

GABA Transmission in the Nucleus Accumbens Is Altered after Withdrawal from Repeated Cocaine

Zheng-Xiong Xi, Sammanda Ramamoorthy, Hui Shen, Russell Lake, Devadoss J. Samuvel, and Peter W. Kalivas

Department of Physiology and Neuroscience, Medical University of South Carolina, Charleston, South Carolina 29425

Repeated cocaine causes enduring changes in dopamine and glutamate transmission in the nucleus accumbens, and dopamine and glutamate terminals synapse on GABAergic accumbens neurons. The present study demonstrates that there are changes in GABA transmission in the accumbens at 3 weeks after discontinuing daily cocaine injections. No-net flux microdialysis revealed a significant increase in the basal levels of extracellular GABA in the accumbens of cocaine-treated rats. The elevated extracellular GABA was normalized by blocking voltage-dependent Na^+ channels and provided increased tone on GABA_B presynaptic autoreceptors and heteroreceptors because blocking GABA_B receptors produced a greater elevation in extracellular GABA, dopamine, and glutamate in cocaine-treated compared with control subjects. For many G-protein-coupled receptors, increased agonist can cause receptor desensitization. Consistent with GABA_B receptor desensitization, baclofen-stimulated GTP γ S binding was reduced, and the reduction in G-protein coupling was accompanied by reduced Ser phosphorylation of the GABA_{B2} receptor subunit. No effect by repeated cocaine was found in the levels of total GABA_{B1} or GABA_{B2} protein. Together, these data demonstrate that withdrawal from repeated cocaine treatment produces an increase in the basal levels of extracellular GABA in the accumbens that depends on neuronal activity. The increase may be mediated in part by functional desensitization of GABA_B receptors, likely the result of diminished Ser phosphorylation of the GABA_{B2} receptor.

Key words: GABA; cocaine; immunoblot; phosphorylation; microdialysis; glutamate

Introduction

Research during the past decade shows that repeated cocaine administration causes a number of alterations in dopamine and glutamate transmission in the nucleus accumbens that may be linked to addiction (Vanderschuren and Kalivas, 2000; Nestler, 2001). These studies are in accord with experiments that point to the accumbens as a critical substrate for both drug reward and the expression of behaviors indicative of cocaine addiction (Everitt and Wolf, 2002). Dopamine and glutamate terminals synapse on GABAergic spiny cells in the nucleus accumbens (Sesack and Pickel, 1990), and spiny cell axons collateralize to provide GABAergic innervation of near adjacent spiny neurons (Pennartz et al., 1994). In addition, there are dense GABAergic afferents to the accumbens, as well as a small population of GABAergic interneurons (Brog et al., 1993; Pennartz et al., 1994). Despite the central physiological role played by GABA in the accumbens, compared with glutamate and dopamine, there is relatively less information on the capacity of repeated cocaine to produce neuroadaptations in accumbens GABA transmission, especially at late withdrawal times when many addiction-related behaviors, such as sensitization, craving, and paranoia continue to be expressed. Understanding long-lasting interactions between repeated cocaine and GABA transmission is especially important, because the cocaine-induced changes in gene expression in GABAergic spiny cells are mediated by dopamine and glutamate and are hypothesized to be primary adaptive events in the development and expression of addiction (Nestler, 2001). Also, there is evidence for recip-

cal presynaptic modulation between GABA, dopamine, and glutamate in the accumbens (Harsing and Zigmond, 1997; Schoffelmeier et al., 2000), posing a role for altered GABA transmission in mediating the cocaine-induced adaptations in dopamine and glutamate transmission (Everitt and Wolf, 2002).

The present study evaluated the possibility that repeated cocaine combined with 3 weeks of withdrawal alters *in vivo* presynaptic GABA transmission in the accumbens. Microdialysis was used to demonstrate that the basal levels of extracellular GABA are elevated by repeated cocaine. This observation generated two additional hypotheses. (1) The elevated basal GABA levels result from a decreased capacity of GABA_B autoreceptors to regulate GABA release. Previous studies supporting this possibility include repeated amphetamine treatment decreases G-protein coupling of GABA_B receptors in the accumbens (Zhang et al., 2000) and repeated cocaine decreases the electrophysiological impact of GABA_B receptor stimulation in the lateral septum (Shoji et al., 1997). (2) Elevated extracellular GABA provides increased tone on presynaptic GABA_B heteroreceptors that decreases the basal level of glutamate. Previous studies have shown a decrease in basal extracellular levels of glutamate in the accumbens after repeated cocaine (Pierce et al., 1996; Hotsenpiller et al., 2001). Also, repeated amphetamine treatment enhances tonic modulation of GABA_B receptors regulating dopamine and glutamate release in the ventral tegmental area (Giorgetti et al., 2002). To evaluate these hypotheses, the level of GABA_B receptor protein and phosphoprotein was measured, as was the efficiency of G-protein coupling. Also, *in vivo* microdialysis was used to examine the capacity of GABA_B receptors to regulate the extracellular levels of glutamate and dopamine.

Materials and Methods

Animals housing and surgery. All experiments were conducted according to specifications of the National Institute of Health guide for the Care and

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Correspondence should be addressed to Dr. Peter Kalivas, Department of Physiology and Neuroscience, Medical University of South Carolina, 173 Ashley Avenue, Suite B5B 403, Charleston, SC 29425. E-mail: kalivas@musc.edu.
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Use of Laboratory Animals. Male Sprague Dawley (Raleigh, NC) rats, weighing between 250 and 300 gm, were individually housed and maintained on a 12 hr light/dark cycle (7:00 A.M., 7:00 P.M.) with access to food and water *ad libitum*. All experimentation was conducted during the light period. Using ketamine (100 mg/kg) and xylazine (3 mg/kg) anesthesia, dialysis guide cannulas (20 gauge, 14 mm; Small Parts, Roanoke, VA) were implanted over the nucleus accumbens [+1.6 mm anterior to bregma, \pm 1.6 mm mediolateral, and -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 cannulas 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. 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). On days 2–6 the rats received saline or 30 mg/kg cocaine and, on day 7, received 15 mg/kg cocaine. Brain dissection or microdialysis was performed after 3 weeks withdrawal from the last saline or cocaine injection. This treatment regimen has been shown previously 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 neuroadaptations mediating the long-lasting behavioral effects of cocaine (for review, see White and Kalivas, 1998; Wolf, 1998).

