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Long-Lasting Impairment of mGluR$_5$-Activated Intracellular Pathways in the Striatum After Withdrawal of Cocaine Self-Administration

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Abstract

Background: Cocaine addiction continues to be a major heath concern, and despite public health intervention there is a lack of efficient pharmacological treatment options. A newly identified potential target are the group I metabotropic glutamate receptors, with allosteric modulators showing particular promise.
Methods: We evaluated the capacity of group I metabotropic glutamate receptors to induce functional responses in ex vivo striatal slices from rats with (1) acute cocaine self-administration, (2) chronic cocaine self-administration, and (3) 60 days cocaine self-administration withdrawal by Western blot and extracellular recordings of synaptic transmission.
Results: We found that striatal group I metabotropic glutamate receptors are the principal mediator of the mGluR$_{1/5}$ agonist (*RS*)-3,5-dihydroxyphenylglycine-induced cAMP responsive-element binding protein phosphorylation. Both acute and chronic cocaine self-administration blunted group I metabotropic glutamate receptor effects on cAMP responsive-element binding protein phosphorylation in the striatum, which correlated with the capacity to induce long-term depression, an effect that was maintained 60 days after chronic cocaine self-administration withdrawal. In the nucleus accumbens, the principal brain region mediating the rewarding effects of drugs, chronic cocaine self-administration blunted group I metabotropic glutamate receptor stimulation of extracellular signal-regulated protein kinases 1/2 and cAMP responsive-element binding protein.

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Significance Statement

The National Institute of Health estimated the US to have 1.6 million cocaine users in 2012, a number staying high due to the lack of efficient treatment options. One of the major challenges in cocaine addiction are the long-lasting changes and memories that develop with repeated drug consumption in the striatum and nucleus accumbens, 2 brain structures important for goal-directed learning and drug reward, respectively. Recent advances have identified group I metabotropic glutamate receptor modulators as potential targets for the treatment of cocaine addiction. We show here that prolonged cocaine consumption, through abstinence, blunts group I metabotropic glutamate receptor responses in the striatum and accumbens. In addition, changes in group I metabotropic glutamate receptor signaling specifically in the accumbens, the brain structure mediating cocaine reward, suggest that blockade of the group I metabotropic glutamate receptor in this structure could be a potential new target in the treatment of addiction-like behavior.

Interestingly, the group I metabotropic glutamate receptor antagonist/inverse-agonist, 2-methyl-6-(phenylethynyl)pyridine hydrochloride, led to a specific increase in cAMP responsive-element binding protein phosphorylation after chronic cocaine self-administration, specifically in the nucleus accumbens, but not in the striatum.

Conclusions: Prolonged cocaine self-administration, through withdrawal, leads to a blunting of group I metabotropic glutamate receptor responses in the striatum. In addition, specifically in the accumbens, group I metabotropic glutamate receptor signaling to cAMP responsive-element binding protein shifts from an agonist-induced to an antagonist-induced cAMP responsive-element binding protein phosphorylation.

Keywords: extracellular signal-regulated protein kinase1 and 2, cAMP response element binding protein, cocaine self-administration withdrawal, striatum, long term depression.

Introduction

Cocaine addiction is a complex multifaceted condition, which thus far has impeded the development of efficient pharmacological treatment options (Shorter et al., 2015; Wolf, 2016). Repeated cocaine consumption decreases expression of metabotropic glutamate 5 receptors ($mGluR_5$) in the striatum, a subcortical region critical in mediating the effects of drugs of abuse (Ghasemzadeh et al., 2009; Knackstedt et al., 2010; Martinez et al., 2014; Milella et al., 2014; Pomierny-Chamiolo et al., 2015; Terbeck et al., 2015). The critical role of the glutamatergic system in humans is supported by preclinical studies that suggest that striatal glutamate homeostasis is disrupted following cocaine exposure (Martinez et al., 2014), an observation made earlier in rodents (Knackstedt and Kalivas, 2009; Moussawi et al., 2009). Indeed, glutamate transmission, and particularly $mGluR_{1/5}$, are central in mood disorders as well as the regulation of the behavioral and molecular responses to cocaine (Chiamulera et al., 2001; Swanson et al., 2001; Kalivas et al., 2005; Kenny et al., 2005; Volkow et al., 2005; Kumaresan et al., 2009; Moussawi et al., 2009; Akkus et al., 2014; Terbeck et al., 2015; Perry et al., 2016). In recent years, $mGluR_5$ have come in focus as promising targets for the treatment of various aspects of cocaine abuse; positive allosteric modulators of $mGluR_5$ facilitate extinction of cocaine-associated contextual memory (Cleva et al., 2011; Perry et al., 2016), whereas negative allosteric modulators, pharmacological blockade, or genetic deletion decrease CSA, cocaine seeking, and conditioned place preference (Chiamulera et al., 2001; Kenny et al., 2005; Kumaresan et al., 2009; Moussawi et al., 2009; Wang et al., 2013; Knackstedt et al., 2014; Gould et al., 2015; Schmidt et al., 2015; Mihov and Hasler, 2016).

Repeated drug administration affects synaptic transmission and induces the formation of long-lasting drug-associated memories, changes maintained after prolonged cocaine withdrawal (Hyman et al., 2006; Collingridge et al., 2010; Knackstedt et al., 2010; Pomierny-Chamiolo et al., 2015; Wolf, 2016). The neuronal adaptations induced by cocaine intake can be evidenced by altered receptor responses, signal transduction, and gene expression, which finally affect synaptic transmission and plasticity (McClung and Nestler, 2008; Kalivas, 2009; Knackstedt et al., 2010). Certainly, chronic exposure to cocaine profoundly alters both long-term potentiation and long-term depression (LTD) at excitatory cortico-striatal and cortico-accumbal synapses. More particularly, LTD inhibition appears to be prominent in the induction of addictive behaviors. As a matter of fact, the loss of the ability to trigger N-methyl-D-aspartate-dependent LTD at excitatory cortico-accumbal synapses is crucial for the transition to addiction and vulnerability to relapse after prolonged cocaine intake (Kasanetz et al., 2010). Nevertheless, this is not restricted to N-methyl-D-aspartate-dependent electrically induced LTD, as chemically induced LTD by $mGluR_{1/5}$ stimulation ($mGluR_{1/5}$-LTD) is also dramatically inhibited at excitatory synapses by cocaine exposure in the dorsal striatum and remains inhibited even 3 weeks after withdrawal (Knackstedt et al., 2014).

