Modulation of Group II Metabotropic Glutamate Receptor Signaling by Chronic Cocaine

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ABSTRACT
Repeated cocaine produces enduring neuroadaptations in glutamate transmission in the nucleus accumbens that are thought to contribute to addiction. Group II metabotropic glutamate autoreceptors (mGluR2/3) regulate glutamate release, and this study investigates whether repeated cocaine injection produces long-lasting alterations in mGluR2/3 content, phosphorylation, and physiology. Rats were administered cocaine daily for 1 week, and 3 weeks after the last injection, mGluR2/3 protein levels were altered in the accumbens and prefrontal cortex (PFC) but not in the dorsal striatum or ventral tegmental area. The level of mGluR2/3 dimer was elevated in the accumbens and PFC and the monomer was reduced in the PFC only. Furthermore, the relative Ser phosphorylation state of the monomer was elevated in both the accumbens and PFC of cocaine-pretreated subjects, whereas the dimer demonstrated negligible phosphorylation in either treatment group. These changes in mGluR2/3 level and phosphorylation state were associated with reduced mGluR2/3 agonist-induced guanosine 5′-3-O -(thio)triphosphate binding in the accumbens and PFC, but not in the dorsal striatum. Stimulation of mGluR2/3 reduces extracellular glutamate by inhibiting Ca2+-dependent and cystine/glutamate antiporter-mediated glutamate release. The capacity of the mGluR2/3 agonist 2R,4R-4-aminoopyrrolidine-2,4-dicarboxylate (APDC) to inhibit [35S]cystine uptake via cystine/glutamate antiporter in accumbens tissue slices was reduced by repeated cocaine. Also, the capacity of APDC to reduce the basal and potassium-stimulated extrasynaptic glutamate was significantly blunted in the accumbens of cocaine-pretreated subjects. Together, these data demonstrate that repeated cocaine produces an enduring reduction in mGluR2/3 function in the nucleus accumbens.

Neuroplasticity induced by repeated exposure to cocaine involves excitatory amino acid transmission and is manifested as drug-induced paranoia and relapse to drug-taking (Wolf, 1998; Berke and Hyman, 2000; Ungless et al., 2001). The most well-established enduring cellular adaptations produced by repeated cocaine have been discovered in the nucleus accumbens, a region known to be important for drug reward and drug-induced relapse (Koob and LeMoal, 2001; Nestler, 2001). Included among the neuroadaptations produced by repeated cocaine are both pre- and postsynaptic changes in glutamate transmission (White and Kalivas, 1998; Wolf, 1998). Within the nucleus accumbens, repeated cocaine produces a general blunting of basal glutamate transmission reflected as a decrease in extrasynaptic glutamate concentrations (Pierce et al., 1996; Bell et al., 2000; Hotsenpiller et al., 2001) and decreased response to ionotropic and group I metabotropic glutamate receptor (mGluR) stimulation (Swanson et al., 2001; Thomas et al., 2001). However, in rats pretreated with repeated cocaine the release of glutamate evoked by a cocaine injection or an environmental stimulus associated with repeated cocaine is augmented (Pierce et al., 1996; Bell et al., 2000; Hotsenpiller et al., 2001). One possible mechanism mediating the increased releasability of glutamate is a decreased capacity of glutamate autoreceptors to regulate presynaptic glutamate release.

It is well known that group II mGluRs (mGluR2/3) function as glutamate autoreceptors to modulate presynaptic glutamate release (Conn and Pin, 1997; Anwyl, 1999; Cartmell and Schoepp, 2000). There is a moderate density of mGluR2/3 identified in the nucleus accumbens (Ohishi et al., 1993a,b; Testa et al., 1998), and stimulating mGluR2/3 inhibits the presynaptic and glial release of glutamate, in part, by inhibiting N-type calcium channels (Manzoni et al., 1997; Pagni et al., 2000; Xi et al., 2002). Group II mGluR agonists also
reduce the extrasynaptic concentration of glutamate by inhibiting the heteroexchange of extracellular cystine for intracellular glutamate through the cystine/glutamate antiporter (Baker et al., 2002; Xi et al., 2002). To evaluate a hypothesis that repeated cocaine administration produces enduring changes in mGluR2/3 autoreceptors, the protein level and phosphorylation state of mGluR2/3 in the nucleus accumbens was measured in rats pretreated 3 weeks earlier with a week of daily cocaine injections that is known to produce behavioral sensitization and changes in glutamate transmission (Pierce et al., 1996; Bell et al., 2000; Swanson et al., 2001). In addition, the effect of daily cocaine injections on the coupling of mGluR2/3 to G proteins and the regulation of [35S]cystine uptake through the cystine/glutamate antiporter was examined. Finally, the reduction in mGluR2/3 function that was identified in the ex vivo experiments outlined above was verified in vivo using microdialysis to assess the capacity of mGluR2/3 receptors to reduce basal and K+ -stimulated extracellular levels of glutamate.