Microdialysis. Dialysis probes were constructed as described by Robinson and Whishaw (1988) with some modifications (including using smaller silica tubing). The active region of the dialysis membrane is 0.8–1.5 mm in length and \sim 175 μ m in diameter. Probes were inserted into the accumbens at least 12 hr before the experiment to minimize the effects of damage-induced dopamine and glutamate release during the experiment. On the day of the experiment, dialysis buffer (5 mM glucose, 2.5 mM KCl, 140 mM NaCl, 1.4 mM CaCl₂, 1.2 mM MgCl₂, and 0.15% PBS, pH 7.4) was perfused through the probe (2.0 μ l/min) for at least 2 hr before sample collection. Dialysis samples were collected every 20 min into 10 μ l of dialysis buffer (for glutamate and GABA) or mobile phase (for dopamine) containing an internal standard.

The GABA_B agonist *R*(+)-baclofen and the GABA_B antagonist 2-OH-saclofen were purchased from Tocris Cookson (Ballwin, MO). GABA and tetrodotoxin (TTX) were purchased from Sigma (St. Louis, MO). Both baclofen and 2-OH-saclofen were initially dissolved in 1 equivalent NaOH (Sigma) and neutralized with 0.1N HCl (Sigma) to a concentration of 10^{-2} M. Other drugs are directly dissolved in filtered dialysis buffer. Working concentrations were then made by diluting with filtered buffer.

Quantification of GABA. The concentration of GABA in each sample was determined using a modified "Fast Analysis of GABA" provided by ESA (Bedford, MA). The dialysis samples were collected into 10 μ l of dialysis buffer (plus 40 μ l of sample in 20 min) containing DL- α -amino-*n*-butyric acid (DL-ABA) as the internal standard for GABA. Methanol (15%) mobile phase containing 100 mM NaH₂PO₄, pH 4.6, and a reversed-phase column (4.6 \times 80, 3 μ m; model HR-80; Bioanalytical Systems, West Lafayette, IN) were used to separate the amino acids. A coulometric electrochemical detection system using three electrodes [preinjection guard electrode, +0.7 V; reduction electrode 1 (E1), 0.4 V; reduction electrode 2 (E2), 0.65 V] was used for quantification. Area under the curve of the GABA and DL-ABA peaks was measured with an ESA 501 Chromatography Data System. GABA values were normalized to the internal standard DL-ABA and compared with an external standard curve for quantification.

Quantification of glutamate. Glutamate in the dialysis sample was measured using an HPLC system with fluorescent detector. The dialysis samples were collected into 10 μ l of 0.05 M HCl containing 2 pmol of homoserine as an internal standard. The mobile phase consisted of 13% acetonitrile (v/v), 100 mM NaH₂PO₄, and 0.1 mM EDTA, pH 6.0. A reversed-phase column (10 cm, 3 μ m C-18 reversed phase; Bioanalytical Systems) was used to separate the amino acids, and precolumn derivatization of amino acids with *o*-phthalaldehyde was performed using an ESA model 540 autosampler. Glutamate was detected by a fluorescence spectrophotometer (LINEAR FLOUR LC 305; ESA) using an excitation wavelength of 336 nm and an emission wavelength of 420 nm. Glutamate

content in each sample was quantified with the ESA 501 Chromatography Data System.

Quantification of dopamine. For the measurement of extracellular dopamine, samples were collected into 10 μ l of mobile phase (4.76 mM citric acid, 150 mM NaH₂PO₄, 50 μ M EDTA, 3 mM SDS, 10% methanol (v/v), and 15% acetonitrile (v/v), pH 5.6, plus 2.0 pmol of dihydroxybenzylamine as an internal standard), and all samples were frozen at -80° C. The samples were subsequently thawed and placed in an ESA model 540 autosampler connected to an HPLC system with electrochemical detection. Dopamine was separated using a 10 cm C18 reversed-phase column (Bioanalytical Systems) and oxidized–reduced using coulometric detection (ESA). Three electrodes were used: a preinjection port guard cell (+0.4 V) to oxidize the mobile phase, an oxidation analytical electrode (E1, -0.1 V), and a reduction analytical electrode (E2, +0.2 V). The area under curve of the dopamine peak was measured with ESA 501 Chromatography Data System. Dopamine values were normalized to the internal standard dihydroxybenzylamine and compared with an external standard curve for quantification.

[³⁵S]GTP γ S binding assay. Membrane proteins were prepared according to the method described by Sim et al. (1996). Three weeks after cocaine or saline pretreatment, the nucleus accumbens (core and shell) was removed and homogenized in 20 vol 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 \times *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 Bio-Rad (Hercules, CA) DC protein assay and then stored at -80° C for binding assay.

The [³⁵S]GTP γ S binding assay was modified from the procedures described by Schaffhauser et al. (2000). Briefly, 12 \times 75 mm polystyrene test tubes had 1 ml of assay buffer containing 30 μ g of proteins, 30 μ M GDP, 1 U of adenosine deaminase, 0.1 nM [³⁵S]GTP γ S (Amersham Biosciences, Arlington Heights, IL), and various concentrations of baclofen (10^{-8} to 10^{-4} M). Basal binding was measured in the absence of agonist, and nonspecific binding was measured in the presence of 10 μ M unlabeled GTP γ S. The reaction was then terminated by filtration under vacuum through Whatman (Maidstone, UK) GF/B glass fiber filters, 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 represented as mean \pm SEM of three experiments, each performed in duplicate.