In most neuronal cell types, $mGluR_{1/5}$-LTD depends on the activation of extracellular signal-regulated protein kinases 1/2 (ERK1/2) (Gallagher et al., 2004; Grueter et al., 2006). ERK1/2 is important for the regulation of gene expression through the transcription factors cAMP responsive-element binding protein (CREB) and E-26 like protein. ERK1/2 and CREB activation are associated with in vivo effects of cocaine. ERK1/2 is linked to cocaine reward (Valjent et al., 2000) as well as the development of locomotor sensitization (Valjent et al., 2006), whereas CREB is critical for the rewarding effects of cocaine (Self et al., 1998; McClung and Nestler, 2003; Larson et al., 2011). In light of the potential changes cocaine can cause to cell signaling and neuronal function, we sought to understand the impact of several stages of CSA on $mGluR_{1/5}$ function in the dorsal and ventral striatum, with a particular focus on the above pathways.

Methods

All experiments were carried out in accordance with the European Community Council Directive of September 22, 2010 (2010/63/UE) as well as the European Communities Council

Directive 86/609/EEC. The cocaine self-administration (CSA) and following signal-transduction pathway studies were carried out in Barcelona and approved by the Ethics Committee for Human and Animal Research of the Autonomous University of Barcelona. Electrophysiological recordings as well as signal-transduction studies were carried out in Montpellier and approved by the local section of the Comité National de Réflexion Ethique sur l'Expérimentation Animale' (C2EA-36), Montpellier.

Subjects

Male Sprague-Dawley rats (including OFA-Sprague Dawley) weighing 210 to 250 g were obtained from the Animal Service, Universitat Autònoma de Barcelona, Spain and the Centre d'Elevage Depré, France. Rats were housed individually in a climate-controlled environment at 19°C to 23°C on a 12-h-light/-dark cycle (lights on 8 AM). Behavioral studies were carried out during the light cycle except initial overnight sucrose training. Rats were allowed ad libitum access to food and water except during sucrose training that was facilitated by partial food restriction for 1 week to maintain rat bodyweight at 85% of the theoretical weight.

Surgery and Behavioral Conditioning

A detailed description of the surgery and conditioning paradigms has been described previously (Hoffmann et al., 2012). Rats were maintained at 85% bodyweight during sucrose training (Figure 1). Rats were equally distributed into 2 groups of operant chambers (LE 1005, Panlab S.L.U.) with either the right or left lever active to obtain sucrose pellets. One active lever press gave one pellet (45 mg Bio-Serv, Frenchtown, NJ) and was associated with 5 seconds of light on over the active lever. Following the initial 5 seconds of light on was a 10-second period with the active lever inactive and house lights off. Inactive lever press had no programmed consequence. Overnight sessions permitted consumption of a maximum 100 pellets in 14 hours. Lever pressing was considered acquired when rats obtained 100 sucrose pellets during 2 additional 2-hour sessions in the light cycle. After sucrose training, the rats were allowed to recover weight before surgery. To implant a jugular catheter, rats were anesthetized with isoflurane and the catheter was implanted in the jugular vein, passed under the skin to the back where it exited. To relieve postoperational pain s.c. meloxicam (1.5 mg/kg Metacam 0.3 mL/kg, Boehringer) was injected daily for 3 days. Rats were allowed a minimum 1 week of recovery before they started saline self-administration (SSA) or CSA. Intravenous infusions of saline or cocaine were administered by a syringe pump (Samford, CT). Behavioral equipment and data collection were controlled by the Packwin Software (Panlab S.L.U). Rats self-administered saline or cocaine during a single 2-h session (acute SSA, acute CSA) or for daily sessions (chronic CSA) on a fixed ratio 1 schedule (FR1) (supplementary Figure 1A). The cues were as for sucrose training. However, one active lever press delivered i.v. saline or cocaine (0.5 mg/kg/0.1 mL for 5 seconds) during the 2-h session with a maximum of 50 injections per session. Then 16 to 20 hours after acute SSA and acute CSA, rats were killed by decapitation (Figure 1). Chronic CSA rats had one cocaine session daily 5 d/wk. Chronic CSA rats were maintained a minimum 2 weeks on a FR1 schedule and then changed to a FR2 and FR3 schedule (2 and 3 lever presses/cocaine delivery) and finally to FR5 on which they were maintained for 3 to 5 weeks before killing (Figure 1; supplementary Figure 1). Total length of CSA varied from 6 to 12 weeks (7.8 ± 1.7 weeks, equivalent to 30 to 60 self-administration sessions) depending on rat performance and time required by experimenters to process brain slices. We previously described that cocaine administered at 0.5 mg/kg/injection, 2 h/d at 5 d/wk for a minimum of 6 weeks permitted to select rats with a high and stable CSA (Hoffmann et al., 2012). These settings permitted us to obtain "high-responding rats," which are likely to reflect the main characteristics of cocaine addiction (Deroche-Gamonet et al., 2004). These rats self-administered cocaine on a FR1 schedule from the first CSA sessions (Figure 1A; supplementary Figure 1). Rats maintained for the study had an average of 24±0.5 lever presses per session (FR1-FR5) in contrast to an average lever pressing rate of 11.6±1.3 in low-responding rats (supplementary Figure 1B). Thirty-one percent of the studied rats did not meet the set criteria within the first 7 days of the study and were consequently

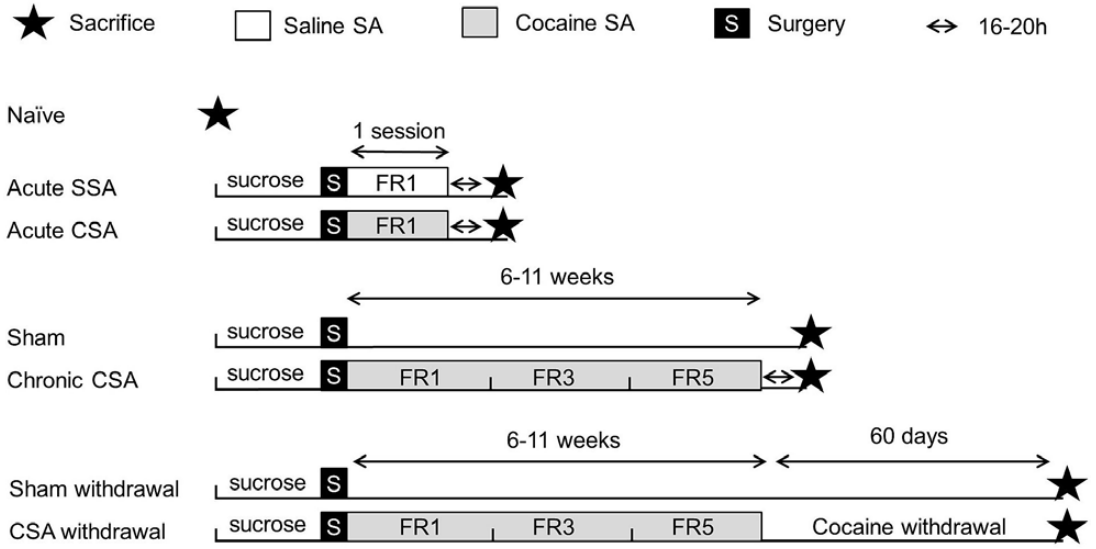

Figure 1. Behavioral conditioning. Rats who successfully acquired sucrose self-administration (sucrose) underwent surgery (S) where an i.v. catheter was implanted. After recovery, sucrose was substituted for i.v. saline (SSA) or cocaine self-administration (CSA, 0.5 mg/kg in 0.1 mL) on a fixed ratio (FR) 1 schedule. Rats were introduced into an operant chamber during a single session (acute) or >6 weeks (chronic) to SSA or CSA. At 16 to 20 hours after the last self-administration session, rats were killed by decapitation, except cocaine withdrawal rats, which were killed 60 days after last CSA (CSA withdrawal).