Materials and Methods

Animal Housing and Surgery. All experiments were conducted according to specifications of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Harlan, Raleigh, NC), weighing between 250 and 300 g, were individually housed and maintained on a 12:12-h light/dark cycle (7:00 AM/7:00 PM) with free access to food and water. All experiments were conducted during the light period. Using ketamine (100 mg/kg) and xylazine (3 mg/kg) anesthesia, dialysis guide cannulae (20 gauge, 14 mm; Small Parts, Roanoke, VA) were implanted over the nucleus accumbens (+1.6 mm anterior to bregma, ±1.6 mm mediiodlateral, −4.7 mm ventral to the skull surface according to the atlas of Paxinos and Watson, 1986) using a 6° angle from vertical. The guide cannulae were fixed to the skull with four stainless steel skull screws (Small Parts) and dental acrylic.

Repeated Cocaine Treatment. Cocaine was donated by the National Institute on Drug Abuse (Bethesda, MD). One week after arrival in the animal facility, rats were treated with either cocaine (15 mg/kg i.p.) or the same volume (1.0 ml/kg i.p.) of saline (day 1) in the home cages of the animal room. On days 2 through 6, the rats received saline or 30 mg/kg cocaine, and on day 7, they received 15 mg/kg cocaine. Brain dissection or microdialysis was performed after 3 weeks withdrawal from the last saline or cocaine injection. This treatment regime has previously been shown to produce enduring behavioral sensitization and changes in extracellular glutamate levels (Pierce et al., 1996). In addition, examining 3 weeks of withdrawal potentially provides a better estimate of the enduring neuroadaptations mediating the long-lasting behavioral effects of cocaine (for reviews, see White and Kalivas, 1998; Wolf, 1998).

mGluR2/3 Immunoblotting. Three weeks after the last daily injection of saline or cocaine rats were decapitated, and the brains were rapidly removed and dissected into coronal sections on ice. The brain regions were dissected on an ice-cooled Plexiglas plate using a 15-gauge tissue punch, including the medial prefrontal cortex (PFC), parietal cortex, ventral tegmental area, dorsolateral striatum, and nucleus accumbens. Brain punches were immediately frozen on dry ice and stored at −80°C until homogenized for immunoblotting. The dissected brain punches were homogenized with a hand-held tissue grinder in homogenization medium (0.32 M sucrose, 2 mM EDTA, 1% SDS, 50 μM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, pH 7.2), subjected to low-speed centrifugation (2000 × g of extract overnight at 4°C with the addition of the specific antibody against mGluR2/3 (3 μg; Upstate Biotechnology), followed by 3-h incubation at 4°C with Protein A-Sepharose beads (3 mg in 100 μl of radioimmuno precipitation buffer containing 100 mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% SDS; 1% Triton X-100; 1% sodium deoxycholate; 1 μg/ml aprotinin; 1 μg/ml leupeptin; 1 μM pepstatin; and 1 mg/ml soybean trypsin inhibitors, 1 mM iodoacetamide, and 250 μM phenylmethylsulfonyl fluoride). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (8%), and immunoblot was performed using p-phospho)-Ser-specific monoclonal antibodies (1:1000; Chemicon International, Temecula, CA). Immunoblot of mGluR2/3 or p-Ser-specific antibodies from immunoprecipitated mGluR2/3 were quantified using computer-assisted densitometry (NIH Image 1.60).

[35S]GTPγS Binding Assay. Membrane proteins were prepared according to the method described by Schaffhauser et al. (2000). Three weeks after cocaine or saline pretreatment, the nucleus accumbens, PFC, and striatum were dissected (see above) and homogenized in 20 volumes of buffer containing 50 mM Tris-HCl, 3 mM MgCl2, and 1 mM EGTA, pH 7.4. The homogenate was centrifuged twice at 48,000 × g at 4°C for 10 min and resuspended in assay buffer (50 mM Tris-HCl, 3 mM MgCl2, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4). Proteins were assayed by using the DC protein assay (Bio-Rad) and then stored at −80°C for binding assay.

The [35S]GTPγS binding assay used was modified from the procedures described by Schaffhauser et al. (2000). Briefly, 1 ml of assay buffer was combined with 30 μg of proteins, 30 μg GDP, 1 U of adenosine deaminase, 0.1 nM [35S]GTPγS (0.1 μCi; Amersham Biosciences), and various concentrations of APDC (10−6–10−14 M). Basal binding was measured in the absence of agonist, and nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The reaction was then terminated by filtration under vacuum through GF/B glass fiber filters (Whatman, Maidstone, UK), followed by three washes with cold Tris-HCl buffer. After transfer of the filters into glass vials containing 10 ml of Ecolite scintillation fluid, the radioactivity was measured by liquid scintillation spectrophotometry. Data are presented as mean ± S.E.M. of at least three experiments, which were each performed in duplicate.