GABA_B 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 nucleus accumbens (containing both core and shell) was dissected on an ice-cooled Plexiglas plate using a 15 gauge tissue punch. 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 a buffer containing 100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ M pepstatin, 1 mg/ml soybean trypsin inhibitors, 1 mM iodoacetamide, 250 μ M PMSF, sodium fluoride, sodium pyrophosphate, sodium orthovanadate, and okadaic acid. Insoluble materials were removed from lysates by centrifugation at 22,000 \times *g* for 20 min at 4°C. Protein determinations were performed using the Bio-Rad DC protein assay according to the instructions of the manufacturer. Samples (30 μ g) were subjected to SDS-PAGE (8%) using a mini-gel apparatus (Bio-Rad), transferred via semidry apparatus (Bio-Rad) to nitrocellulose membrane, and probed for the proteins of interest (one gel per protein per brain region). GABA_B receptors were identified using a rabbit anti-rat antibody against GABA_{B1} (1:2000) or guinea pig anti-rat antibody against GABA_{B2} receptor (1:2000) purchased from Chemicon (Temecula, CA) that was made against a peptide containing the C terminus of GABA_{B1} or GABA_{B2} subunit. In control experiments, a synthesized peptide having the same 21 amino acid sequence on the C-terminal of GABA_{B1} was used to competitively inhibit the binding of antibody to GABA_{B1}. Skeletal muscle extract was used as a negative control for GABA_{B2} receptor-specific detection. Labeled proteins were detected using an HRP-conjugated anti-rabbit

secondary IgG diluted 1:30,000 (Upstate Biotechnology, Lake Placid, NY) or goat anti-guinea pig secondary IgG diluted 1:10,000 (Jackson ImmunoResearch, West Grove, PA) and visualized with enhanced chemiluminescence (Amersham Biosciences). Assurance of even transfer and equal amount of protein loading was evaluated with Ponceau S (Sigma), followed by destaining with de-ionized water, and the blots were reprobed with anti-calnexin (BD Transduction Laboratories, Lexington, KY). Blots were stripped (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β -mercaptoethanol) for 30 min at 50°C, washed with PBS twice for 10 min, reblocked in 5% dry milk for 1 hr, and probed with anti-calnexin (1:2000; BD Transduction Laboratories), followed by goat anti-rabbit HRP-conjugated secondary antibody (1:10,000). In no gel did calnexin density indicate differences in protein loading (for representative calnexin immunostaining, see Fig. 6A). Immunoreactive levels were quantified by integrating band density \times area using computer-assisted densitometry (NIH Image version 1.60). GABA_B densities were divided by the corresponding calnexin densities. The resulting values were averaged over three control samples for each gel, and all bands were normalized as percentage of the control values.

Immunoprecipitation of GABA_{B2}. Brain tissues were homogenized in cold radioimmunoprecipitation assay (RIPA) buffer containing 100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate, supplemented with protease inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ M pepstatin, 1 mg/ml soybean trypsin inhibitors, 1 mM iodoacetamide, and 250 μ M PMSF). Phosphatase inhibitors such as sodium fluoride, sodium pyrophosphate, sodium orthovanadate, and okadaic acid were used in RIPA buffer to preserve the phosphorylation state of GABA_{B2} receptor proteins. Receptor proteins were immunoprecipitated from 400 μ g of extract overnight at 4°C by the addition of the specific antibodies against GABA_{B2} (3 μ g; Chemicon), followed by 3 hr incubation at 4°C with Protein A Sepharose beads (3 mg in 100 μ l of RIPA buffer). The immunoprecipitates were washed three times with RIPA buffer, and the immunoprecipitated proteins were eluted and subjected to SDS-PAGE (8%). Immunoblotting was performed using phospho (p)-Ser-specific monoclonal antibodies (1:1000; Chemicon). To verify that the identified band was a phosphoprotein, the blots were stripped as described above and incubated with phosphatase (5 U) in 50 mM Tris-HCl, pH 9.3, and 1 mM MgCl₂ for 6 hr at 37°C. The blots were washed and probed with p-Ser antibody. Also, in a separate experiment, nucleus accumbens extracts from control subjects were treated with phosphatase (5 U) at 37°C for 6 hr, followed by immunoprecipitation and Western analysis with p-Ser antibody. The previously observed immunoreactivity of p-GABA_{B2} was absent, suggesting that assay is detecting p-GABA_{B2} receptors. Immunoblots with p-Ser-specific antibodies from immunoprecipitated GABA_B receptors were quantified using computer-assisted densitometry (NIH Image version 1.60).

Histology. After the dialysis experiments, rats were administered an overdose of pentobarbital (>100 mg/kg, i.p.) and transcardially perfused with 0.9% saline, followed by 10% Formalin solution. Brains were removed and placed in 10% Formalin for at least 1 week to ensure proper fixation. The tissue was blocked, and coronal sections (100 μ m thick) were made through the site of dialysis probe with a vibratome. The brains were then stained with cresyl violet to verify anatomical placement according to the atlas of Paxinos and Watson (1986).

Statistical analysis. The StatView statistics package was used to estimate statistical significance. A one-way ANOVA with repeated measures over dose was used to determine the effect of individual drugs on extracellular dopamine, glutamate, or GABA levels. A two-way ANOVA with repeated measures over time or dose was used to compare between treatments. During identification of statistical significance, *post hoc* comparisons were made with a Fischer's PLSD test.

Results

Cocaine withdrawal elevates the basal levels of extracellular GABA

The *in vivo* basal levels of extracellular GABA in the nucleus accumbens were determined using no-net flux microdialysis (Parsons et al., 1991). After collecting five 20 min samples different concentrations of GABA (2.5, 25, 50, 100 nM) were passed

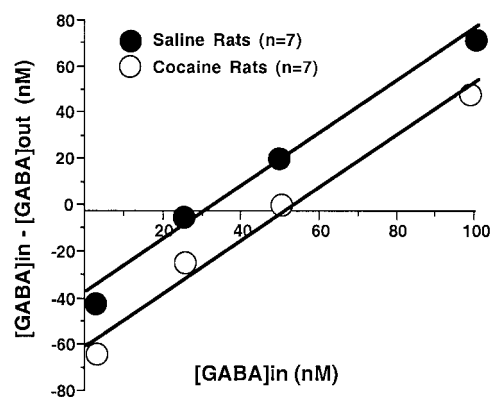


Figure 1. No-net flux microdialysis showing the increase in basal level of extracellular GABA in the accumbens in chronic cocaine- versus saline-treated rats. After collecting five 20 min baseline samples, 2.5, 25, 50, and 100 nM GABA was added to the dialysis buffer, and the net loss or gain in GABA in the collected dialysis buffer was quantified. The resulting linear equation estimates the basal level of extracellular GABA (concentration of no-net flux, i.e., $y = 0$) and the rate of elimination of GABA from the extracellular space (i.e., the slope of the line) in each subject (Parsons et al., 1991). The average of the basal levels of extracellular GABA was significantly increased in chronic cocaine-treated rats (32.7 ± 4.0 nM in saline-treated rats vs 50.3 ± 6.6 nM in cocaine-treated rats; a two-tail Student's *t* test; $p < 0.05$), whereas the elimination rates of GABA from extracellular space (the slopes) were not different (1.14 ± 0.08 in saline-treated rats vs 1.19 ± 0.05 in chronic cocaine-treated rats).