excluded (supplementary Figure 1B, low responders) (Hoffmann et al., 2012). A total of 15 chronic CSA rats were used: 10 were killed 16 to 20 hours after the last CSA session and 5 were destined for the chronic CSA withdrawal group (Figure 1). The CSA withdrawal rats were maintained in their home cage for 60 days before euthanasia. Eleven sham rats were handled daily and killed pair-wise with the paired CSA rats. Sham rats received the same training, surgery, and treatment as rats destined for self-administration, except that they never self-administered saline or cocaine. Naïve rats were not subject to conditioning or surgery before killing. All killings were carried out between 9 and 11 AM under isoflurane anesthesia.

Brain Slice Preparation and Incubation for Immunoblotting

Brain slice preparation and Western-blot conditions were described previously in detail (Moreno et al., 2011; Hoffmann et al., 2012). Immunoblotting was carried out using primary antibodies against tyrosine hydroxylase (TH, 1:5000, Chemicon AB1542), phospho Thr202 and Tyr204 ERK (1:6000, Cell Signaling 9101), total ERK (1:5000, Cell Signaling 4695), phospho Ser133 CREB (1:3500, Millipore 06-519), GAPDH (1:12000, Cell Signaling 2118), total CREB (1:750, Cell Signaling 9197), phospho-ERK 1/2 antibody (1:2500, Sigma, M8159, Steinheim, Germany), and ERK 1/2 antibody that recognizes both phosphorylated and nonphosphorylated ERK 1/2 (1:40000, Sigma, M5670). Horseradish peroxidase-coupled secondary antibodies used were goat anti-rabbit (1:1000, Cell Signaling 7074) and donkey anti-sheep (1:3000, Chemicon AP147P). Some ERK1/2 and phosphorylated ERK1/2 blots were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10000, Sigma) and IRDye 680 (anti-rabbit) antibody (1:10000, Sigma). After correction for loading variability, Western-blot results were normalized to controls and expressed as means ± SEM. Each rat gave rise to various brain slices that were incubated under control and treated conditions generally in duplicate or triplicate. The ventro-dorsal, anterior-posterior localization of each incubated slice was noted, and treatments were distributed throughout the different regions, thus masking any possible regional differences in ligand-induced signaling transduction. The number of replicates, n, refers to individual slice incubations from individual rats. Data was analyzed by ANOVA. 95% CI was used to determine statistical significance.

Electrophysiological Recordings

The protocol used was adapted from (Escobar et al., 2011; Marta González-Sepúlvedaa, 2013). In brief, brains from naïve rats (5-9 weeks of age) or rats previously used in behavioral experiments (30-32 weeks of age) were extracted and submerged into ice-cold Krebs buffer containing 124 mM NaCl, 3.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM glucose, and 10 mM HEPES (pH 7.4) and cut into sagittal slices (350 μm). Recordings were done at 30°C to 32°C in Krebs buffer containing 2 mM $CaCl_2$. Field potentials were evoked every 20 seconds in the striatum by stimuli intensity ranging from 100 to 800 μA for a duration of 80 to 800 microseconds. Hippocampal field potentials were evoked every 15 seconds by stimuli intensity ranging from 50 to 250 μA for a duration of 50 to 250 microseconds. Field potentials were recorded by a patch-clamp amplifier (Axopatch 200 B, Axon Instruments) and data collected by Win LTP (Dr. W. Anderson, University of Bristol, UK). Experiments were systematically terminated by applying kynurenic acid (4 mM). Field potential amplitudes were normalized to baseline and expressed as percentage of variation. Each data point is the mean of 6 consecutive field potentials for striatum and 8 for hippocampus. Data represent means ± SEM.

Drugs

(*RS*)-3,5-Dihydroxyphenylglycine (DHPG), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), kynurenic acid, 7-(Hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester, (S)-(+)-alpha-Amino-4-carboxy-2-methylbenzeneacetic acid, 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(f)quinoxaline, 1,4-Diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene (U0126), and kynurenic acid were obtained from Tocris or Ascent. All dilutions were done in Krebs buffer. Antagonists were applied 10 minutes prior to agonists. For immunoblot analysis, U0126 was added 20 minutes prior to DHPG. Stock solution of U0126 was prepared in DMSO. Cocaine hydrochloride was obtained from the Ministerio de Sanidad y Consumo, Spain, and dissolved in physiological saline and sterile filtered for i.v. administration.

Results

Metabotropic $GluR_{1/5}$ Promote ERK1/2 Phosphorylation in Ex Vivo Striatal Slices

To determine how $mGluR_{1/5}$ coupling to ERK1/2 was affected over time during CSA (Figure 1), we first validated our experimental set-up. To establish the time frame and concentration of ligand, we applied the selective $mGluR_{1/5}$ agonist DHPG (10 and 100 μM) to striatal slices from naïve rats. We found that DHPG 10 μM increased ERK1/2 phosphorylation to a greater degree than DHPG 100 μM at the studied time points, reaching maximal levels after 5 to 10 minutes of incubation (Figure 2A-B). Based on this, we used 10 μM for the remaining studies. The striatum is enriched with dopaminergic terminals containing the dopamine-synthesizing enzyme TH. To control for correct tissue dissection, we systematically verified the slices were enriched with TH. Approximately 2% of slices were discarded due to low or absent TH. To establish whether DHPG-associated responses in striatal slices were dependent on both $mGluR_1$ and $mGluR_5$, we applied either receptor antagonists prior to DHPG and evaluated ERK1/2 activation. In the presence of the selective $mGluR_1$ antagonist, 7-(Hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester, CPCCOEt (100 μM, 20 minutes) or the $mGluR_5$ antagonist, MPEP (20 μM, 20 minutes), DHPG-induced ERK1/2 phosphorylation was abolished (Figure 2C).