[35S]cystine Uptake. Rats were decapitated and the nucleus accumbens was rapidly dissected and cut into slices (350 × 350 × 350 μm) using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Gosham, Surrey, UK). The slices were then washed five times for 10 min at 37°C in oxygenated Krebs-Ringer phosphate
buffer (KRP; 118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5.0 mM HEPES, and 10 mM glucose, pH 7.4). The slices were incubated at 37°C in oxygenated KRP containing 1.0 mM [³⁵S]cystine (0.1 μCi) for 15 min. Cystine uptake can also occur via two other mechanisms, Xₐ₅G and γ-glutamyl transpeptidase (Knickelbein et al., 1997). To isolate cystine uptake to cystine glutamate antipporter, the Xₐ₅G inhibitor aspartate (1 mM) and the γ-glutamyl transpeptidase inhibitor avicin (1 mM) were added to the incubation buffer. Incubation was terminated by rapidly washing the tissue three times using ice-cold KRP. Slices were then solubilized using 1% SDS and the level of radioactivity was determined using a liquid scintillation counter. Radioactivity counts from known concentrations of [³⁵S]cystine were used to determine the concentration of [³⁵S]cystine in tissue slices. Protein content in the slices was measured using the Bradford assay. Cystine uptake in the presence of unlabeled 1 mM cystine was used to identify nonspecific labeling and was subtracted from all data.

In Vivo Microdialysis. The night before the experiment, concentric microdialysis probes (with 2 mm of active membrane) were inserted 3 mm beyond tips of the guide canulae into the nucleus accumbens. Dialysis buffer (5 mM KCl, 140 mM NaCl, 1.4 mM CaCl₂, 1.2 mM MgCl₂, 5.0 mM glucose, plus 0.2 mM phosphate-buffered saline to give a pH of 7.4) was advanced through the probe at a rate of 1.2 mM MgCl₂, 5.0 mM glucose, plus 0.2 mM phosphate-buffered saline to give a pH of 7.4) was advanced through the probe at a rate of 2 μl/min via syringe pump (Biosanalytical Systems, West Lafayette, IN). Beginning at 2 h after turning on the pump at 8:00 AM the next morning, baseline samples were collected at 10- or 20-min intervals for 100 min. After collecting the baseline samples various drugs were administered via reverse dialysis into the nucleus accumbens. The group II mGluR agonist (2R,4R)-aminopyrrolidine-2,4-dicarboxylate (APDC), and the antagonist LY341495 and (R,S)-1-amino-5-phosphonomindan-1-carboxylic acid (APICA) were purchased from Tocris Cookson (Ballwin, MO). Pertussis toxin (PTX) was purchased from Sigma-Aldrich. They were initially dissolved in 1 equivalent of NaOH (Sigma-Aldrich) and neutralized with 0.1 N HCl (Sigma-Aldrich) to a concentration of 10⁻² M. Working concentrations were then diluted with filtered buffers in the different experiments (see above). In some experiments 80 mM KCl was used to stimulate glutamate release and in these experiments NaCl was reduced proportionally to retain iso-osmolarity.

Quantiﬁcation of Glutamate. The concentration of glutamate in the dialysis samples was determined using high-performance liquid chromatography with fluorometric detection. The dialysis samples were collected into 10 μl of 0.05 M HCl containing 2 pmol of homoserine as an internal standard. The mobile phase consisted of 250 mM Na₂HPO₄, pH 6.04. A reversed-phase column (10 cm, 3 μm ODS; Bioanalytical Systems) was used to separate the amino acids, and precolumn derivatization of amino acids with o-phthalaldehyde was performed using a model 540 autosampler (ESA, Chelmsford, MA). Glutamate was detected by a fluorescence spectrophotometer (Linear Fluor LC 305; ESA) using an excitation wavelength of 336 nm and an emission wavelength of 420 nm. The area under curve of the glutamate and homoserine peaks was measured with the 501 Chromatography Data System (ESA). Glutamate values were normalized to the internal standard homoserine and compared with an external standard. Protein content was measured with the Coomassie blue method.

Statistical Analysis. The StatView statistics package was used to estimate statistical significance. A two-way ANOVA was used to determine the effect of individual drugs on [³⁵S]cystine uptake and [³⁵S]GTPγS binding. A two-way ANOVA with repeated measures over drug was used to evaluate the microdialysis data. Upon identification of statistical significance, post hoc comparisons were made with a Fisher’s protected least significant difference test. Immunoﬂuorescence blot data were analyzed using a two-tailed Student’s t test.

