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A Critical Interaction between Dopamine D2 Receptors and Endocannabinoids Mediates the Effects of Cocaine on Striatal GABAergic Transmission

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Compelling evidence indicates that endocannabinoids are implicated in drug addiction. In the present study, we have addressed the interaction between cocaine and endocannabinoid system by means of neurochemical and neurophysiological experiments in rat brain slices. Using gas chromatography–electron impact mass spectrometry, we have found that cocaine increased the levels of the endocannabinoid anandamide in the striatum, a brain area primarily involved in the compulsive drug-seeking and drug-taking behaviors typical of addiction. This effect was attenuated by pharmacological inhibition of D2-like receptors but not D1-like receptors, and it was mimicked by D2-like but not D1-like receptor stimulation. The cocaine-induced increase in anandamide concentrations was attributable to both stimulation of its synthesis and inhibition of its degradation, as suggested by the ability of cocaine and quinpirole, a D2-like receptor agonist, to enhance the activity of NAPE-phospholipase D and to inhibit fatty acid amide hydrolase. By means of electrophysiological recordings from single striatal neurons, we have then observed that the ability of cocaine to inhibit, via D2-like receptors, GABA transmission was partially prevented following blockade of cannabinoid receptors, suggesting that endocannabinoids may act as downstream effectors in the action of cocaine in the striatum. Understanding the molecular and physiological effects of drugs of abuse in the brain is essential for the development of effective strategies against addiction.
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INTRODUCTION

Cannabinoid system has been implicated in a number of neuropsychiatric conditions, including dopamine (DA)-related disorders such as schizophrenia (Emrich *et al*, 1997), Parkinson's disease (Gubellini *et al*, 2002; Maccarrone *et al*, 2003a), and drug addiction (Ledent *et al*, 1999; De Vries *et al*, 2001; Maldonado and Rodriguez de Fonseca, 2002). The involvement of endocannabinoids in drug addiction is likely to reflect the effects of these compounds in the so-called 'reward circuitry', which includes midbrain DA neurons and their target structures (Berke and Hyman, 2000; Everitt and Wolf, 2002; Maldonado and Rodriguez de Fonseca, 2002). With respect to the role of endocannabinoids in opiate addiction, it has been reported that the rewarding effects of morphine are blocked by both pharmacological (Chaperon *et al*, 1998) and genetic inactivation of cannabinoid CB1 receptors (Martin *et al*, 2000), while blockade of CB1 receptors abolishes morphine intravenous self-administration (Ledent *et al*, 1999), and precipitates withdrawal symptoms in morphine-dependent rats (Navarro *et al*, 1998).

Although matter of less extensive investigations, also the involvement of cannabinoid system in psychostimulant addiction has been addressed. Δ^9-tetrahydrocannabinol (THC) self-administration in monkeys is greatly facilitated after previous acquisition of cocaine self-administration (Tanda *et al*, 2000; Maldonado and Rodriguez de Fonseca, 2002), while chronic cannabinoid administration produces

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cross-sensitization not only to the locomotor effects of opioids (Pontieri *et al*, 2001) but also of amphetamine (Gorriti *et al*, 1999). Finally, pharmacological blockade of cannabinoid CB1 receptors prevents relapse to cocaine-seeking behavior induced by re-exposure to the drug, while stimulation of these receptors facilitates it (De Vries *et al*, 2001).

Although some of these results might indicate that endocannabinoids act as critical downstream effectors of the cocaine action, the molecular and cellular bases of the cocaine–endocannabinoid interaction are still unknown. The striatum contains high levels of both cannabinoid CB1 receptors and DA receptors (Piomelli, 2003), and represents therefore an ideal structure to study the interplay between these two transmitter systems with respect to cocaine effects. Accordingly, the effects of cocaine in the brain are mainly mediated by the stimulation of DA receptors (Berke and Hyman, 2000), which are important modulators of synaptic transmission in the striatum (Calabresi *et al*, 2000). We have recently shown that both cocaine and amphetamine inhibit GABA transmission in this structure, an effect primarily attributable to the activation of DA D2 receptors located on presynaptic terminals of GABAergic interneurons (Centonze *et al*, 2002). Since GABAergic terminals and GABAergic interneurons seem to be also the preferential location of CB1 receptors in the striatum (Piomelli, 2003), a functional interaction between cannabinoid and DA receptors might occur at this level.

In the present study, therefore, we addressed this important issue by means of biochemical and electrophysiological experiments in rat brain slices. Our results show that cocaine, through the stimulation of DA D2-like receptors, increases the levels of the endocannabinoid anandamide (AEA) in the striatum, a brain area primarily involved in the compulsive drug-seeking and drug-taking behaviors typical of addiction (Berke and Hyman, 2000; Everitt and Wolf, 2002; Gerdeman *et al*, 2003). The cocaine-induced increase in AEA concentrations was attributable to both stimulation of its synthesis and inhibition of its degradation, and facilitated, through the activation of cannabinoid receptors, the DA D2-receptor-dependent inhibition of GABA transmission induced by cocaine.

MATERIALS AND METHODS

Adult male Wistar rats (150–250 g) were used for all the biochemical and electrophysiological experiments. All the experiments were conducted in conformity with the European Communities Council Directive of November 1986 (86/609/EEC).