Chronic CSA and Prolonged CSA Withdrawal Disrupts $mGluR_{1/5}$ Coupling to ERK1/2 in the Dorsal Striatum

In agreement with others (Lenoir et al., 2007; Schmidt et al., 2015), we found sucrose self-administration to be highly rewarding in rats, as evidenced by the high number of lever presses on the active lever (+) during the first session of self-administration after substituting sucrose by saline (Figures 1 and 3A, saline). The high responding on the active lever (+) in the acute SSA group likely reflects a dissatisfying search for sucrose. In line with this, the subjective effects of cocaine were revealed by the lower number of active lever (+) presses in the acute CSA group compared with the acute SSA (Figure 3A). The acute CSA animals did show preference for the active lever (+), although the total number of lever presses was significantly lower than in the acute SSA group (Figure 3A, cocaine). To establish how different stages of CSA affected $mGluR_{1/5}$ coupling to ERK1/2, we evaluated

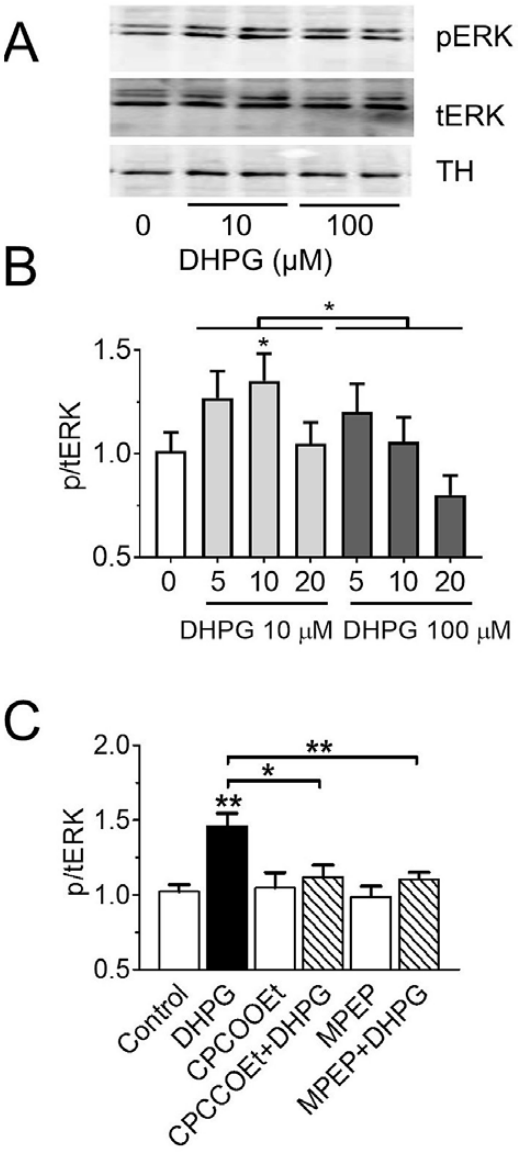

Figure 2. (*RS*)-3,5-Dihydroxyphenylglycine (DHPG)-induces extracellular signal-regulated protein kinases 1/2 (ERK1/2) phosphorylation in ex vivo striatal slices of naive rats. DHPG-mediated ERK1/2 (A-B) phosphorylation in the presence or absence (C) of group I metabotropic glutamate receptors ($mGluR_1$) antagonist 7-(Hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester (CPCCOEt) (100 μM) or mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) (20 μM). The analysis of ERK1/2 phosphorylation was performed on 9 to 30 slices from 5 to 6 animals. Statistical analysis was done by 1-way or 2-way ANOVA, $^*P < .05$; $^{**}P < .01$ compared with control or as indicated by bar.

DHPG-mediated ERK1/2 activation in striatal slices from acute CSA, chronic CSA, and cocaine withdrawal rats. Interestingly, there was a time-dependent effect on the capacity for DHPG to induce ERK1/2 phosphorylation, where acute CSA had no effect (Figure 3B-C), chronic CSA decreased or abolished (Figure 3D-E) and a 60-day withdrawal from chronic CSA abolished DHPG-ERK1/2 activation (Figure 3F-G). It has been reported that $mGluR_5$ can acquire constitutive activity (Ango et al., 2001; Muhlemann et al., 2005). To explore this possibility, we used the $mGluR_5$ antagonist/inverse agonist MPEP. Application of MPEP (10 μM, 10 minutes) did not elicit any changes of basal phosphorylation of ERK1/2 in any of the studied conditions (Figure 3C, E, G, MPEP).

CSA Abolishes DHPG-LTD and DHPG-Induced CREB Phosphorylation in the Dorsal Striatum

Cocaine addiction induces long-lasting effects on cellular function, synaptic plasticity, and memory formation (Huang et al., 2011). These effects are in part mediated by glutamate and induction of transcription factors, such as CREB. We therefore asked if the loss of DHPG-induced ERK1/2 phosphorylation following CSA would lead to long-lasting changes of synaptic plasticity. Due to the technical difficulty to distinguish between dopamine D1 and D2 receptor-expressing medium spiny neurons of the rat striatum, we decided to evoke field potentials at cortico-striatal afferents in the dorsal striatum to obtain a general image of $mGluR_{1/5}$-mediated synaptic plasticity. The stimulation of cortico-striatal afferents evoked both positive and negative going extracellular field potentials in the striatum of naive rats, which were sensitive to AMPA/kainate receptor blockade with the selective antagonist, 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (10 μM, 10 minutes) (supplementary Figure 2A) and with the nonselective glutamate ionotropic receptor antagonist, kynurenic acid (Figure 4F). This confirmed the requirement of the synaptic release of glutamate for their occurrence. The application of DHPG (10 μM, 10 minutes) resulted in a long-lasting reduction of field potentials (supplementary Figure 2B; Fig. 4A, DHPG). On average field potential amplitude decreased to 69 ± 7% (n = 9) of baseline (102 ± 2%) during perfusion of DHPG and remained reduced for >40 minutes after DHPG washout (47 ± 2% of baseline, n = 9). DHPG-LTD was observed on both negative and positive going field potentials (supplementary Figure 2B-C). The analysis of the paired-pulse ratios revealed an increased ratio after DHPG application (supplementary Figure 2D), indicating a presynaptic expression of DHPG-LTD. DHPG-LTD in other brain regions depends on ERK1/2 phosphorylation (Gallagher et al., 2004; Grueter et al., 2006). Thus, we next evaluated the role of ERK1/2 in the induction of DHPG-LTD in the dorsal striatum. Application of the specific mitogen-activated protein kinase kinase inhibitor U0126 prior to DHPG prevented DHPG-LTD expression (Figure 4A) and abolished ERK1/2 and CREB phosphorylation (Figure 4B-C, nuclear localization of CREB was confirmed; supplementary Figure 3). DHPG-LTD required activation of both $mGluR_1$ and $mGluR_5$ (Figure 4D-E) as evidenced by the capacity of the $mGluR_1$ antagonist, (S)-(+)-alpha-Amino-4-carboxy-2-methylbenzeneacetic acid (50 μM), and the $mGluR_5$ antagonist, MPEP (10 μM), to inhibit DHPG-LTD (LY367386: 98 ± 5%, n = 6; MPEP: 98 ± 4%, n = 7 compared with baseline). The requirement of ERK1/2 activation for DHPG-LTD and the lack of DHPG-induced ERK1/2 phosphorylation after CSA withdrawal led us to hypothesize that DHPG-LTD would be abolished after CSA withdrawal. Indeed, 60 days after CSA withdrawal, DHPG was unable to induce a cortico-striatal LTD (Figure 4F). In contrast, DHPG-induced LTD in the hippocampal CA1 area was normal in slices from the same group of animals (supplementary Figure 4). The loss of both DHPG-induced ERK1/2 phosphorylation and LTD in the striatum after chronic CSA withdrawal suggest that CREB, a downstream target of ERK1/2, could be involved in the maintained effects of CSA on synaptic plasticity. Surprisingly, both acute and chronic CSA had a blunting effect on the capacity of DHPG to induce CREB phosphorylation in the dorsal striatum (Figure 5A-D), whereas MPEP had no effect in any of the studied groups (Figure 5B, D, MPEP). In contrast to DHPG-induced ERK1/2 phosphorylation (Figure 2C), DHPG-induced CREB phosphorylation relied principally on $mGluR_5$ (Figure 5E).