Results

Cocaine Alters the Amount and Phosphorylation of mGluR2/3. Figure 1 confirms previous observations that mGluR2/3 is present in brain tissue at two molecular weights most likely corresponding to monomer and dimer forms of the receptor (Testa et al., 1998; Schaffhauser et al., 2000; Xi et al., 2002). The two bands appear in the molecular mass positions of around 97 and 200 kDa in the expanded electrophoresis gel, and the appearance of both bands was competitively blocked by a synthesized peptide that had the same 21 amino acid sequence with the C terminals of mGluR2/3 (data not shown; Xi et al., 2002). Three weeks after completion of a week of daily cocaine injections the level of mGluR2/3 monomer was unaltered in the nucleus accumbens but was significantly reduced in the PFC. In both the nucleus accumbens and PFC the dimer form of the protein was significantly increased. In contrast, there was no effect by repeated cocaine on the content of either the monomer or dimer of mGluR2/3 in the dorsal striatum or ventral tegmental area (Table 1). Cocaine pretreatment also did not alter the level of mGluR2/3 dimer in parietal cortex, whereas the monomer was present in amounts too low to quantify. Comimmunoblotting of calnexin, an internal marker protein, did not show differences in protein loading in either the nucleus accumbens or the PFC experiments.

It was shown previously that PKA phosphorylates Ser residues of mGluR2/3 (Schaffhauser et al., 2000), and an enduring increase in PKA activity in the nucleus accumbens after repeated cocaine has been reported (Nestler, 2001). The Ser phosphorylation state of mGluR2/3 was examined in the accumbens and PFC. In neither brain region was the dimer phosphorylated sufficiently to be quantified (Fig. 1). In the accumbens the monomer form of mGluR2/3 was significantly more phosphorylated in the cocaine-pretreated subjects. The amount of phosphorylated monomer was not increased by cocaine in the PFC. However, because the total amount of mGluR2/3 monomer was significantly reduced by cocaine the ratio of phosphorylated to total monomer for each sample was calculated and found to be significantly augmented in cocaine-pretreated animals.

Repeated Cocaine Reduces mGluR2/3-Induced GTPγS Binding. Increased PKA-mediated Ser phosphorylation of mGluR2/3 has been shown in vitro to promote receptor desensitization (Macek et al., 1998; Schaffhauser et al., 2000; De Blasi et al., 2001). To determine whether increased phosphorylation after repeated cocaine administration had desensitized mGluR2/3, GTPγS binding in nucleus accumbens homogenates was examined. Similar to a previous report using hippocampal tissue (Schaffhauser et al., 2000), the mGluR2/3 agonist APDC induced a dose-dependent increase in GTPγS binding in the striatum, nucleus
Fig. 1. Chronic cocaine administration produces an enduring change in the expression and Ser phosphorylation state of mGluR2/3 in the nucleus accumbens and PFC. A, data from the nucleus accumbens (including the core and shell together to get sufficient proteins to run the immunoprecipitation experiment). Representative immunoblots of mGluR2/3 and phosphorylated mGluR2/3 from the same subjects are shown. Note that the putative dimer form of mGluR2/3 (molecular mass of ~200 kDa) is not appreciably phosphorylated. S5, S6, C9, and C12 refer to individual rats in chronic saline (S) or cocaine (C) treatment groups. The bar graph shows the mean ± S.E.M. percentage change from the saline group for the putative dimer, monomer, and phosphorylated monomer (p-monomer). B, data obtained from PFC tissues. P-M/M shows the ratio of p-monomer to monomer of mGluR2/3 in the same sample of chronic saline or cocaine treated rats. The number in each bar graph is the number of determinations in each group. *p < 0.05, comparing the saline and cocaine groups using a two-tailed Student’s t test.

TABLE 1

Effects of repeated cocaine treatment on the levels of brain mGluR2/3 proteins by immunoblotting