Determination of Anandamide Levels

The endogenous levels of anandamide (AEA) in the striatum were determined by gas chromatography–electron impact mass spectrometry (GC/MS) (Maccarrone *et al*, 2001). Immediately after decapitation, corticostriatal brain slices (200 μm) were prepared with the use a vibratome and kept in artificial cerebrospinal fluid (ACSF), whose composition was (in mM): (126) NaCl, (2.5) KCl, (1.2) $MgCl_2$, (1.2) NaH_2PO_4, (2.4) $CaCl_2$, (11) glucose, (25) $NaHCO_3$. The temperature of ACSF was maintained at 35°C and it was gassed with O_2/CO_2 (95/5%). Slices were bathed for 10 min in the presence or in the absence of specific pharmacological treatments, then they were washed in phosphate-buffered saline (precooled at 4°C), frozen in liquid nitrogen and kept at −70°C until processed. Lipids were extracted from frozen tissues and were injected into a Carlo Erba model HRGC5160 gas chromatograph (Rome, Italy), equipped with a BP5 silica capillary column (30 m × 0.25 mm i.d.) from SGE (Milan, Italy), and interfaced with a VG Micromass model QUATTRO spectrometer (Manchester, UK). Analyses were performed in a 'split-less' mode at temperatures rising from 70 to 250°C, at a rate of 30°C/min. The identity of AEA was assessed by comparison of the retention times and the mass spectra recorded at 70 eV with those of authentic standards. Quantitation of AEA was achieved by isotope dilution with AEAd4. Calibration solutions and calibration curves were obtained as described (Maccarrone *et al*, 2001).

Determination of AEA Metabolism and Binding

The uptake of [^{3}H]AEA by the AEA membrane transporter (AMT) was assayed in synaptosomes prepared from corticostriatal slices as reported (Maccarrone *et al*, 2001, 2003a). Tissues were resuspended in ice-cold 0.32 M sucrose, 5 mM Tris-HCl buffer (pH 7.4) and were gently disrupted by 10 up-and-down strokes in a Teflon-glass homogenizer (weight : volume ratio = 1 : 20). The homogenates were centrifuged at 1000*g* for 5 min, at 4°C, then supernatants were centrifuged again at 17 000 *g* for 15 min, at 4°C. The final pellets were resuspended in 136 mM NaCl, 5 mM KCl, 0.16 mM $CaCl_2$, 0.1 mM EGTA, 1.3 mM $MgCl_2$, 10 mM glucose, and 10 mM Tris-HCl buffer (pH 7.4), at a protein concentration of 3 mg/ml. The activity of AMT was measured using 100 μl synaptosomes and 300 nM [^{3}H]AEA per test.

Anandamide hydrolase (arachidonoylethanolamide amidohydrolase, EC 3.5.1.4; fatty acid amide hydrolase, FAAH) activity was assayed in homogenates of corticostriatal slices by reversed-phase high-performance liquid chromatography, using 5 μM [^{3}H]AEA as substrate (Maccarrone *et al*, 2003a). FAAH activity was expressed as pmol arachidonate released per min per mg protein.

The activity of *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4; NAPE-PLD) was assayed in homogenates of corticostriatal slices (50 μg/test) according to Moesgaard *et al* (2000), using 100 μM *N*-[^{3}H]arachidonoyl-phosphatidylethanolamine ([^{3}H]NArPE) as substrate (Okamoto *et al*, 2004). NAPE-PLD activity was expressed as pmol [^{3}H]AEA released per min per mg protein.

The binding of the synthetic cannabinoid [^{3}H]CP55,940 to striatal membranes was determined by using rat membrane fractions prepared, quickly frozen in liquid nitrogen and stored at −80°C for no longer than 1 week as reported (Maccarrone *et al*, 2003a). These membrane fractions were used in rapid filtration assays with 400 pM agonist, a dose that gives a well-detectable binding in rat striatal membranes (Gubellini *et al*, 2002; Maccarrone *et al*, 2003a). The apparent dissociation constant (K_d) and maximum binding

(B_{max}) values of [^{3}H]CP55,940 were calculated from saturation curves (in the range 0–800 pM) through non-linear regression analysis with the Prism 3 program (GraphPAD Sofware for Science, San Diego, CA, USA) (Maccarrone *et al*, 2003a). [^{3}H]AEA (223 Ci/mmol) and [^{3}H]CP55,940 (126 Ci/mmol) were from NEN DuPont de Nemours (Köln, Germany); [^{3}H]NArPE (200 Ci/mmol) was from ARC (St Louis, MO, USA). Biochemical data were expressed as the mean ± SD, and statistical analysis was performed by the Student's *t*-test (ST), through the InStat 3 program (GraphPAD Software for Science, San Diego, CA, USA). The significance level was established at $p < 0.05$.

Electrophysiology

Corticostriatal coronal slices (200 μm) were prepared from tissue blocks of the rat brain with the use of a vibratome (Centonze *et al*, 2002; Picconi *et al*, 2003). A single slice was then transferred to a recording chamber and submerged in a continuously flowing ACSF (35°C, 2–3 ml/min) gassed with 95% O_2–5% CO_2. The composition of the control solution was (in mM): (126) NaCl, (2.5) KCl, (1.2) $MgCl_2$, (1.2) NaH_2PO_4, (2.4) $CaCl_2$, (11) Glucose, (25) $NaHCO_3$.

The striatum could be readily identified under a low-power magnification, whereas individual neurons were visualized *in situ* using a differential interference contrast (Nomarski) optical system. This employed an Olympus BX50WI (Japan) noninverted microscope with × 40 water immersion objective combined with an infrared filter, a monochrome CCD camera (COHU 4912), and a PC compatible system for analysis of images and contrast enhancement (WinVision 2000, Delta Sistemi, Italy). Recording pipettes were advanced toward individual striatal cells in the slice under positive pressure and, on contact, tight GΩ seals were made by applying negative pressure. The membrane patch was then ruptured by suction and membrane current and potential monitored using an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Whole-cell access resistances measured in voltage clamp were in the range of 5–30 MΩ prior to electronic compensation (60–80% was routinely used).