through the dialysis probe permitting extrapolation to the concentration of no net GABA flux across the dialysis membrane, which corresponds to the basal extracellular concentration. The slope of the line reflects the relative activity of processes eliminating GABA from the extracellular space by uptake, diffusion and enzymatic degradation. Figure 1 reveals a significant increase in the basal level of GABA from 32.7 ± 4.0 nM in saline rats to 50.3 ± 6.6 nM in cocaine-treated subjects after 3 weeks withdrawal. In contrast, the slopes of the two lines were statistically equivalent indicating no difference in GABA elimination from the extracellular space.

Increased extracellular GABA by chronic cocaine is from neurons

To determine the source of the extracellular GABA, voltage-dependent ion channel blockers were infused into the nucleus accumbens. Figure 2A shows that blockade of the voltage-dependent Na⁺ channel by TTX (1 μ M) or Ca²⁺ channels by either ω -conotoxin GVIA (N-type blocker) or ω -conotoxin MVIIC (P/Q-type blocker) lowered the basal levels of extracellular GABA by $\sim 25\%$. This is consistent with other studies showing that the majority of extracellular GABA measured by microdialysis was not derived from neuronal or vesicular pools (for review, see Timmerman and Westerink, 1997).

In contrast to the basal levels of extracellular GABA in control subjects, Figure 2B shows that the elevated extracellular GABA associated with cocaine-treated subjects was reduced substantially by 1 μ M TTX and approached the level of extracellular GABA in control subjects. Increasing the dose from 1 to 10 μ M TTX failed to produce additional reductions in extracellular GABA in either treatment group.

Enhanced GABA tone on GABA_B autoreceptors and heteroreceptors by chronic cocaine

Figure 3 shows that reverse dialysis of the GABA_B receptor antagonist 2-OH-saclofen into the accumbens elevated the extracellular content of GABA, dopamine, and glutamate in cocaine-

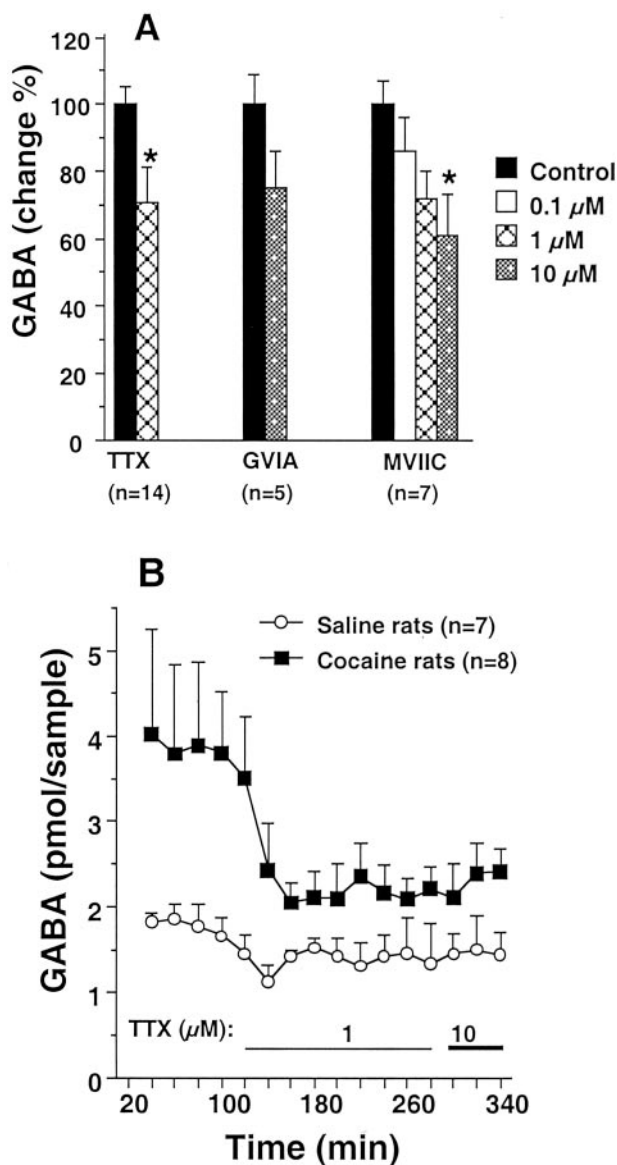


Figure 2. Blockade of the voltage-dependent Na⁺ channels markedly reduces the elevated GABA levels measured after repeated cocaine treatment. *A* shows that the basal levels of extracellular GABA in control subjects is only marginally reduced by TTX (Na⁺ channel blocker), ω-conotoxin GVIA (GVIA; N-type Ca²⁺ channel blocker), or ω-conotoxin MVIIC (MVIIC; P/Q-type Ca²⁺ channel blocker). The value in each column represents the average of three 20 min dialysis samples collected for each drug or dose. *B* shows that blockade of the voltage-dependent Na⁺ channels by TTX reversed the increase in extracellular GABA observed in chronic cocaine-treated rats. A two-way ANOVA with repeated measurements revealed a significant difference between the treatment (chronic saline vs cocaine) ($F_{(1,12)} = 6.62$; $p = 0.025$) and a significant interaction between treatment and time ($F_{(16,192)} = 3.85$; $p = 0.0001$). * $p < 0.05$ compared with the average of the baseline samples (Control).

treated rats. In contrast, in saline-treated subjects, much lower GABA tone was evident on GABA_B presynaptic receptors. Reverse dialysis of 2-OH-saclofen produced no effect on the extracellular levels of dopamine or glutamate in control animals, whereas relatively small increases were measured in extracellular GABA. The repeated cocaine treatment group had elevated basal levels of GABA (1.40 ± 0.23 pmol/sample for saline; 2.22 ± 0.24 for cocaine; $p < 0.05$) and reduced basal levels of extracellular glutamate (115.4 ± 20.4 pmol/sample for saline; 72.6 ± 12.6 for cocaine; $p < 0.05$). In contrast the basal levels of dopamine were

not significantly altered by repeated cocaine (36.8 ± 6.0 fmol/sample for saline; 25.6 ± 7.6 for cocaine).