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>mGluR2/3</th>
<th>Saline Rats</th>
<th>Cocaine Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal cortex</td>
<td>Dimer</td>
<td>100 ± 22.6</td>
<td>166.7 ± 21.3*</td>
</tr>
<tr>
<td></td>
<td>Monomer</td>
<td>100 ± 5.7</td>
<td>55.9 ± 9.7*</td>
</tr>
<tr>
<td></td>
<td>Dimer + monomer</td>
<td>100 ± 12.5</td>
<td>125.6 ± 15.9</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>Dimer</td>
<td>99.99 ± 9.8</td>
<td>96.2 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>Monomer</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Striatum</td>
<td>Dimer</td>
<td>100 ± 11.1</td>
<td>113.7 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>Monomer</td>
<td>100 ± 8.9</td>
<td>101.0 ± 10.6</td>
</tr>
<tr>
<td></td>
<td>Dimer + monomer</td>
<td>100 ± 9.9</td>
<td>110.8 ± 7.9</td>
</tr>
<tr>
<td>Accumbens, shell</td>
<td>Dimer</td>
<td>100 ± 8.2</td>
<td>130.9 ± 6.7*</td>
</tr>
<tr>
<td></td>
<td>Monomer</td>
<td>100 ± 12.4</td>
<td>85.3 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>Dimer + monomer</td>
<td>100 ± 9.5</td>
<td>124.8 ± 5.6*</td>
</tr>
<tr>
<td>Accumbens, core</td>
<td>Dimer</td>
<td>100 ± 7.3</td>
<td>132.8 ± 10.1*</td>
</tr>
<tr>
<td></td>
<td>Monomer</td>
<td>100 ± 6.5</td>
<td>103.2 ± 14.5</td>
</tr>
<tr>
<td></td>
<td>Dimer + monomer</td>
<td>100 ± 5.8</td>
<td>121.3 ± 7.6*</td>
</tr>
<tr>
<td>VTA</td>
<td>Dimer</td>
<td>100 ± 14.8</td>
<td>126.4 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>Monomer</td>
<td>100 ± 13.5</td>
<td>108.4 ± 12.3</td>
</tr>
<tr>
<td></td>
<td>Dimer + monomer</td>
<td>100 ± 14.4</td>
<td>119.5 ± 6.2</td>
</tr>
</tbody>
</table>

N.D., not detected; VTA, ventral tegmental area.

* p < 0.05, compared with the saline control group with two-tailed Student’s t test.

acumbens, and PFC (Fig. 2, A, C, and D). The increased GTPγS binding by APDC in the nucleus accumbens was antagonized by pretreatment with the mGluR2/3 antagonists APICA and LY341495. Neither antagonist alone altered the basal GTPγS binding (−9 ± 3.4% for 300 nM LY341495; −10 ± 4% for 100 μM APICA). Ribosylation and inactivation of Gαβγ by pretreatment with PTX also prevented APDC-induced increase in GTPγS binding (Fig. 2B), whereas PTX alone did not alter the basal GTPγS binding significantly (data not shown). In the nucleus accumbens of the cocaine-treated group, the capacity of APDC to increase GTPγS binding was significantly blunted compared with the saline group (Fig. 2A). Similarly, the APDC-induced increase in GTPγS in the PFC was blunted in the cocaine pretreatment group (Fig. 2C). In contrast, no significant difference was observed in striatal homogenates between saline- and cocaine-pretreated animals (Fig. 2D). No significant difference in the basal [35S]GTPγS binding was observed in either the nucleus accumbens (7.59 ± 0.58 versus 8.19 ± 0.48), the PFC (8.8 ± 0.59 versus 9.49 ± 0.72), or the dorsal striatum (8.24 ± 0.75 versus 9.27 ± 0.6 fmol/μg of protein) between the saline- and cocaine-treated rats, respectively.

Effect of mGluR2/3 Agonist on Cystine-Glutamate Antiporter Is Blunted after Repeated Cocaine. Previous studies have shown that mGluR2/3-induced reduction of glutamate release in the accumbens involves the inhibition of both Ca2+-dependent vesicular and cystine-glutamate antiporter-mediated nonvesicular release of glutamate (Manzoni et al., 1997; Baker et al., 2002; Xi et al., 2002). Figure 3 shows that the capacity of APDC to reduce [35S]cysteine uptake through the cystine/glutamate antiporter in tissue slices from the nucleus accumbens of cocaine-pretreated rats is decreased compared with the saline-treated group. Although
10 μM APDC significantly reduced [35S]cystine uptake in saline-pretreated subjects, 100 μM APDC was required in cocaine-pretreated animals. No difference in basal uptake was observed between the two groups (17.43 ± 2.29 fmol/μg of proteins in saline rats versus 18.93 ± 3.57 fmol/μg of proteins in cocaine-treated rats).

**Effect of mGluR2/3 Agonist on Extracellular Glutamate Is Blunted after Repeated Cocaine.** Experiments were conducted to determine whether the enduring decrease in mGluR2/3 coupling to G proteins induced by repeated cocaine had functional consequences in vivo on the capacity of mGluR2/3 to inhibit basal or high K+ stimulated vesicular glutamate release. Figure 4 shows that the capacity of the mGluR2/3 agonist APDC to reduce the basal levels of extracellular glutamate in the nucleus accumbens was blunted 3 weeks after discontinuing repeated cocaine administration. Figure 4A shows the data expressed as picomoles per sample, whereas Fig. 4B shows the data normalized to percentage of change from the basal levels. The minimum effective dose for a statistically significant APDC-induced decrease in extracellular glutamate was at least 10-fold higher in the cocaine compared with the saline treatment group.