Whole-cell patch clamp recordings were made with borosilicate glass pipettes (1.8 mm o.d.; 3–5 MΩ), both in voltage-clamp and current-clamp configuration. To study spontaneous $GABA_A$-mediated inhibitory postsynaptic currents (IPSCs), the recording pipettes were filled with internal solution of the following composition (mM): CsCl (110), K^+-gluconate (30), ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid (EGTA; 1.1), HEPES (10), $CaCl_2$ (0.1), Mg-ATP (4), Na-GTP (0.3). MK-801 and CNQX were added to the external solution to block, respectively, NMDA and non-NMDA glutamate receptors. Spontaneous $GABA_A$-mediated IPSCs were stored by using P-CLAMP 8 (Axon Instruments) and analyzed offline on a personal computer with Mini Analysis 5.1 (Synaptosoft, Leonia, NJ, USA) software. The detection threshold was set at twice the baseline noise. The fact that no false events would be identified was confirmed by visual inspection for each experiment. Offline analysis was performed on spontaneous synaptic events recorded during a fixed time epoch (3–5 min), sampled every 5 or 10 min before (two samplings) and after (3–6 samplings) the application of the drugs. Only cells that exhibited stable IPSC frequencies in control (less than 20% changes during the two control samplings) were taken into account. For IPSC kinetic analysis, events with peak amplitude between 10 and 30 pA were grouped, aligned by half-rise time, and normalized by peak amplitude. Events with complex peaks were eliminated. In each cell, all events between 10 and 30 pA were averaged to obtain rise times, decay times, and half-widths.

For data presented as the mean ± SEM, statistical analysis was performed using a paired or unpaired Student's *t*-test or Wilcoxon's test. When comparing differences between two cumulative distributions, the Kolmogorov–Smirnov (K–S test) was used. The significance level was established at $p < 0.05$.

Drugs

For both biochemical and electrophysiological experiments, drugs were applied by dissolving them to the desired final concentration in the bathing ACSF. The concentrations of the various drugs were chosen according to previous *in vitro* studies on corticostriatal brain slices (Centonze *et al*, 2002; Gubellini *et al*, 2002), and were, unless otherwise specified in the text or in the figures, as follows: CNQX (10 μM), HU 210 (1 μM), SCH23390 (10 μM), MK-801 (30 μM) (from Tocris Cookson, Bristol, UK), bicuculline (10 μM), SKF38393 (10 μM), quinpirole (10 μM) (from Sigma-RBI, St Louis, USA), cocaine (10 μM), L-sulpiride (3 μM) (from Sigma, Milan, Italy), SR141716A (1 μM) (from Sanofi Recherche, Montpellier, France).

RESULTS

Effects of Cocaine on AEA Synthesis, Transport, and Degradation

Cocaine treatment significantly increased the levels of endogenous AEA. Also the activity of NAPE-PLD was increased following cocaine treatment, while FAAH activity decreased and AMT activity was unchanged (Figure 1). Taken together, these results suggest that the cocaine-induced increase in AEA levels was secondary to both increased synthesis and decreased degradation. Accordingly, NAPE-PLD plays a crucial role in AEA synthesis from membrane lipids, while FAAH is responsible for AEA breakdown following its intracellular transport mediated by AMT (Di Marzo *et al*, 1994; Cadas *et al*, 1997; Iversen, 2003). It seems noteworthy that the NAPE-PLD activity shown here in rat striatum extends a recent report on mouse brain, which appeared during the preparation of this manuscript (Okamoto *et al*, 2004).

Remarkably, the cocaine effects on AEA levels and metabolism (NAPE-PLD, AMT, and FAAH activity) were almost completely prevented by blocking DA receptors through the co-application of SCH23390 plus L-sulpiride, antagonists of D1- and D2-like receptors, respectively. The isolated application of L-sulpiride, but not of SCH23390, mimicked this effect, indicating that it was entirely mediated by the stimulation of D2-like receptors (Figure 1). Accordingly, the D2-like receptor agonist quinpirole, but not the D1-like receptor agonist SKF38393,