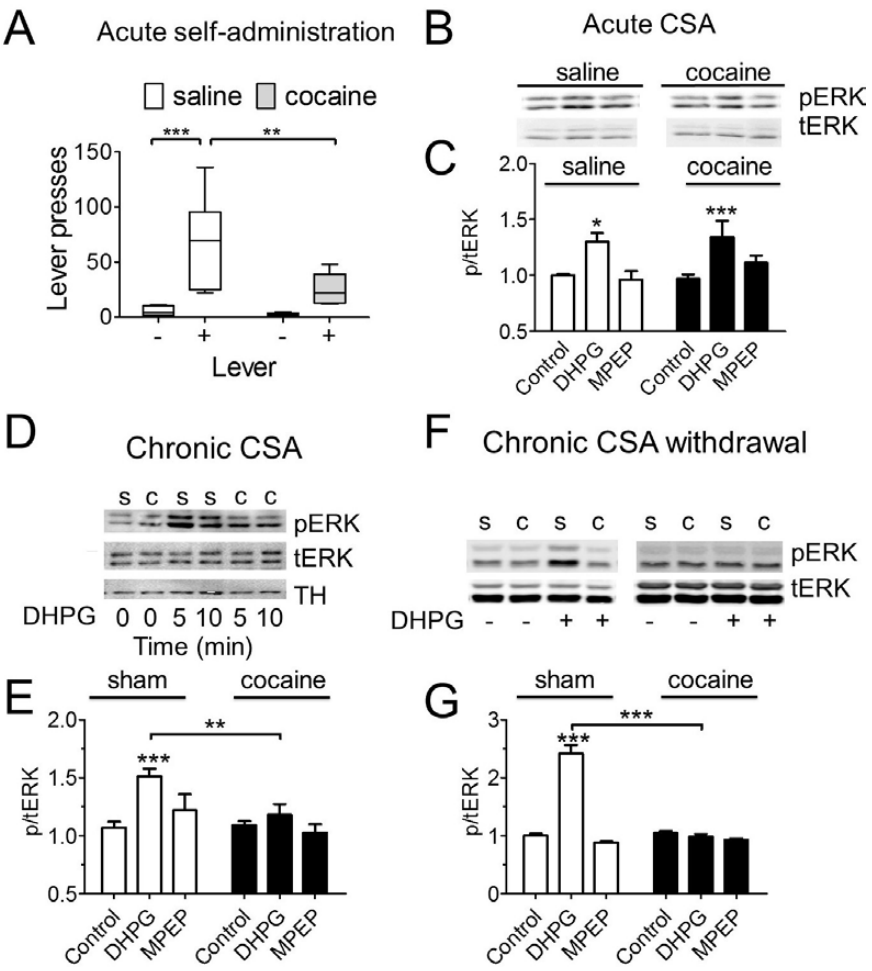

Figure 3. Chronic cocaine self-administration (CSA) specifically abolished (RS)-3,5-Dihydroxyphenylglycine (DHPG)-induced ERK1/2 phosphorylation in the striatum. (A) Cumulated number of lever presses during the initial session of 2 hours of saline (SSA) or cocaine (CSA) at fixed ratio 1 (FR1) after replacing sucrose with cocaine. Representative immunoblots (B, D, F) and histograms (C, E, G) of DHPG and 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP)-mediated ERK1/2 phosphorylation in striatal slices from acute (B-C), chronic (D-E), and cocaine withdrawn (F-G) rats. +, active lever; -, inactive lever; s, sham; c, cocaine. n = 6 to 10 rats. Two-way ANOVA, $^{*}P<.05$; $^{**}P<.01$; $^{***}P<.001$ compared with control or as indicated by bar.

Signaling Shift of $mGluR_{1/5}$ in the Nucleus Accumbens by Chronic CSA

Numerous studies have shown evidence of alterations of synaptic transmission in the nucleus accumbens after cocaine administration (Backstrom and Hyytia, 2007; Moussawi et al., 2009). We therefore decided to examine whether $mGluR_{1/5}$ signaling was also affected in our conditions. Due to the size of the accumbens, we did not separate the core and shell regions for the biochemical analysis. The data obtained thus reflects the overall effect of treatment of both accumbal subregions. In the accumbens, chronic CSA strongly decreased DHPG-induced signaling to ERK1/2 and CREB (Figure 6A-D). To assess a possible constitutive activity of $mGluR_5$ after chronic CSA, we applied MPEP to the slices. MPEP (10 µM, 10 minutes) did not alter basal phosphorylation of ERK1/2 or CREB in sham rats (Figure 6B, D, white columns). Surprisingly, chronic CSA led to a strong enhancement of CREB phosphorylation induced by MPEP (10 µM, 10 minutes) (Figure 6D).

Discussion

We provide evidence here that depending on the CSA stage (acute, chronic, withdrawal), the capacity of $mGluR_{1/5}$ to stimulate specific downstream signaling cascades is altered. Interestingly, specifically in the nucleus accumbens, chronic CSA also leads to a gain of $mGluR_5$ function that could be interpreted as an increase in its constitutive activity.