Because the basal level of extracellular glutamate was lower in the cocaine-pretreated subjects (Fig. 4A; Pierce et al., 1996; Bell et al., 2000), the decreased capacity of mGluR2/3 to reduce the basal levels of extracellular glutamate is blunted after repeated cocaine.
Cocaine Blunts Group II mGluRs

Fig. 4. Repeated cocaine treatment blunted the capacity of APDC to reduce the extracellular concentration of glutamate in the nucleus accumbens. A, mGluR2/3 agonist APDC decreased extracellular glutamate in rats 3 weeks after discontinuing from 7 days of saline control treatment. Increasing doses of APDC were added to the dialysis buffer every hour. A two-way ANOVA with repeated measures over time revealed a significant difference over the dose (time course) [F(12,156) = 12.98, p < 0.05] and a treatment × time interaction between two groups of rats [F(12,156) = 4.36; p < 0.05]. B, data from A were normalized to percentage of change from baseline. A two-way ANOVA with repeated measures over dose revealed a significant treatment (saline versus cocaine) × time (APDC doses) interaction [F(4,52) = 3.13, p < 0.05]. C, repeated cocaine treatment decreased the capacity of APDC to inhibit K+-evoked glutamate release in the nucleus accumbens. Dialysis samples were collected every 10 min. After obtaining baseline samples each animal received in sequence for 30 min each a dose of APDC (0, 0.5, 5, or 50 μM), followed by that dose of APDC plus K+ (80 mM) followed by a return to normal dialysis buffer for 60 min. Each rat received two doses of APDC randomly with 1 h of time intervals. The data are shown as normalized to the percentage of change from the average of three 10-min baseline samples obtained before introducing APDC. The line illustrates the value of the normalized baseline (e.g., 100%). Each column represents the average percentage of change under each condition. Each dose of APDC was evaluated normalized baseline (e.g., 100%). Each column represents the average per-

mate may not accurately reveal the functional state of mGluR2/3. Rather, the blunted decrease may reflect that the levels of glutamate in the accumbens are already reduced to near minimum in the cocaine treatment group. Figure 4C demonstrates that the capacity of APDC to inhibit the K+-stimulated increase in vesicular glutamate release was also significantly reduced in rats pretreated with daily cocaine injections. After obtaining baseline levels of glutamate, a dose of APDC (0, 0.5, 5.0, or 50 μM) was perfused through a dialysis probe in the nucleus accumbens, and 30 min later the buffer was switched to high K+ buffer (80 mM) plus the same concentration of APDC for 30 min. The buffer was then switched back to normal dialysis buffer for 60 min. Figure 4C shows the data normalized to the average of three baseline samples obtained before introducing APDC. Similar to the study on [35S]cystine uptake, the capacity of APDC to inhibit K+-induced release of glutamate in vivo was reduced by about 10-fold in the cocaine treatment group. This is seen most clearly at 5 μM APDC, which blocked the K+-induced rise in extracellular glutamate in the saline group, but was without effect in the cocaine group.

Discussion

The present study reveals that repeated exposure to cocaine produces marked alterations in the content, phosphorylation state, and physiological function of mGluR2/3 autoreceptors. The focus of the study was in the nucleus accumbens where glutamate transmission is known to be an important effector of cocaine-induced behavioral neuroadaptations, such as sensitization and drug- or cue-primed relapse (Wolf, 1998; Cornish et al., 1999; Bell et al., 2000; Di Ciano and Everitt, 2001; Hotsenpiller et al., 2001). Taken together, the changes in protein content, phosphorylation state, and GTPγS binding suggest that repeated cocaine is reducing the efficiency of mGluR2/3 signaling. This is supported by the reduced capacity of mGluR2/3 stimulation to inhibit both the cystine/glutamate antiporter and the in vivo release of glutamate by potassium. The functional down-regulation of mGluR2/3 observed in the present study could be associated with the increased release of glutamate in response to a cocaine challenge injection or a cocaine-paired cue during cocaine withdrawal (Pierce et al., 1996; Reid and Berger, 1996; Bell et al., 2000; Hotsenpiller et al., 2001). However, it is important to note that mGluR2/3 receptors are located both pre- and postsynaptic, as well as on glia, and none of the assays in this report can distinguish between these receptor populations (Petraila et al., 1996).

Protein Phosphorylation and G Protein Coupling.