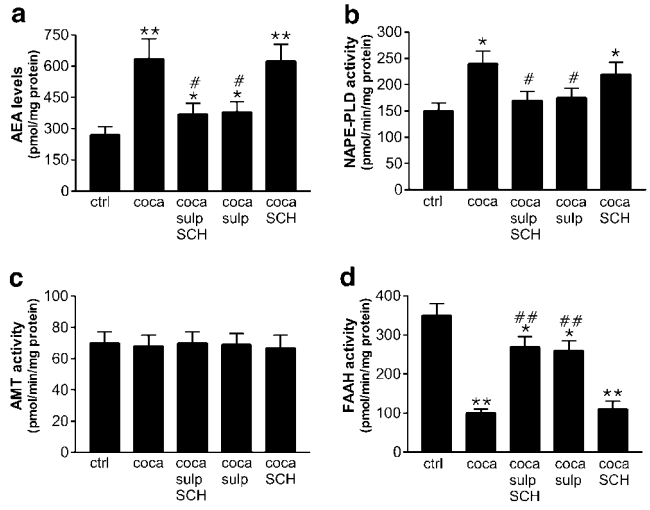

Figure 1 Cocaine increases AEA levels in the striatum by stimulating its synthesis and inhibiting its degradation. (a) The graph shows that 10 μM cocaine increases the levels of AEA in the striatum. Blockade of both D1- and D2-like receptors by SCH23390 (10 μM) plus L-sulpiride (3 μM) significantly reduced this effect. The cocaine effect was also reduced by 3 μM L-sulpiride, but not by 10 μM SCH23390, applied alone. (b) Cocaine increased the activity of NAPE-PLD, an effect inhibited by the blockade of both D1- and D2-like receptors, or by the selective blockade of D2-like receptors. (c, d) The activity of AMT was unaffected by cocaine, while the activity of FAAH was significantly inhibited through the stimulation of DA D2-like receptors. Drugs were applied for 10 min; $n=4$ for each experiment; *means $p<0.05$ compared to the control value; **means $p<0.01$ compared to the control value; # means $p<0.05$ compared to the cocaine value; ## means $p<0.01$ compared to the cocaine value; no symbol means nonsignificant compared to the control value.

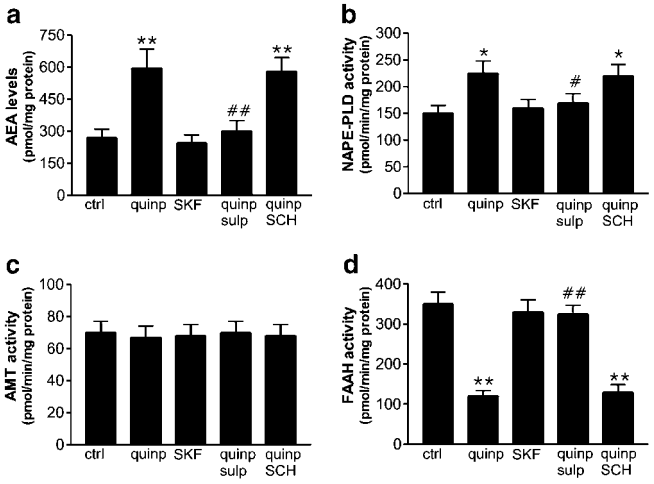

Figure 2 Stimulation of DA D2-like receptors mimics the effects of cocaine on AEA levels and metabolism. Application of the DA D2-like receptor agonist quinpirole (10 μM), but not the DA D1-like receptor agonist SKF38393 (10 μM), increased AEA levels in the striatum (a), by stimulating AEA synthesis (b) and inhibiting AEA degradation (d). L-Sulpiride (3 μM), but not SCH23390 (10 μM), prevented the quinpirole effects. Drugs were applied for 10 min; $n=4$ for each experiment; *means $p<0.05$ compared to the control value; **means $p<0.01$ compared to the control value; # means $p<0.05$ compared to the quinpirole value; ## means $p<0.01$ compared to the quinpirole value; no symbol means nonsignificant compared to the control value.

increased AEA levels by stimulating NAPE-PLD and inhibiting FAAH, an effect prevented by L-sulpiride but not by SCH23390 (Figure 2).

Effects of Cocaine, Quinpirole, and SKF38393 on Cannabinoid CB1 Receptor Binding

We also tested the effects of cocaine, quinpirole, and SKF38393 on the binding of the CB1 receptor agonist [^{3}H]CP55,940 to rat striatal membranes. As with AEA levels and metabolism, our results indicate that cocaine and quinpirole, but not SKF38393, significantly altered CB1 receptor function, by increasing its binding properties. Again, these effects were prevented by DA D2- but not D1-like receptor blockade (Figure 3). Analysis of the binding of [^{3}H]CP55,940 to striatal membranes, untreated or treated for 10 min with 10 μM cocaine, showed saturation curves superimposable to those recently reported in the same brain area (Maccarrone *et al*, 2003a). From these saturation curves, apparent K_d values of 340 ± 70 and 310 ± 91 pM ($n=4$; $p>0.05$) and B_{max} values of 330 ± 40 and 499 ± 57 fmol/mg protein ($n=4$; $p<0.05$) were calculated in controls and cocaine-treated samples, respectively.

Spontaneous Gabaergic Activity in Striatal Neurons

Electrophysiological recordings with cesium chloride-containing pipettes allowed detecting, at −80 mV holding

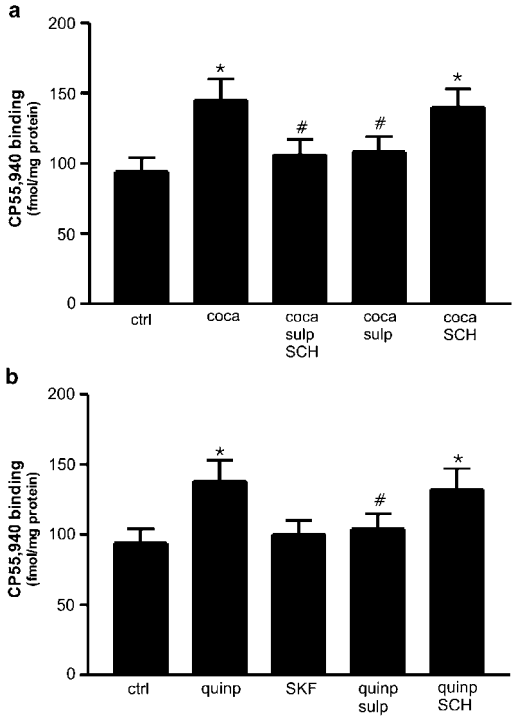

Figure 3 Cocaine and quinpirole increase CB1 receptor binding in the striatum. (a) The graph shows that 10 μM cocaine increases the binding of CP55,940 in the striatum. Blockade of both D1- and D2-like receptors by SCH23390 (10 μM) plus L-sulpiride (3 μM) blocked this effect. The cocaine effect was also reduced by L-sulpiride, but not by SCH23390, applied alone. (b) Application of quinpirole (10 μM), but not the DA D1-like receptor agonist SKF38393 (10 μM), mimicked the effects of cocaine on striatal CP55,940 binding, which were prevented by 3 μM L-sulpiride but not by 10 μM SCH23390. Drugs were applied for 10 min; $n=4$ for each experiments. Symbols as in Figures 1 and 2.