Limitations of Rodent CSA Paradigms and Their Validity as Study Models for Human Addiction

Repeated CSA is a complex behavior, where the rewarding effects of this drug change with its concentration as well as mode and speed of administration. Depending on the specific rodent drug self-administration paradigm used, specific features of human drug abuse can be evaluated (Panlilio et al., 2005; Sughondhabirom et al., 2005; Panlilio and Goldberg, 2007). Although it has been established that repeated cocaine consumption triggers long-lasting changes in brain plasticity, which contribute to drug craving during abstinence and might induce relapse into cocaine-seeking behavior, we still have a very limited understanding of the exact molecular changes in the brain, which is reflected by the poor success in developing efficient drugs for cocaine addiction (Shorter et al., 2015; Wolf, 2016). To increase our understanding of the changes taking place in the striatum from the first CSA to chronic CSA through CSA withdrawal, we studied the progression of $mGluR_{1/5}$ responses in striatal slices. Although the validity of the chronic CSA model has proven to reflect numerous aspects of human cocaine addiction

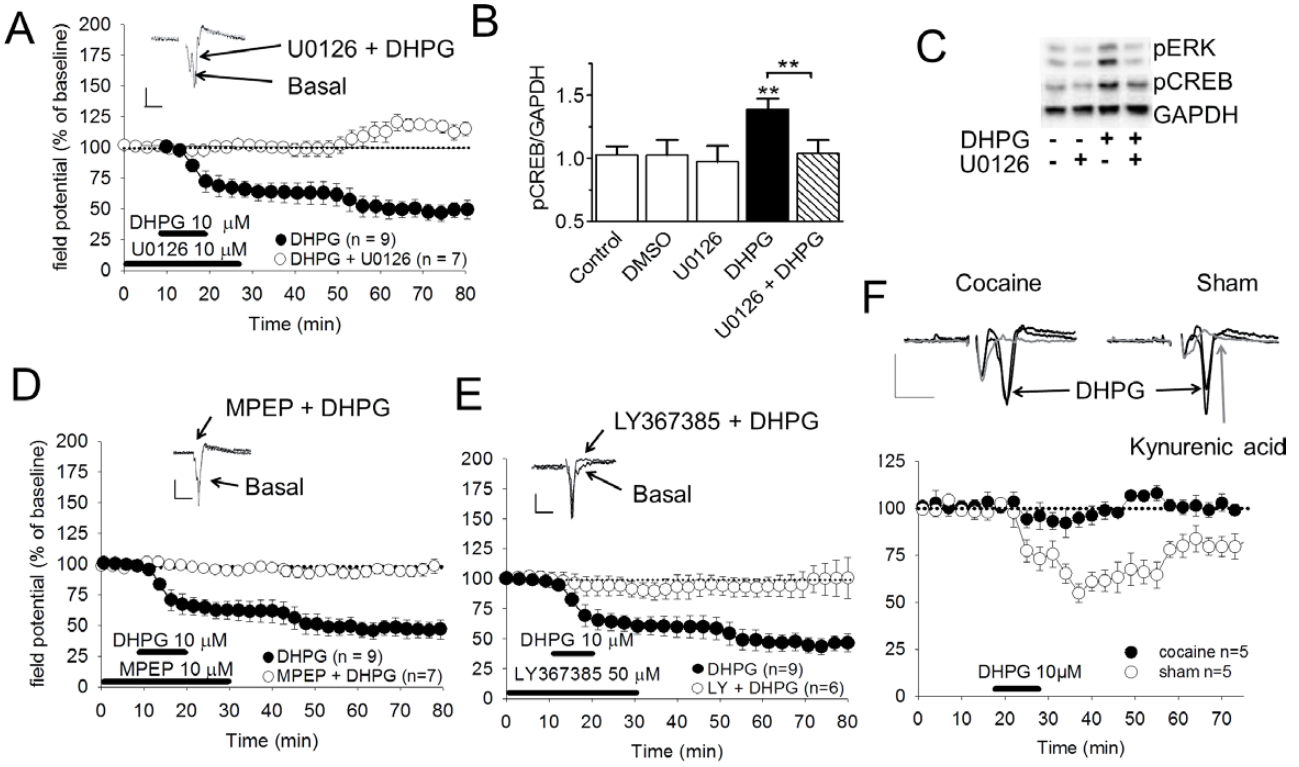

Figure 4. Cocaine withdrawal leads to loss of group I metabotropic glutamate receptors ($mGluR_{1/5}$)-mediated long-term depression (LTD). The MEK inhibitor U0126 abolished (RS)-3,5-Dihydroxyphenylglycine (DHPG)-induced LTD (A), CREB phosphorylation (pCREB) (B-C), and ERK1/2 phosphorylation (pERK) (C) in striatal slices of naive rats. LTD was blocked with the metabotropic glutamate 5 receptor (mGluR5) antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) (D) as well as with the mGluR1 antagonist (S)-(+)-alpha-Amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) (E) in naive rats. (B) The analysis of pCREB was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and is representative of 8 to 12 slices from 4 to 6 animals. Statistical analysis by 1-way ANOVA, $^{**}P < .01$ compared with control or as indicated by bar. (A,D-F) Data are expressed as normalized field potential amplitude to baseline and expressed as percentages. They are means ± SEM. Inset, representative field potential traces during baseline recording and 40 minutes after wash-out, horizontal bar indicates 5 milliseconds, vertical bar 50 µV. (F) Sixty-day withdrawal from chronic cocaine self-administration (CSA) abolished DHPG-LTD in the striatum.

(Deroche-Gamonet et al., 2004; Grueter et al., 2006; Kasanetz et al., 2010; Mihov and Hasler, 2016; Perry et al., 2016), it is still unclear whether a forced i.p. cocaine administration or a single CSA session best reflects the acute effects of cocaine. We believe CSA best reflects the motivation to administer drugs, as seen in humans, and the final dose self-administered is chosen by the animal and thus it would hardly be aversive. We therefore chose a single CSA session over forced i.p. delivery. There are, however, important limitations using this model that need to be taken into account. While rats in our study were allowed control of the cocaine dose received during the first session they expected to receive sucrose, not cocaine. To discriminate cocaine effects from sucrose expectation, we compared self-administration behavior in acute SSA and acute CSA rats. The high number of active lever presses in the SSA group indicates that rats undergo sucrose extinction (Knackstedt et al., 2010). However, acute SSA (or a sucrose extinction session) did not alter the capacity of $mGluR_{1/5}$ to elicit ERK1/2 or CREB phosphorylation. This indicates that $mGluR_{1/5}$ coupling to these 2 proteins is intact after an acute SSA.