In contrast to the differential effects produced by antagonizing GABA_B autoreceptors and heteroreceptors in repeated cocaine and saline animals, stimulating GABA_B receptors with baclofen produced approximately equivalent reductions in the extracellular content of GABA, glutamate, or dopamine in both treatment groups (Fig. 4). The basal levels of extracellular GABA were significantly elevated in the repeated cocaine group (1.30 ± 0.29 pmol/sample for saline; 2.16 ± 0.24 for cocaine; $p < 0.05$), whereas the basal levels of neither glutamate (135.6 ± 17.8 pmol/sample for saline; 102.8 ± 20.2 for cocaine) nor dopamine (28.2 ± 5.2 fmol/sample for saline; 22.8 ± 4.4 for cocaine) were significantly affected.

Repeated cocaine reduces the functional capacity of GABA_B receptors

Elevated levels of extracellular GABA could arise from desensitization of GABA_B receptors. To evaluate this possibility, the coupling of GABA_B receptors to intracellular G-proteins was determined by measuring baclofen-stimulated GTPγS binding in membranes obtained from the accumbens of cocaine- and saline-treated rats. Figure 5 shows that baclofen elicited a dose-dependent increase in [³⁵S]GTPγS binding to G-proteins in both saline- and cocaine-treated rats. However, the maximum stimulation of binding was significantly reduced in the cocaine-treated subjects.

GABA_{B1} and GABA_{B2} receptors

Figure 6*A* shows a representative immunoblot of GABA_{B2} protein in the nucleus accumbens. There was no significant difference in the levels of total GABA_{B2} protein between the cocaine and saline treatment groups (Fig. 6*C*). Similar to observations made by Couve et al. (2002) in neuronal cultures, the GABA_{B2} receptor is substantially Ser-phosphorylated *in vivo*. Cocaine treatment markedly reduced the amount of Ser-phosphorylated GABA_{B2} (p-GABA_{B2}) measured in the nucleus accumbens (Fig. 6*B, C*). Figure 6*B* also shows the results of reprobing the blot with p-Ser antibody after stripping and incubating with phosphatase. The lack of immunostaining verifies that the original staining was a phosphoprotein.

Akin to the GABA_{B2} subunit, there was no difference in total GABA_{B1} protein content between saline- and cocaine-treated subjects (Fig. 6*D*). The amount of p-GABA_{B1} was not sufficient to quantify in either cocaine or saline animals (data not shown).

Histology

All dialysis probe placements used for data analysis had >50% of the active membrane within the boundaries of the nucleus accumbens, as defined by Paxinos and Watson (1986). The probe placements were generally at the interface between the core and shell compartments of the nucleus accumbens. When a portion of the probe was outside the nucleus accumbens, it was in the ventrolateral septum, ventromedial diagonal band of Broca, or ventromedial striatum.

Discussion

The present study demonstrates that, 3 weeks after discontinuing treatment with repeated cocaine injections, the basal level of extracellular GABA in the nucleus accumbens is increased. Because the Na⁺ channel blocker TTX substantially reduced the increase in basal extracellular GABA, the majority of the increase in cocaine-treated subjects is derived from a neuronal source.