Immunoblotting revealed that the level and/or the phosphorylation state of mGluR2/3 was altered in brain regions known to be critical for the expression of cocaine-induced behavioral plasticity, including the nucleus accumbens and PFC (Berke and Hyman, 2000; Nestler, 2001). mGluR2/3 contains a consensus phosphorylation sequence for a number of Ser kinases, including cAMP-dependent and calcium-dependent protein kinases (PKA and PKC, respectively), as well as calcium-calmodulin kinase (calcium-calmodulin kinase II is most common in brain). Both PKA and PKC phosphorylation of group II mGluRs in vitro results in the inhibition of mGluR2/3-signalized events, such as reduced excitatory synaptic transmission, inhibition of voltage-dependent Ca2+-
channels, and blunted mGluR2/3 agonist-induced GTPγS binding (Tyler and Lovingier, 1995; Schaffhauser et al., 2000; De Blasi et al., 2001). There is evidence for an enduring up-regulation of PKA and calcium-calmodulin kinase II in the nucleus accumbens of animals pretreated with daily injections of cocaine or amphetamine (for reviews, see Gnegy, 2000; Nestler, 2001), whereas PKC is apparently unchanged (Steketee et al., 1998), posing the former two kinases as potential mediators of the increased Ser phosphorylation of mGluR2/3.

Consistent with the cocaine-induced increases in Ser phosphorylation reducing mGluR2/3 signaling, repeated cocaine treatment decreased the capacity of APDC to stimulate GTPγS binding in the accumbens and PFC. Altered mGluR2/3 protein content could also directly contribute to the reduced GTPγS binding in cocaine-pretreated animals. Supporting this possibility, there was anatomic concordance between cocaine-induced increases in dimer protein content and blunted GTPγS binding. Thus, the stimulation of GTPγS binding by mGluR2/3 agonist was reduced and the putative mGluR2/3 dimer was increased in the accumbens and PFC, but neither neuroadaptation was present in the striatum. Also, the increase in dimer content in the PFC produced by repeated cocaine administration was accompanied by reduced monomer content, perhaps indicating a shift from active monomer to a relatively inactive dimer form of mGluR2/3. Although the inverse relationship between dimer content and GTPγS binding may indicate that the dimer is a relatively inactive form of the receptor, the function of mGluR2/3 dimers has never been evaluated in vitro. Moreover, group I mGlur dimerization is thought to stabilize the active state, thereby increasing agonist-stimulated signaling (for review, see De Blasi et al., 2001). Also, little or no Ser phosphorylation of the mGluR2/3 dimer could be demonstrated (Fig. 1B), suggesting that the dimer is not being endogenously modulated by Ser kinases. Because the dimer can be phosphorylated in vitro by PKA (Schaffhauser et al., 2000), this observation may be consistent with the dimer being a more active form of the receptor because Ser phosphorylation reduces mGluR2/3 signaling.

Other cocaine-induced neuroadaptations could also contribute to the decrease in GTPγS binding by APDC. For example, mGluR2/3 couples to Gia proteins (Conn and Pin, 1997), and the level of Gia is reduced in the nucleus accumbens at 3 weeks after discontinuing daily cocaine injections (Striplin and Kalivas, 1993). Also, it was recently shown that repeated cocaine produces an enduring increase in AGS-3 (activator of G protein signaling, isoform 3) in the nucleus accumbens and PFC (Bowers et al., 2001), and an increase in AGS-3 has been shown in vitro to decrease GTPγS binding by sequestering the inactive (GDP-bound) form of Gia (Bernard et al., 2001). Thus, in addition to changes in mGluR2/3 content and phosphorylation, repeated cocaine produces more general changes in Gia-receptor coupling by reducing Gia content and increasing the selective Gia binding protein AGS-3. Indeed, both of these latter neuroadaptations may contribute to the recent report that repeated psychostimulants reduce GABAA-mediated GTPγS binding (Zhang et al., 2000).

