends both on p (the probability of release) and n (the number of active release sites). Because we used postsynaptic AMPA receptor-mediated currents to probe glutamate synapses, n could be reduced either through presynaptic or postsynaptic silencing of synaptic sites. Indeed, such a mechanism has been described in the hippocampus, where DHPG application is thought to produce the internalization of synaptic AMPA receptors to result in a postsynaptic silencing and an effective decrease in n. Consistent with this idea,
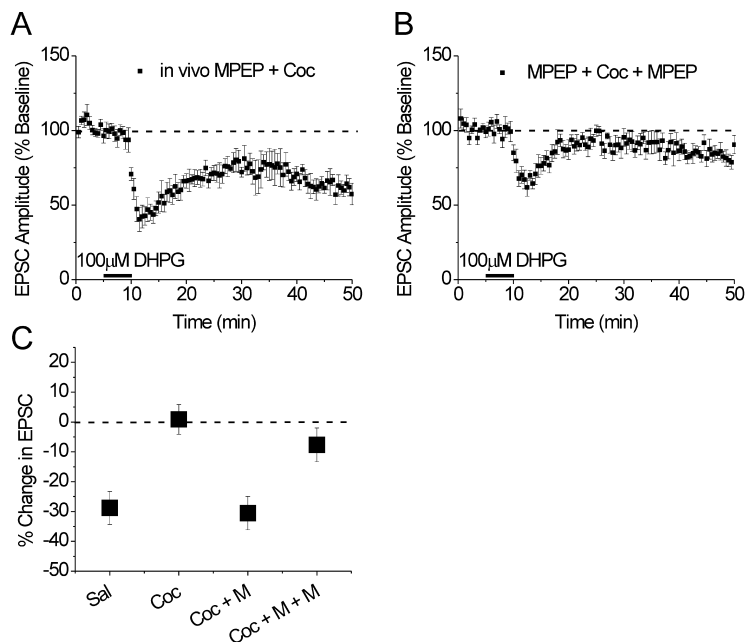

Figure 9. A single cocaine exposure alters mGluR5 LTD in BNST slices in an mGluR5-dependent manner on an acute (30 min after cocaine) time scale. *A–B*, Average time courses of normalized EPSC amplitudes evoked by local stimulation in the BNST in acute slice preparations. DHPG (100 μM) was applied for 5 min as indicated. *A*, Intraperitoneal MPEP (10 mg/kg) followed 30 min later by intraperitoneal cocaine (Coc) (20 mg/kg). In *B*, mice were conditioned as in *A*, and MPEP (10 μM) was then applied to the slice 10 min before DHPG application. *C*, Summary graph in which values represent percentage change in EPSC ± SEM, 30 min after DHPG washout. *$p < 0.05$ (Bonferroni and Tukey test after one-way ANOVA). Error bars indicate SEM. Values taken from time courses in *A* and *B* and Figure 6, *A* and *B*.

we find that the inclusion of a peptide designed to disrupt dynamin–amphiphysin interactions, and thus clathrin-dependent endocytosis, or stabilization of actin, which has been shown to prevent endocytosis, greatly diminished DHPG-LTD in the BNST (Xiao et al., 2001). Thus, in total, our data are most consistent with a postsynaptic silencing mechanism for the maintenance of DHPG-LTD.

mGluR5 activation *in vivo* contributes to cocaine-induced disruption of *ex vivo* DHPG-LTD induction

A second major finding of this study was that pretreatment of mice with the mGluR5 antagonist MPEP before cocaine administration rescued subsequent *ex vivo* induction of mGluR5-dependent LTD. Data from a number of behavioral studies using mice with targeted deletions of mGluR5 or homer isoforms, as well as pharmacological inhibition of mGluR5, suggest that this receptor plays a critical role in behavioral responses to psychostimulants as well as other addictive substances such as ethanol (Chiamulera et al., 2001; Olive et al., 2005). However, it is unclear from these studies whether the involvement of mGluR5 reflects an acute action of the receptor or a more constitutive background role. We recently demonstrated that acute *ex vivo* induction of DHPG-LTD is not due to constitutive activation of mGluR5 (Grueter et al., 2006). Similarly the present data are most consistent with mGluR5 being acutely activated in response to cocaine administration to participate in cocaine-induced synaptic and behavioral effects, inasmuch as MPEP treatment in the absence of cocaine resulted in no effect on *ex vivo* DHPG-LTD.

Previous studies in the nucleus accumbens suggested that cocaine administration disrupts group I mGluR LTD via an NMDA- and D1 dopamine receptor-mediated recruitment of the scaffolding protein homer 1a and internalization of mGluR5 (Fourgeaud et al., 2004). Our findings that *in vivo* MPEP admin-

istration restores MPEP-sensitive LTD *ex vivo* indicates that in the BNST, cocaine disrupts group I mGluR signaling through a homotypic process that involves recruitment of mGluR5 activity. These data lend further support to the idea that mGluR5 signaling plays an integral role in behavioral responses to psychostimulants and provides data consistent with the idea that mGluR5 activation occurs after *in vivo* cocaine administration, adding to a growing body of literature suggesting that mGluR5 may represent a useful therapeutic target in the treatment of addiction.

The use of *in vivo* application of a receptor antagonist to rescue subsequent *ex vivo* effects of an agonist at the same receptor represents a novel strategy to explore CNS targets in disorders. The finding that pretreatment with MPEP can alter effects of cocaine on synaptic function in the CNS may provide a window of opportunity for the study of mechanistic insights into the interactions of cocaine with glutamatergic synapses in key regions in the CNS. In future studies, it is anticipated that this experimental design can be further used to test the role of a variety of different signaling processes that may participate in cocaine-induced regulation of synaptic plasticity. Moreover, this assay may provide a means to aid in therapeutic discovery. MPEP is one of a class of allosteric regulators of mGluR5. Both positive and negative allosteric regulators of mGluR5 have been developed, and can be tested in a manner analogous to the MPEP experiments described in this study to test their effectiveness in shifting the efficacy of cocaine in regulating glutamatergic function.

Behavioral implications

Evidence suggests that the BNST plays an important role both in mediating the acute rewarding effects of cocaine (Epping-Jordan et al., 1998; Carboni et al., 2000) and in stress-induced reinstatement of cocaine-seeking behavior (Erb and Stewart, 1999; Erb et al., 2001; Leri et al., 2002). In the reinstatement models, in particular, the data suggest that the BNST initiates circuit activity ultimately responsible for the behavior. Thus, alterations in the efficacy of the glutamate inputs to the BNST that would be predicted to drive this activation would potentially alter the likelihood that the BNST would provide output to key downstream nuclei involved in reinstatement, such as dopaminergic neurons and the lateral hypothalamus. Although it is difficult to speculate at this point on the precise role of this modification of mGluR5 action by cocaine on behavior, our data suggest that cocaine impairs the ability of mGluR5 LTD to occur at some of these glutamate inputs, preventing a potential "brake" on the system from being engaged.

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