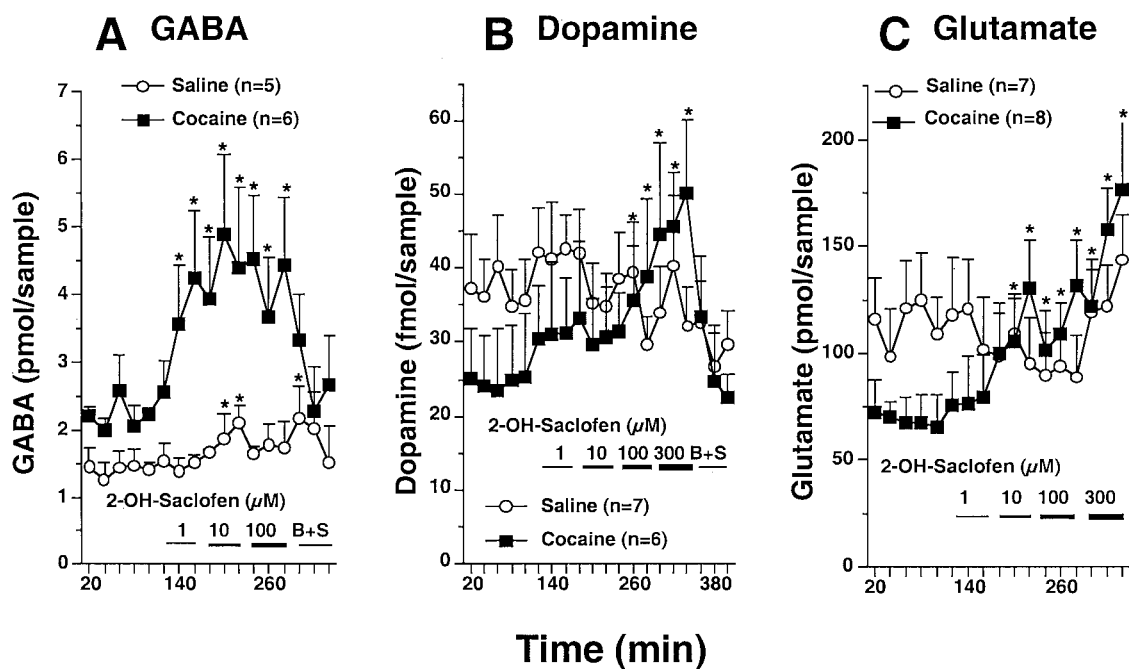


Figure 3. Blockade of GABA_B receptors by 2-OH-saclofen produced an enhanced elevation of extracellular GABA (A), dopamine (B), and glutamate (C) in the nucleus accumbens of chronic cocaine-treated rats. Increasing concentrations of 2-OH-saclofen were added to the dialysis buffer, and, in some experiments, 300 μ M baclofen was added to the final concentration of 2-OH-saclofen to reverse the GABA_B receptor blockade (B+S). A two-way ANOVA with repeated measurements over dose reveals a significant effect of baclofen dose ($F_{(16,126)} = 3.44, p = 0.0001$ for GABA in A; $F_{(16,126)} = 2.14, p = 0.005$ for dopamine in B; and $F_{(19,247)} = 6.59, p = 0.0001$ for glutamate in C) and a time \times treatment interaction for GABA ($F_{(16,144)} = 2.66; p = 0.001$), dopamine ($F_{(19,190)} = 1.89; p = 0.017$), or glutamate ($F_{(19,247)} = 2.07; p = 0.0063$). * $p < 0.05$ compared with the average of the last three baseline samples using a PLSD *post hoc* comparison.

Moreover, the increase in extracellular GABA may be derived in part from the functional uncoupling of GABA_B autoreceptors, perhaps attributable to reduced Ser phosphorylation of the GABA_{B2} receptor subtype.

The origin of extracellular GABA in the accumbens

Confirming work by others (for review, see Timmerman and Westerink, 1997), extracellular GABA levels in control subjects were found to be derived primarily from Na⁺- and Ca²⁺-insensitive, presumably nonsynaptic, sources. However, the portion of extracellular GABA that was increased after repeated cocaine was exquisitely sensitive to blockade of Na⁺ channels, indicating primarily action potential-dependent, neuronal origin. Moreover, identical slopes in the no-net flux study indicate the involvement of release rather than elimination of GABA in mediating the increased levels (Parsons et al., 1991). Further supporting the role of release mechanisms, repeated cocaine administration does not alter GABA synthesis or presynaptic GABA content in the accumbens (Sorg et al., 1995; Meshul et al., 1998).

There are three potential neuronal sources of extracellular GABA in the accumbens. The majority of the accumbens neurons are medium-sized spiny GABAergic projection cells that possess extensive recurrent collaterals (Smith and Bolam, 1990; Pennartz et al., 1994). In addition, the accumbens contains ~5% medium-sized aspiny GABAergic interneurons. Because these interneurons fire at a relatively high frequency (Kawaguchi et al., 1995), they may also contribute to extracellular GABA. Finally, GABAergic afferents from other brain nuclei, such as the ventral tegmental area, olfactory nucleus, and ventral pallidum, can contribute to extracellular GABA in the accumbens (Brog et al., 1993). Which of these potential sources of extracellular GABA that may be affected by repeated cocaine administration cannot be defined by microdialysis. Although spiny cells are thought to

undergo a number of cocaine-induced neuroadaptations that could promote transmitter release, such as increased PKA and calcium/calmodulin-dependent protein kinase II signaling (Gnegy, 2001; Nestler, 2001), electrophysiological studies indicate that the spiny cells may be relatively hyperpolarized and less active after repeated psychostimulant administration (Zhang et al., 1998; Thomas et al., 2001). Although this may signify less contribution by spiny cell recurrent collaterals, cocaine-induced neuroadaptations in dopamine-regulated GABA release in the ventral tegmental area could stimulate the activity of GABAergic neurons projecting to the accumbens (Bonci and Williams, 1996).

Elevating basal extracellular GABA increases tone on GABA_B presynaptic receptors

Consistent with functionally significant elevations in extracellular GABA by repeated cocaine, blockade of GABA_B autoreceptors and heteroreceptors caused an augmented increase in the extracellular levels of GABA, dopamine, and glutamate in the accumbens of cocaine-treated rats. These data not only support the presence of increased GABA tone on GABA_B receptors but also provide *in vivo* evidence that the accumulation of extracellular GABA after withdrawal from repeated cocaine can influence near-adjacent heterosynapses to tonically inhibit the release of other neurotransmitters (for review of *in vitro* evidence, see Isaacson, 2000). Similar to this finding, Giorgetti et al. (2002) recently found that chronic amphetamine treatment increased GABAergic tone on GABA_B receptors regulating extracellular glutamate and dopamine in the ventral tegmental area. These studies suggest that increased GABA release could be a relatively prevalent feature of psychostimulant abuse and offers an explanation for the widespread reduction in basal metabolic activity produced in brain by repeated psychostimulants in both experimental ani-