potential (HP) and in the presence of 10 μM CNQX plus 30 μM MK-801, spontaneous synaptic events that could be blocked by the $GABA_A$ receptor antagonist bicuculline (Centonze *et al*, 2003) ($n = 14$). Most events had amplitudes ranging between 5 and 30 pA and had kinetic properties (rise times, dacay time constants, and half-widths) significantly different from glutamate-mediated excitatory postsynaptic currents (not shown). The frequency of spontaneous IPSCs recorded in control conditions ranged between 1.4 and 3.2 Hz.

Involvement of Endocannabinoids in the Effects of Cocaine on Spontaneous IPSCs

According to a previous report showing that psychostimulants presynaptically inhibit GABA inputs to striatal neurons (Centonze *et al*, 2002), cocaine (5–10 min) dose-dependently reduced the frequency of spontaneous GABAergic IPSCs recorded from striatal slices ($n = 17$). This effect, fully reversible at the wash of the drug, was prevented by preincubation (5–7 min) with the D2-like receptor antagonist L-sulpiride ($n = 4$), and was mimicked by the D2-like receptor agonist quinpirole (5–10 min, $n = 15$) but not by the D1-like receptor agonist SKF38393 (5–10 min, $n = 7$), confirming that it was mediated by the stimulation of D2-like receptors (Delgado *et al*, 2000; Centonze *et al*, 2002) (Figure 4). As expected for a presynaptic site of action, neither cocaine nor quinpirole altered IPSC mean amplitude, rise time, decay time, and half-width (Figure 5a–d).

Cocaine has been reported to modulate striatal neuron firing activity through a direct interaction with voltage-dependent sodium channels (Kiyatkin and Rebec, 2000). In a further set of experiments, therefore, we wanted to see whether this drug, at the concentrations employed in the present study, was able to modulate repetitive firing activity of striatal neurons in an anesthetic-like manner. As shown in Figure 5e, 30 μM cocaine (10 min, $n = 4$) failed to affect both firing activity and hyperpolarizing response in striatal neurons recorded in the current-clamp mode.

To investigate the possible involvement of endocannabinoids in the D2-mediated effects of cocaine in the striatum, we measured the action of this drug in the presence of the cannabinoid CB1 receptor antagonist SR141716A. Although SR141716A failed to affect the frequency of sIPSCs recorded from striatal neurons ($n = 8$, 7–10 min), it significantly attenuated the cocaine effects. Further application of L-sulpiride was required to restore fully spontaneous IPSC frequency to control values ($n = 4$) (Figure 4).