Progressive Loss of $mGluR_{1/5}$ Function in the Dorsal Striatum during CSA and CSA Withdrawal

Cocaine administration is accompanied by a decreased expression of $mGluR_5$ in the striatum of rodents (Ghasemzadeh et al., 2009; Knackstedt et al., 2010; Pomierny-Chamiolo et al., 2015) and humans (Martinez et al., 2014; Milella et al., 2014) and has been shown to reduce $mGluR_5$ responses (Swanson et al., 2001; McCutcheon et al., 2011). Although repeated cocaine administration decreases dopamine D2 receptor expression in the ventral striatum, paradoxically they couple more efficiently to downstream cascades, leading to increased signal-transduction (Bailey et al., 2008; Hoffmann et al., 2012). Thus, to increase our understanding of how $mGluR_{1/5}$ functions in states of disease, such as cocaine addiction, it is important to address both receptor expression and isoforms as well as their capacity to signal and modulate synaptic plasticity. It has previously been shown that $mGluR_{1/5}$ promote ERK1/2 and CREB phosphorylation (Mao et al., 2008), 2 kinases associated with both acute and prolonged effects of cocaine. CREB activity affects learning and memory processes by regulating experience-based gene transcription amending to long-lasting neuro-adaptations (McClung and Nestler, 2008). In addition, it is believed that CREB activation in the dorsal striatum controls drug-associated memory (Fasano et al., 2009) as well as cocaine reinforcement (Hollander et al., 2010). The important role of the glutamatergic system in the progression to addiction-like behavior is supported by our data showing a lessened function of $mGluR_{1/5}$ signaling to ERK1/2 and CREB in the dorsal striatum after CSA. Interestingly, DHPG-induced CREB activation was more sensitive to CSA than ERK1/2 activation. Only chronic CSA, and its withdrawal, was able to abolish ERK1/2 phosphorylation, whereas a blunting of DHPG-induced CREB phosphorylation was seen as early as the first CSA, an effect that appeared more pronounced after chronic CSA and withdrawal. Our data suggest that acute CSA only affects DHPG-mediated CREB phosphorylation due to the ability of both $mGluR_1$ and $mGluR_5$ to regulate

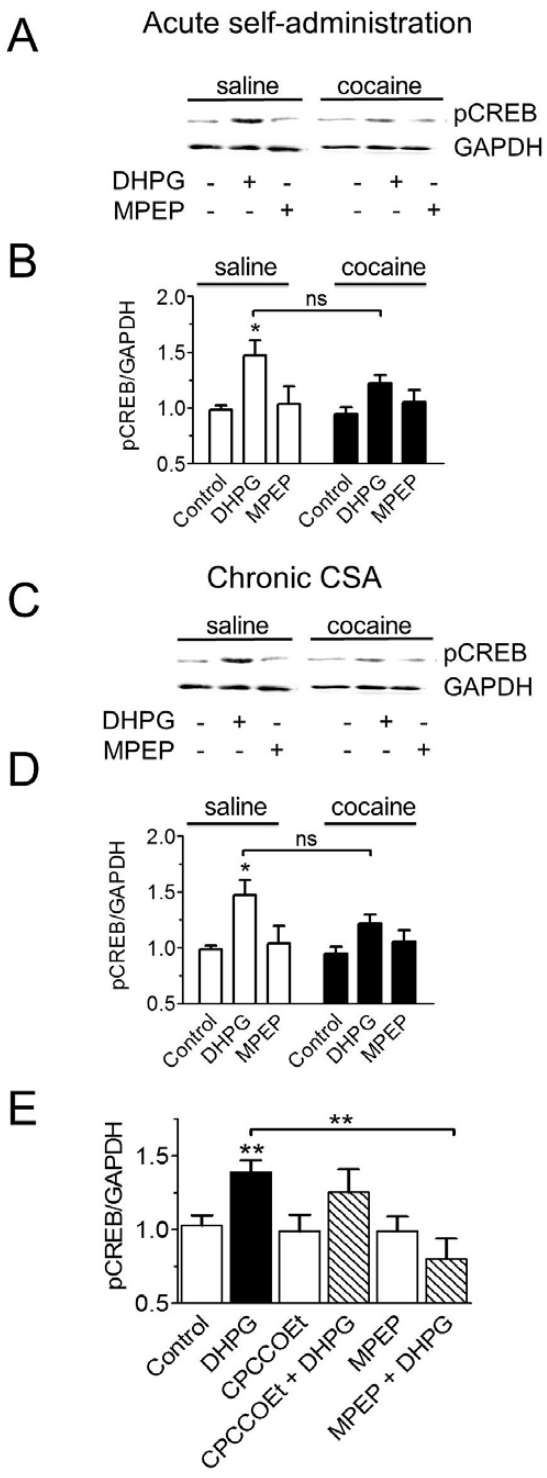

Figure 5. Loss of (*RS*)-3,5-Dihydroxyphenylglycine (DHPG)-mediated CREB phosphorylation (pCREB) with cocaine self-administration (CSA). Illustrative immunoblots (A,C) and histograms (B-E) of DHPG-induced pCREB in striatal slices from acute (A-B), chronic CSA (C-D), and naïve (E) rats. (E) Striatal metabotropic glutamate 5 receptor (mGluR5)-induced pCREB was blocked with the mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), but not the mGluR1 antagonist 7-(Hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester (CPCCOEt). Two-way ANOVA,*$P < .05$; *** $P < .001$ compared with control or as indicated by horizontal bar; n.s., nonsignificant ($P > .05$). n = 5 to 7.

ERK1/2 phosphorylation, whereas CREB phosphorylation solely relies on $mGluR_5$. The mechanisms of the blunted induction of ERK1/2 and CREB phosphorylation agree with the decrease of $mGluR_5$ observed by others in this structure (Ghasemzadeh et al., 2009; Knackstedt et al., 2010; Pomierny-Chamiolo et al., 2015). Taken together, this progressive loss of signaling through the $mGluR_5$ pathway after prolonged CSA and withdrawal suggest a critical role of $mGluR_5$ in cocaine addiction. As negative allosteric modulators are promising drugs for the treatment of cocaine addiction (Panlilio et al., 2005; Mihov and Hasler, 2016), it will be interesting to further the current study and determine on a molecular level how these drugs function in the addicted brain.

Prolonged Cocaine Withdrawal Abolishes DHPG-Mediated LTD and ERK1/2 Phosphorylation in the Dorsal Striatum

The expression of striatal LTD shapes goal-directed learning, and its loss correlates with the progression to addiction-like behavior (Moussawi et al., 2009; Kasanetz et al., 2010; Huang et al., 2011). Metabotropic $GluR_{1/5}$ are central in the modulation of striatal plasticity, which involves both these receptor subtypes, mutually with dopamine and endocannabinoid CB1 receptors (Sung et al., 2001; Mao et al., 2005; Chepkova et al., 2009). Repeated involuntary administration of cocaine leads to a progressive inhibition of DHPG-induced $mGluR_{1/5}$ activation and LTD in the core nucleus accumbens, lasting up to 28 days after cocaine administration (Huang et al., 2011), whereas 3 weeks of cocaine abstinence after short-term self-administration abolishes striatal LTD expression (Knackstedt et al., 2014). Both the loss of DHPG-LTD and a reduction of CREB activation could be permissive to cocaine addiction-like behavior (Martin et al., 2006; Fasano et al., 2009; Hollander et al., 2010; Kasanetz et al., 2010; Milella et al., 2014). Consistent with this, we found that the induction of DHPG-LTD in striatal slices was abolished after a 60-day CSA withdrawal. Repeated administration of cocaine has previously been associated with loss of LTD in various brain structures (Grueter et al., 2006; Backstrom and Hyytia, 2007; Moussawi et al., 2009; Kasanetz et al., 2010). Our study expands these findings by showing that in the striatum, both $mGluR_{1/5}$-LTD and $mGluR_{1/5}$-ERK1/2 phosphorylation were lost after chronic CSA withdrawal.