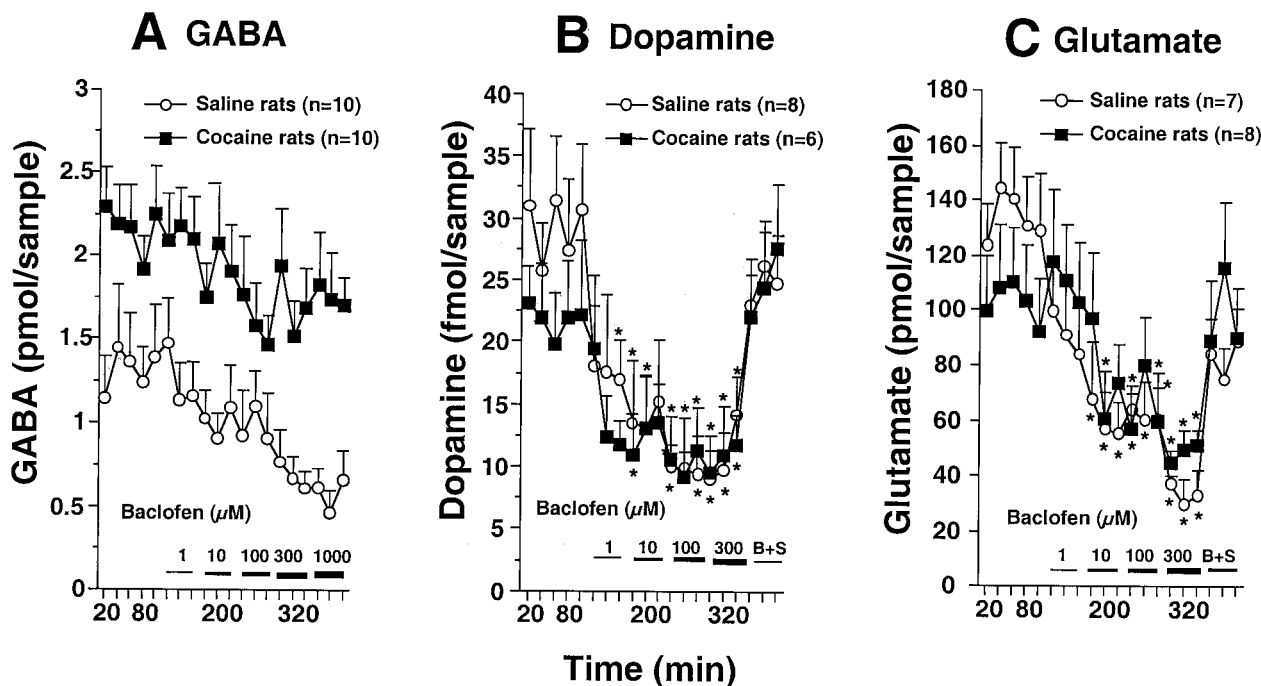


Figure 4. Effects of activation of GABA_B receptors by reverse dialysis of baclofen on nucleus accumbens levels of GABA, dopamine, and glutamate in chronic saline- and cocaine-treated rats. Increasing concentrations of baclofen were added to the dialysis buffer, and, in some experiments, 300 μM 2-OH-saclofen was added to the final concentration of baclofen to block the stimulation of GABA_B receptors (B + S). *A*, Baclofen produced parallel effects on GABA in both treatment groups. A two-way ANOVA with repeated measures showed a significant difference between treatment (chronic saline vs cocaine; $F_{(1,13)} = 15.45$; $p = 0.0017$) and over dose ($F_{(16,208)} = 2.03$; $p = 0.0128$) but no time × treatment interaction ($F_{(16,208)} = 0.373$; $p = 0.987$). *B*, Baclofen significantly decreased extracellular dopamine levels in both groups of rats. A two-way ANOVA with repeated measurement shows a significant difference over dose ($F_{(17,204)} = 8.05$; $p = 0.0001$) but no difference between treatments ($F_{(1,12)} = 0.48$; $p = 0.51$) or a time × treatment interaction ($F_{(17,204)} = 0.654$; $p = 0.845$). *C*, Baclofen similarly decreased extracellular glutamate levels in both the saline- and cocaine-treated groups. A two-way ANOVA with repeated measurements shows a significant difference over dose ($F_{(19,247)} = 5.42$; $p = 0.0001$) but no effect of treatment ($F_{(1,13)} = 0.1$; $p = 0.98$) or the time × treatment interaction ($F_{(19,247)} = 1.09$; $p = 0.366$). * $p < 0.05$ compared with the average of the last three of the five baseline samples.

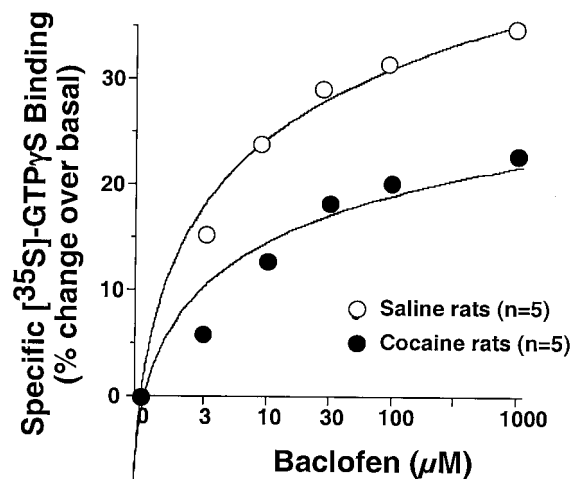


Figure 5. Chronic cocaine treatment decreased the baclofen-stimulated [³⁵S]GTPγS binding to G-proteins in the nucleus accumbens. A two-way ANOVA with repeated measurements revealed a significant effect of repeated cocaine treatment ($F_{(5,40)} = 2.79$; $p = 0.029$).

mals and human addicts (Volkow et al., 1993; Breiter et al., 1997; London et al., 1999).

Although the enhanced capacity of GABA_B receptor blockade to elevate extracellular GABA indicates that the GABA_B receptors are functional and may not be desensitized, repeated or prolonged exposure to agonist often desensitizes the responsiveness of G-protein-coupled receptors by altering receptor density, conformation, or trafficking (for review, see Tsao and von Zastrow, 2000; Ferguson, 2001). The present study demonstrated a de-

crease in baclofen-stimulated [³⁵S]GTPγS binding in the accumbens that was associated with increased extracellular GABA levels. This observation is consistent with the finding that repeated amphetamine caused a reduction in GABA_B coupling to G_{iα} in the nucleus accumbens, indicating that GABA_B receptors may be desensitized (Zhang et al., 2000). The total protein content of GABA_{B1} or GABA_{B2} receptors was unaltered in the cocaine group, indicating that changes in overall protein synthesis or degradation are not mediating the apparent desensitization. Similarly, a previous study found no significant alteration in GABA_{B1} receptor protein in repeated cocaine-treated rats (Li et al., 2001). However, GABA_{B1} forms a heterodimer with GABA_{B2} to make an active receptor (Bowery and Enna, 2000), and, although p-GABA_{B1} protein could not be detected in either the saline or cocaine treatment groups, a marked reduction in the amount of p-GABA_{B2} was measured in the cocaine group.