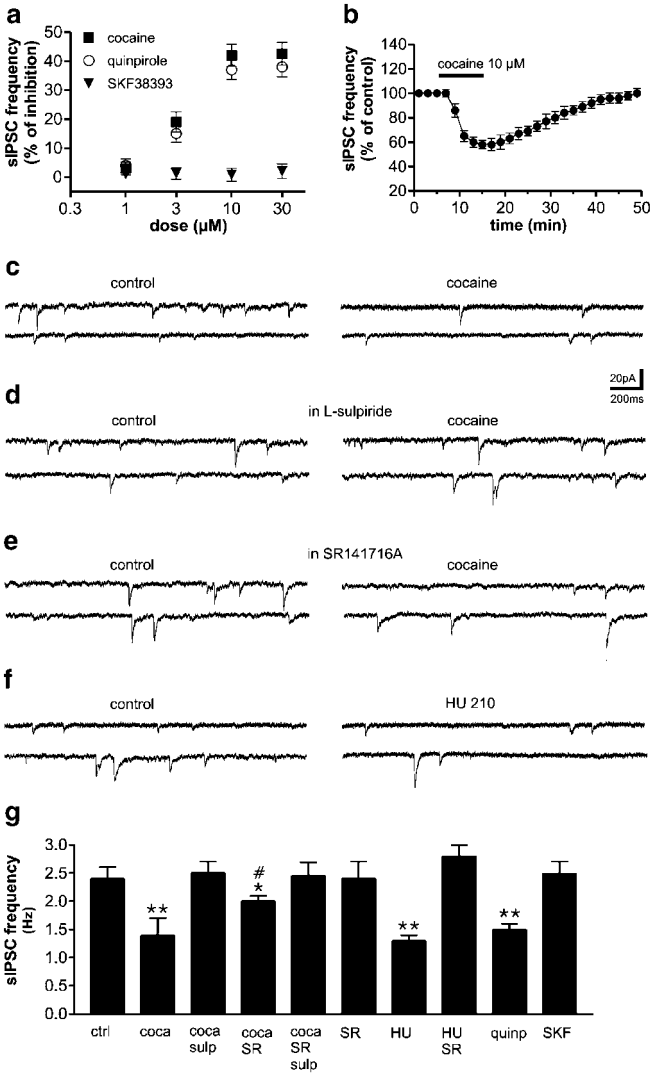

Figure 4 Cocaine depresses inhibitory synaptic transmission in the striatum in a CB1 receptor-dependent manner. (a) Cocaine and quinpirole, but not SKF38393, dose-dependently inhibited GABA transmission in the striatum. (b) This graph shows the time course of the effects of cocaine on sIPSC frequency. (c) The electrophysiological traces show that 10 μM cocaine reduced the frequency of spontaneous GABA-mediated synaptic currents (downward deflections). (d) In another striatal neuron, preincubation with 3 μM L-sulpiride abolished the cocaine effect. (e) The cocaine effect was also significantly attenuated by the CB1 receptor antagonist SR141716A (1 μM). (f) Also the CB1 receptor agonist HU 210 (1 μM) depressed sIPSCs recorded from striatal neurons. In (c–f), upper and lower traces on the right and on the left are from single experiments. (g) The histogram describes the effects of several pharmacological treatments on sIPSC frequency. For each experiment $n =$ at least 4. Symbols as in Figure 1.

Effects of the Cannabinoid CB1 Receptor Agonist HU 210 on Striatal Inhibitory Transmission

To further address the role of cannabinoid CB1 receptors in the modulation of striatal GABA transmission, we also investigated the electrophysiological effects of HU 210, a CB1 receptor agonist, on spontaneous IPSCs recorded from spiny neurons. According to a previous report (Szabo *et al*, 1998), bath application of this agent (7–10 min, $n = 5$) reduced IPSC frequency, without altering IPSC mean amplitude, rise time, decay time, and half-width. Preincubation of the slices with SR141716A (5–7 min, $n = 7$) blocked the HU 210-mediated effects, supporting the conclusion that the effects of this antagonist on cocaine-induced IPSC inhibition were indeed mediated by the blockade of CB1 receptors (Figures 4 and 5).

DISCUSSION

In the present study, we provided evidence that cocaine, through stimulation of DA D2-like receptors, increases

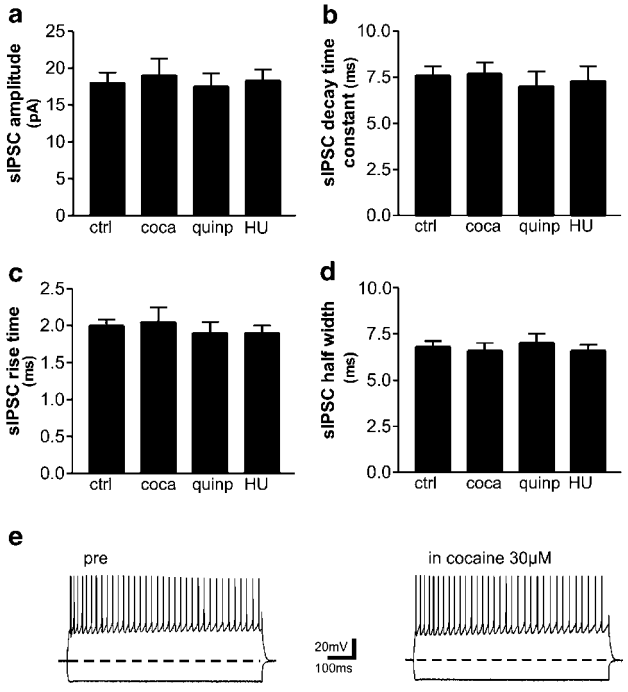

Figure 5 The postsynaptic and intrinsic properties of striatal neurons are unaltered in the presence of cocaine, quinpirole, or HU 210. The histograms describe the effects of 10 μM cocaine, 10 μM quinpirole, and 1 μM HU 210 on sIPSC amplitude (a), decay time (b), rise time (c), and half-width (d). (e) In the current-clamp mode, the firing discharge and the hyperpolarizing response evoked in a striatal neuron by intracellular injection of, respectively, positive and negative current (±600 pA) were unaltered in the presence of 30 μM cocaine. The dotted line indicates the resting membrane potential of this neuron (−86 mV). For the experiments in (a–d), n = at least 4. No symbol means nonsignificant compared to the control value.

striatal levels of the endocannabinoid AEA, an effect attributable to both increased NAPE-PLD activity and inhibition of FAAH. It should be noted that the levels of AEA found here are comparable to those found in the striatum of healthy and parkinsonian rats (Gubellini *et al*, 2002), but higher than those found by others in rat brain (Yang *et al*, 1999). However, our values are in the same range as the endogenous levels of AEA in rat substantia nigra and globus pallidus (Di Marzo *et al*, 2000), in mouse cortex and hippocampus (Maccarrone *et al*, 2001), and in mouse uterus (Schmid *et al*, 1997). In addition, we have recently measured by our GC–MS procedure the levels of AEA in human T lymphocytes treated or not with progesterone, and found differences superimposable to those found in the same samples in Dr Kunos' laboratory at NIAAA-NIH by liquid chromatography–mass spectrometry (reported in Maccarrone *et al*, 2003b). Furthermore, the endogenous levels of AEA that we found in human keratinocytes (Maccarrone *et al*, 2003c) were comparable to those reported by others in mouse epidermal cells (Berdyshev *et al*, 2000). On the other hand, we have recently summarized evidence against possible pitfalls in the extraction procedure and analysis of our samples (Maccarrone *et al*, 2004). Of importance is also the fact that the electrophysiological data reported here demonstrate that neuronal cells were healthy and well, ruling out any post-mortem release of AEA as a result of brain damage or ischemia.