The blunting of DHPG-induced ERK1/2 phosphorylation most likely contributes to the loss of DHPG-LTD, given ERK1/2 is necessary for DHPG-LTD in the striatum. This finding is in agreement with the disappearance of DHPG-LTD in other brain regions after prolonged withdrawal from forced cocaine injections (Grueter et al., 2006; Huang et al., 2011). Interestingly, we found that prolonged withdrawal from chronic CSA specifically abolished DHPG-LTD in the striatum without affecting it in the CA1 region of the hippocampus in these same animals. We propose that the long-term loss of striatal DHPG-LTD is related to the long-term effects of cocaine addiction in the brain, which contributes to the formation of habits established during voluntary drug use. Remarkably, the loss of LTD after prolonged withdrawal of cocaine administration appears unrelated to the way of administration as the loss of DHPG-LTD occurred both after voluntary (current study) as well as involuntary (Huang et al., 2011) cocaine administration. We believe the loss of striatal DHPG-LTD to be responsible for facilitating the enduring memory of cocaine exposure for up to 60 days after the last CSA.

Possible Gain of Constitutive Activity of $mGluR_5$ Specifically in the Accumbens after Chronic CSA

The nucleus accumbens is required for both natural and drug-induced reward (Carlezon et al., 1998; Kalivas et al., 2005; Kalivas and Volkow, 2005). Phosphorylation of accumbal ERK1/2 and CREB participates in cocaine sensitization and reward and

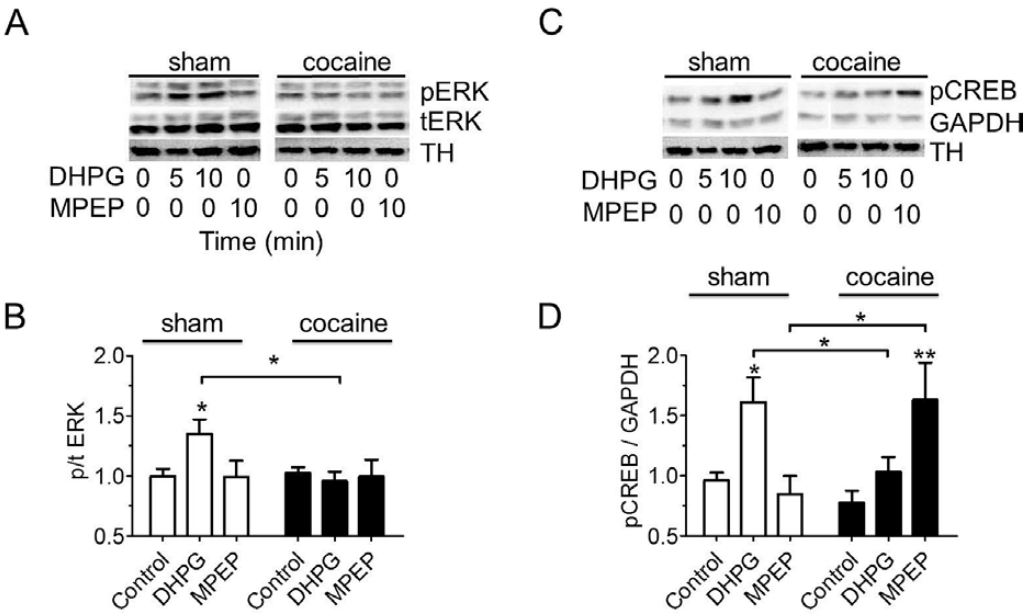

Figure 6. Chronic cocaine self-administration (CSA) shifted metabotropic glutamate 5 receptor (mGluR5) signaling in the nucleus accumbens. Representative immunoblots (A,C) and histograms (B,D) of DHPG-induced ERK1/2 and CREB phosphorylation (pCREB) in nucleus accumbens slices from sham (white) and chronic CSA rats (black). n = 6 to 10. Two-way ANOVA, $^{*}P < .05$; $^{**}P < .01$ compared with control or as indicated by bar.

contributes to motivation for cocaine (Carlezon et al., 1998; Valjent et al., 2000; Valjent et al., 2006; Kong et al., 2011; Larson et al., 2011). Interestingly, we found that CSA reduced agonist-induced $mGluR_5$ responses in the nucleus accumbens and that the $mGluR_5$ antagonist/inverse agonist MPEP specifically increased CREB and not ERK1/2 phosphorylation after CSA. MPEP has previously proven capable of revealing $mGluR_5$ constitutive activity (Ango et al., 2000; Muhlemann et al., 2005). It is thus possible the MPEP mediated increase in CREB phosphorylation in the nucleus accumbens might reflect a gain of constitutive activity of the $mGluR_5$ or increased glutamate levels in these slices after chronic CSA and thus represents a means for cocaine to facilitate cocaine-induced behavior and memory. We did not observe any changes in basal phosphorylation or a proper effect of MPEP in the striatum in any of our experimental groups, supporting the idea that $mGluR_5$ developed a constitutive activity after prolonged CSA. More research is warranted to understand how the switch from an agonist-activated receptor to a constitutive active receptor occurs and what the role is. In addition, alternative recruitment of signaling pathways linked to $mGluR_5$ function must be considered. Both the loss of LTD and the reduction of CREB activation are permissive to cocaine addiction-like behavior (Self et al., 1998; Martin et al., 2006; Fasano et al., 2009; Kasanetz et al., 2010); thus the increased inhibition of CREB through accumbal $mGluR_5$ would favor cocaine addiction behavior. One possibility is a change in the recruitment of scaffolding proteins associated with $mGluR_5$ such as Homer, Shank, and postsynaptic density protein-95, certain of which have been shown to be involved in cocaine-mediated behavior (Swanson et al., 2001; Wang et al., 2013; Bakshi et al., 2014; Martinez et al., 2014). The complexity of cocaine-induced changes to signaling transduction pathways and the validity of rodent models to develop new treatment strategies has recently been reviewed and highlights the importance of continuing our efforts in elucidating the molecular changes in signaling transduction in order to develop better drug targets for the treatment of cocaine addiction (Terbeck et al., 2015; Garcia-Pardo et al., 2016; Mihov and Hasler, 2016).

Supplementary Material

For supplementary material accompanying this paper, visit http://www.ijnp.oxfordjournals.org/

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Statement of Interest

None.

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