Reduced Ser phosphorylation of GABA_{B2} after repeated cocaine

Reduced Ser phosphorylation of GABA_B receptors by PKA or PKC has been shown to both promote (Kamatchi and Ticku, 1990; Taniyama et al., 1992) and desensitize (Couve et al., 2002) GABA_B receptor-mediated effects. The present findings are most consistent with the study by Couve et al. (2002) demonstrating that dephosphorylation of Ser892 in the cytoplasmic tail of GABA_{B2} mediates desensitization of GABA_B receptor coupling to K⁺ channels and that GABA_B agonists inhibit PKA, thereby promoting GABA_B dephosphorylation and desensitization. Thus, the increase in extracellular GABA after cocaine withdrawal would be expected to decrease p-GABA_{B2} and thereby desensitize

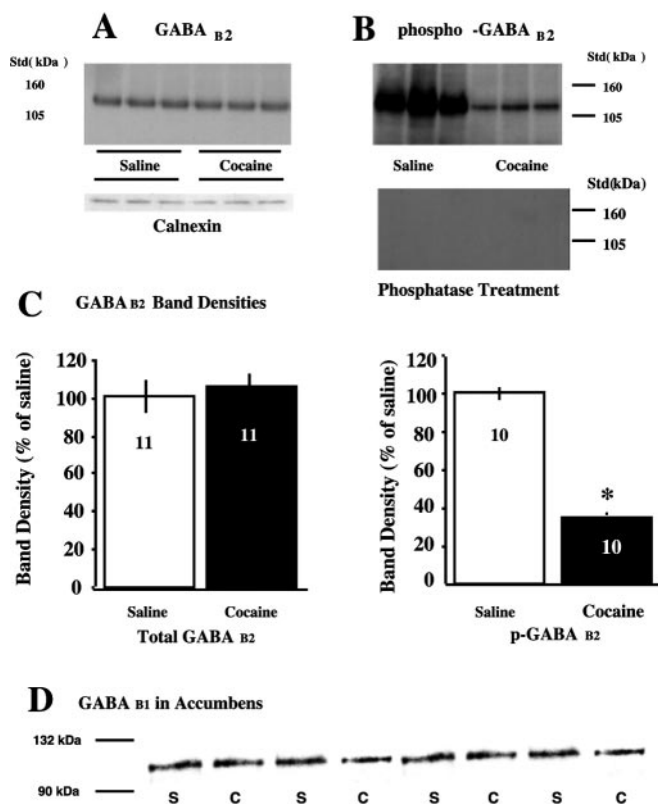


Figure 6. Cocaine treatment reduced p-GABA_{B2} without altering total GABA_B proteins. *A*, Representative immunoblots of GABA_{B2} total protein and reprobing for the loading control calnexin. *B*, Representative immunoblot of p-GABA_{B2} proteins using the same tissue as in *A*. The bottom blot is the top blot after incubation with phosphatase that eliminated immunoreactive staining of phosphorylated protein. *C*, Mean \pm SEM percentage change from saline for total GABA_{B2} and p-GABA_{B2}. *D*, Representative immunoblots of GABA_{B1} total protein showing no difference between treatment groups. * $p < 0.05$ comparing saline with cocaine treatment groups using a two-tailed Student's *t* test.

baclofen stimulation of GTP γ S binding and presynaptic transmitter release. These data appear contradictory to the general consensus that repeated psychostimulant administration increases PKA signaling (for review, see Nestler, 2001). However, it is possible that the intracellular microdomain in the vicinity of GABA_B receptors may not reflect whole-cell PKA activity. For example, an upregulation of whole-cell PKA by chronic cocaine may facilitate vesicular GABA release (Greengard et al., 1993; Trudeau et al., 1996), providing a source of the increased GABA tone on GABA_B autoreceptors. However, GABA_B receptors are G_i coupled, and increased tone on GABA_B receptors will inhibit PKA in the vicinity of the receptor, thereby reducing GABA_B receptor phosphorylation, desensitizing GABA_B receptors, and further facilitating GABA release.

Repeated cocaine effects on GABA_B heteroreceptors

Although the GTP γ S binding assay cannot distinguish between GABA_B autoreceptors and heteroreceptors, the dialysis study indicates that, similar to autoreceptors, the heteroreceptors regulating extracellular glutamate and dopamine levels are affected by repeated cocaine. Thus, akin to GABA autoreceptors, the cocaine-induced elevation in extracellular GABA provided increased GABAergic tone on heteroreceptors regulating glutamate and dopamine release. In one of the experiments, the basal levels of extracellular glutamate were reduced after repeated cocaine. A reduction in extracellular glutamate in the accumbens after re-

peated cocaine has been previously reported (Pierce et al., 1996; Bell et al., 2000; Hotsenpilliar et al., 2001), and blocking GABA_B receptors appeared to normalize the levels of glutamate in cocaine animals to the levels measured in saline animals (Fig. 3C). Basal extracellular glutamate content is derived primarily from the exchange of extracellular cystine with intracellular glutamate (Baker et al., 2002), posing the possibility that GABA_B receptors may regulate extracellular glutamate in part by inhibiting cystine–glutamate exchange. Indeed, the activity of the exchanger is inhibited by reducing PKA activity (Baker et al., 2002).

Conclusions

After 3 weeks of withdrawal from repeated cocaine administration, there is an increase in extracellular GABA in the nucleus accumbens that is derived primarily from neuronal sources. The elevated extracellular GABA provides a corresponding increase in tone to GABA_B autoreceptors and heteroreceptors that regulates the extracellular levels of GABA, glutamate, and dopamine. The increased tone to GABA_B receptors decreases p-GABA_{B2}, which may account for the desensitization of GABA_B receptors, and contributes to the previously observed decrease in basal extracellular levels of glutamate in the accumbens of cocaine-treated animals (Pierce et al., 1996; Bell et al., 2000; Hotsenpilliar et al., 2001). Decreased striatal GABA_A receptor function or subunit composition has also been induced by repeated cocaine in some (Peris, 1996; Suzuki et al., 2000) but not all (Goeders, 1991) studies. This poses the possibility that increased extracellular GABA may downregulate GABA_A as well as GABA_B receptor function. Given the integral role in addiction that has been postulated for neuroadaptations in accumbens GABAergic spiny cells (Nestler, 2001; Everitt and Wolf, 2002), the elevation of extracellular GABA and corresponding impact on neurotransmission regulated by GABA_B receptors may constitute a functionally important step in the development or expression of behaviors associated with psychostimulant addiction.

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