Decreased Function of mGluR2/3 and Glutamate Transmission in Nucleus Accumbens. Consistent with the increased Ser phosphorylation state of mGluR2/3 and the reduction in G protein coupling, repeated cocaine administration decreased the capacity of mGluR2/3 stimulation to affect glutamate transmission. Thus, the ability of APDC to inhibit K+-stimulated levels of extracellular glutamate was markedly reduced by pretreatment with repeated cocaine. This finding is consistent with a recent electrophysiological study in the amygdala showing desensitization of mGluR2/3 after repeated cocaine administration (Neugebauer et al., 2000). The blunted regulation of extracellular glutamate by mGluR2/3 after repeated cocaine administration may contribute to some of the previously observed changes in extracellular glutamate levels associated with repeated cocaine administration. For example, the repeated administration of cocaine has been shown to potentiate the capacity of a subsequent cocaine injection to increase extracellular glutamate (Pierce et al., 1996; Reid and Berger, 1996), especially when the cocaine injection is associated with environmental cues (Bell et al., 2000). Similarly, it was recently shown that a cocaine-associated cue alone enhances extracellular glutamate levels in the nucleus accumbens (Hotsenpiller et al., 2001). The decrease in mGluR2/3 presynaptic autoreceptor tone after repeated cocaine administration could contribute to the increased releasability of glutamate. Because glutamate release in the nucleus accumbens has been shown to be an important trigger in the expression of behavioral sensitization and cocaine-primed reinstatement of drug-seeking behavior (Pierce et al., 1996; Cornish and Kalivas, 2000; Di Ciano and Everitt, 2001), the decreased inhibitory tone by mGluR2/3 may be important in the expression of these addiction-related behaviors. In addition, functional desensitization of Gi-coupled autoreceptors, including groups II and III mGluRs, dopamine D2, and GABAA autoreceptors (Wolf, 1998; Neugebauer et al., 2000), may contribute to the up-regulation of the intracellular cAMP-PKA cascade by a disinhibition mechanism.

The capacity of mGluR2/3 agonist to inhibit [35S]cystine uptake through the cystine/glutamate antiporter was also blunted in the nucleus accumbens of cocaine-pretreated subjects. The cystine/glutamate antiporter is a heteromer found in all cells that exchanges extracellular cystine for intracellular glutamate in a 1:1 stoichiometry at a rate dependent on substrate concentration gradients (Sato et al., 1999; Warr et al., 1999). Recently, glutamate derived from cystine/glutamate antiporter was shown to be the primary contributor to the in vivo levels of extracellular glutamate and, similar to the presynaptic release of glutamate, the cystine/glutamate antiporter is negatively coupled to mGluR2/3 stimulation (Baker et al., 2002).

Although potentially contributing to the increased releasability of glutamate, the finding that the capacity of mGluR2/3 receptor agonist to inhibit cystine/glutamate antiporter was blunted in the accumbens of animals treated with daily cocaine injections is not consistent with the observation that the basal extracellular levels of glutamate are reduced (Pierce et al., 1996; Bell et al., 2000; Hotsenspiller et al., 2001; Fig. 4A). Thus, decreased mGluR2/3 regulation of the cystine/glutamate antiporter would be expected to elevate extrasynaptic levels of glutamate. However, countermanding the reduced mGluR2/3 regulation of the cystine/glutamate antiporter after repeated cocaine administration it was recently found that there is a cocaine-induced down-regulation of the antiporter itself, which may underlie the reduced basal levels of glutamate.
glutamate (Baker et al., 2002). Thus, reduced mGluR2/3 inhibitory regulation of the cystine/glutamate antiporter may contribute to the slow rise in extracellular glutamate observed in response to a cocaine or amphetamine challenge in animals previously treated with daily drug injections (Pierce et al., 1996; Xue et al., 1996; Bell et al., 2000).

**Technical Considerations.** It is interesting that the minimum effective dose of APDC required to produce a significant effect in the GTPγS binding assay (0.1 µM) was approximately 2 orders of magnitude lower than for either the [35S]cystine uptake or K⁺-stimulated glutamate release assays (10 and 5 µM, respectively). This likely reflects the fact that changes in the latter two assays required changes in Gl signaling, presumably inhibition of PKA, whereas increased GTPγS binding is a direct effect of receptor occupancy. Thus, it may require greater receptor occupancy by APDC to manipulate intracellular signaling to the extent that changes in exchanger function and receptor occupancy by APDC to manipulate intracellular signaling may arise from an alteration in the regulatory state of G-proteins. The decreased response to mGluR2/3 stimulation may arise from a change in the relative proportions of receptor dimer and monomer and/or an increase in the relative Ser phosphorylation state of the monomer in cocaine-pretreated subjects. The reduced functional capacity of mGluR2/3 receptors may underlie some of the previously reported changes in glutamate transmission that occur after repeated cocaine administration and could also be involved in neuroadaptations associated with addiction, such as sensitization and craving.

**Conclusions**

The capacity of mGluR2/3 receptors in the nucleus accumens to couple to G proteins and to regulate extracellular glutamate levels undergoes an enduring reduction in animals pretreated with daily cocaine injections. The decreased response to mGluR2/3 stimulation may arise from an alteration in the relative proportions of receptor dimer and monomer and/or an increase in the relative Ser phosphorylation state of the monomer in cocaine-pretreated subjects. The reduced functional capacity of mGluR2/3 receptors may underlie some of the previously reported changes in glutamate transmission that occur after repeated cocaine administration and could also be involved in neuroadaptations associated with addiction, such as sensitization and craving.

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