We also showed that increased levels of AEA significantly contributed to the cocaine-induced presynaptic inhibition of GABA transmission in the striatum, presumably through stimulation of cannabinoid CB1 receptors. This conclusion is mainly based on the finding that SR141716A, a CB1 receptor antagonist, attenuated the effects of cocaine on GABA transmission. Since SR141716A could in principle target additional subtypes of cannabinoid receptors in the striatum, we cannot exclude the involvement of other receptors in the cocaine effects. Another brain cannabinoid receptor different from CB1 has been shown to be blocked by SR141716A (Breivogel *et al*, 2001), leaving open the possibility that also this receptor is modulated by cocaine. Yet, the non-CB1 receptor is blocked by SR141716A with lower potency, and is not activated by HU 210 (Breivogel *et al*, 2001). Therefore, the observations reported here that SR141716A attenuated the cocaine effect at a dose (1 μM) widely used to antagonize genuine CB1 receptors (Pertwee and Ross, 2002) and that also HU 210 depressed sIPSCs recorded from striatal neurons (Figure 4) seem to rule out the involvement of non-CB1 receptors.

Enhanced function of CB1 receptors likely contributed to the effect of cocaine in the striatum, as suggested by the finding that also the binding properties of these receptors increased in response to the administration of this psychostimulant. In particular, the increase of B_{max} values, but not of K_d values, of [^{3}H]CP55,940 binding in cocaine-treated slices suggests that cocaine increased the number of receptors in striatal membranes, but not their affinity for the ligand (Gubellini *et al*, 2002; Maccarrone *et al*, 2003a). This hypothesis is in keeping with the notion that CB1 receptors are recycled into the membrane (Hsieh *et al*, 1999; Coutts *et al*, 2001). In addition, trafficking of CB1 receptors can also explain their fast modulation upon a short time of treatment, like that (10 min) used in this study (Hsieh *et al*, 1999; Coutts *et al*, 2001). In this line it seems noteworthy that the increased number of receptors is paralleled by enhanced levels of the endogenous ligand AEA, suggesting that indeed cocaine treatment engages a larger number of activated CB1 receptors. The possibility that enhancement of AEA levels triggers the increase in receptor number and/or cell-surface expression remains to be explored.

Involvement of the Striatum in Cocaine Addiction

The nucleus striatum is a major component of the basal ganglia, primarily involved in sensorimotor functions and also in cognitive and limbic processes (Calabresi *et al*, 1997; Jog *et al*, 1999; Packard and Knowlton, 2002). Not surprisingly, therefore, alterations of striatal function are involved in a variety of neurological and psychiatric disorders including Parkinson's disease, Tourette's syndrome, schizophrenia, and drug addiction (Berke and Hyman, 2000; Leckman and Riddle, 2000; Obeso *et al*, 2000; Carlsson *et al*, 2001).

Increasing evidence indicates that not only cortical but also subcortical areas are involved in the diverse cognitive, emotional, and motor effects of cocaine and, in particular, the increased locomotor activity and stereotypy caused by

psychostimulants seem to involve specifically the nucleus striatum (Kelly *et al*, 1975; Amalric and Koob, 1993; Berke and Hyman, 2000). More recently, synaptic changes in the striatum have been claimed to be involved in the advanced stages of drug addiction, which are characterized by compulsive drug-seeking and drug-taking behaviors despite serious negative consequences (Gerdeman *et al*, 2003). Accordingly, the striatum plays a critical role in habit learning, a process consisting in the acquisition of increasingly automatic motor actions particularly refractory to devaluation processes (Jog *et al*, 1999; Canales and Graybiel, 2000). In the striatum, therefore, cocaine is believed to co-opt the neuronal mechanisms involved in habit formation, leading to nearly automatic and repetitive drug assumption even when the outcome becomes frankly undesirable (Berke and Hyman, 2000; Hyman and Malenka, 2001; Gerdeman *et al*, 2003).

DA plays a critical role in habit formation and in the effects of cocaine in the striatum (Jog *et al*, 1999; Canales and Graybiel, 2000; Everitt and Wolf, 2002; Gerdeman *et al*, 2003). This brain area receives profuse dopaminergic innervation arising from midbrain DA neurons and has a very high density of DA receptors (Mansour and Watson, 1995; Surmeier *et al*, 1996; Centonze *et al*, 2003). Cocaine increases DA release in the striatum through the blockade of transporter-mediated DA reuptake from nigrostriatal nerve endings and causes rapid induction of striatal c-fos, a commonly used molecular marker for neuronal activity. This effect is fully sensitive to DA receptor blockade (Graybiel *et al*, 1990; Moratalla *et al*, 1993), indicating that the actions of cocaine in the striatum are mediated, largely if not exclusively, by the release of endogenous DA and consequent DA receptor stimulation. Other mechanisms, however, might be involved in the pharmacological actions of cocaine in the striatum. Cocaine, in fact, facilitates serotonin and norepinephrine release (Barker and Blakely, 1995; Johanson and Schuster, 1995), two transmitters found to mediate important physiological effects on striatal neurons (Wilms *et al*, 2001; Pisani *et al*, 2003). In addition, cocaine has also been found to modulate striatal neuron firing activity *in vivo*, independently of transmitter release but through a direct interaction with sodium channels (Kiyatkin and Rebec, 2000). Notably, these non-DA-mediated effects of cocaine might also play a role in the action of this addictive drug on striatal AEA concentrations, since we have observed that blockade of both D1- and D2-like receptors prevented largely, but not entirely, the effects of cocaine on endocannabinoid system.

DA–Cannabinoid Interaction and the Effects of Cocaine in the Striatum

The recruitment of endocannabinoid system that follows DA receptor stimulation might help to explain its involvement in the neuronal processes underlying cocaine addiction. There is evidence that under certain conditions endocannabinoids can counter the action of D2 receptor stimulation in the brain, suggesting that the D2-receptor-dependent stimulation of endocannabinoid system may function as an inhibitory feedback mechanism aimed at limiting the cocaine-mediated effects. In this line, in fact, it has been found that pretreatment with the cannabinoid antagonist SR141716A enhanced the stimulation of motor behavior induced by the D2-like receptor agonist quinpirole (Giuffrida *et al*, 1999), while the inhibition of intracellular transport of AEA reduced its effects (Beltramo *et al*, 2000).

In our experimental condition, however, D2-like receptor-stimulated AEA production enhanced the effects of cocaine in the striatum, through the stimulation of CB1 receptors. The cooperative action of D2-like and CB1 receptors is supported by at least two experimental findings. First, endogenously produced endocannabinoids act as downstream effectors of D2 receptor signalling in the generation of corticostriatal long-term depression, a form of synaptic plasticity believed to underlie specific aspects of psychostimulant addiction (Gerdeman *et al*, 2002, 2003). Second, both D2 and CB1 receptors are expressed on GABA terminals of the striatum (Hermann *et al*, 2002; Delgado *et al*, 2000; Iversen, 2003), and the activation of both receptors reduces cAMP levels and transmitter release through an inhibitory G protein (Stoof and Kebabian, 1981; Pertwee, 1997; Vallone *et al*, 2000; Iversen, 2003).

Other experimental data indicate that cannabinoids and D2 receptors play synergistic actions in the brain. For example, concomitant activation of DA D2-like receptors by endogenous DA has been found to play a role in the THC-induced memory impairment and reduction of extracellular acetylcholine concentration in the hippocampus (Nava *et al*, 2000), while D2, but not D1 receptor agonists, potentiate cannabinoid-induced sedation in non-human primates (Meschler *et al*, 2000). In addition, the alteration in Fos expression that follows the blockade of CB1 receptors has been found to occur via reduced D2-like receptor-mediated function (Alonso *et al*, 1999). Furthermore, D2 (but also D1) receptors participate in the THC-induced activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) in the dorsal striatum (Valjent *et al*, 2001). Finally, differential regulation of AEA by distinct DA receptors has also been reported within mouse limbic forebrain (Patel *et al*, 2003). Interestingly, a recent electrophysiological study showed that CB1 receptors mediate the long-term effects of amphetamine in the amygdala, independently of DA, serotonin, and noradrenaline transmission (Huang *et al*, 2003). Our neurophysiological findings, however, do not contradict the role of anandamide as a stop signal for DA, since the nature of the interaction between these two transmitter systems might ultimately depend on a series of variables, including the physiological state of striatal neurons *in vivo*.

DA–Cannabinoid Interaction in the Modulation of GABAergic Transmission in the Striatum

In the present work, we have found that cocaine-stimulated AEA significantly contributes to inhibit GABA-mediated synaptic transmission in the striatum, thereby favoring neuronal excitation. Striatal neuron firing activity, in fact, is governed by both glutamate-mediated excitatory synaptic inputs and GABA-dependent inhibitory potentials, which efficiently contrast the excitatory action of glutamate (Kita, 1996; Plenz and Kitai, 1998). Accordingly, *in vivo*, blockade of ongoing GABAergic inhibition of striatal neurons has been found to elevate significantly basal activity of these cells (Nisenbaum and Berger, 1992).

GABAergic innervation of striatal cells is essentially intrinsic, as recurrent collaterals of projection neurons and GABAergic interneurons virtually represent the totality of their inhibitory inputs (Plenz and Kitai, 1998; Koos and Tepper, 1999; Tunstall *et al*, 2002). Among the various GABAergic inputs to spiny neurons, those originating from fast-spiking interneurons seem to exert the prominent inhibitory control on the functional activity of these cells (Plenz and Kitai, 1998; Koos and Tepper, 1999). Since these GABAergic interneurons are a preferential location of cannabinoid CB1 receptors in the striatum (Hohmann and Herkenham, 2000), where they act to inhibit GABA release (Iversen, 2003; Piomelli, 2003), it is possible that the IPSCs measured in our study were those originating from the activity of these cells, as they were modulated by cocaine through the engagement of cannabinoid CB1 receptors.

Concluding Remarks

From behavioral studies, it is increasingly clear that endocannabinoids, rather than contributing to the hedonic and psychomotor effects of cocaine assumption, play a central role in the generation and maintenance of addictive behavior, which is commonly believed to reflect the aberrant engagement of synaptic plasticity (long-term depression (LTD) and long-term potentiation (LTP)) at excitatory corticostriatal synapses (Everitt and Wolf, 2002; Gerdeman *et al*, 2003).

The endocannabinoid-dependent inhibition of GABA inputs might favor synaptic plasticity at excitatory corticostriatal synapses by enhancing the level of postsynaptic depolarization required for the induction of both LTP and LTD (Centonze *et al*, 2001). In this line, it has recently been shown that cannabinoid-dependent inhibition of GABA transmission may actually facilitate the emergence of synaptic plasticity at excitatory synapses in the hippocampus (Carlson *et al*, 2002).

In conclusion, we have demonstrated that cocaine significantly perturbs endocannabinoid system in the striatum, and we have identified a possible mechanism by which this perturbation modulates the cellular mechanisms of drug addiction.

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