ABSTRACT

Endocannabinoids are a new class of lipid mediators, which include amides, esters and ethers of long chain polyunsaturated fatty acids. Anandamide (N-arachidonoylethanolamine; AEA) and 2-arachidonoylglycerol (2-AG) are the main endogenous agonists of cannabinoid receptors, able to mimic several pharmacological effects of ∆-9-tetrahydrocannabinol, the active principle of Cannabis sativa preparations like hashish and marijuana. The pathways leading to the synthesis and release of AEA and 2-AG from neuronal and non-neuronal cells are still rather uncertain. Instead, it is known that the activity of AEA is limited by cellular uptake through a specific membrane transporter (AMT), followed by intracellular degradation by a fatty acid amide hydrolase (FAAH). Together with AEA and congeners these proteins form the “endocannabinoid system”. Here, we reported that there are important interactions between the endocannabinoid and dopaminergic systems and that the consequences of these connections become evident in pathological conditions in which one of the two systems is likely to be malfunctioning.

We focused on the involvement of AEA in Parkinson’s disease, demonstrating that changes in endocannabinoid system may partecipate in symptom generation or are part of a compensatory mechanism to counteract the unbalance in basal ganglia physiology. In these studies we have reported that alterations in the endocannabinoid system, associated to a neurological disorder, are restricted to the brain area responsible for this disorder and are reversed by a treatment which corrects the symptoms of the disease.

The interaction endocannabinoid-dopaminergic systems affect also the mesocorticolimbic pathway, involved in the acquisition of behaviour which is reinforced by natural rewarding and drugs of abuse. In this context, we provide evidence that cocaine significantly perturbs endocannabinoid system in the striatum, and we identify a possible mechanism by which this perturbation modulates the cellular mechanisms of drug addiction.
RIASSUNTO

Gli endocannabinoidi sono una nuova classe di mediatori lipidici, a cui appartengono ammidi, esteri ed eteri di acidi grassi poliinsaturi a lunga catena. L'anandamide (N-arachidonooiletanolammina; AEA) ed il 2-arachidonoiilglicerolo (2-AG) sono i due principali agonisti dei recettori cannabici. Legandosi ad essi, AEA e 2-AG sono in grado di mimare alcuni effetti farmacologici del Δ-9-tetraidrocannabinolo, il principio attivo della Cannabis sativa, presente in alcune droghe leggere quali hashish e marijuana. Le vie metaboliche che portano alla sintesi ed al rilascio di AEA e 2-AG dalle cellule neuronali e non-neuronali, necessitano di ulteriori approfondimenti sperimentali per essere definite. Fino ad oggi, è noto che l’attività dell’AEA a livello dei recettori è limitata dal trasporto cellulare attraverso uno specifico trasportatore di membrana (AMT), seguito da una degradazione intracellular ad opera di una idrolasi specifica, “fatty acid amide hydrolase” (FAAH). Queste proteine, insieme con AEA e congeneri, costituiscono il “sistema endocannabinoide”.

In questa tesi abbiamo sottolineato l’importanza delle interazioni esistenti tra il sistema endocannabinoide e quello dopaminergico ed abbiamo evidenziato che le conseguenze di queste interazioni diventano evidenti in alcune condizioni patologiche in cui uno dei due sistemi non funzioni correttamente. Nella prima parte dello studio, abbiamo riportato che alterazioni nel sistema endocannabinoide, associate ad un disordine neurologico, quale il Morbo di Parkinson, sono ristrette all’area del cervello responsabile della patologia e sono revertite dallo stesso trattamento farmacologico usato nella cura della malattia.

L’interazione tra i due sistemi influenza anche il cammino mesocorticolumbico, coinvolto principalmente nelle vie di apprendimento e comportamento, rinforzate dall’abuso di droghe.

Infatti nell’ultima parte della tesi, si evidenzia che la cocaina perturba in modo significativo il sistema endocannabinoide nella regione dello striato e si identifica un possibile meccanismo attraverso il quale questa perturbazione modula il meccanismo cellulare di assuefazione alla droga.
INTRODUCTION

The isolation of a pure form of a psychoactive Cannabis principle, (-)-\(\Delta^9\)-tetrahydrocannabinol ((-)-\(\Delta^9\)-THC), by Gaoni and Mechoulam (Gaoni et al., 1964) prompted several researchers to study its possible mechanisms of action. Structure activity relationships (SAR) studies and the finding that the (+)-\(\Delta^9\)-THC was far less potent than the (-)-\(\Delta^9\)-THC enantiomer (Mechoulam et al., 1992) suggested the presence of a specific receptor for this molecule. The identification of type 1 (CB1) cannabinoid receptors (Devane et al., 1988) confirmed the hypothesis that brain must have its own endogenous compounds able to bind and activate these receptors, rather than synthesize new receptors for exogenous constituents of plants.

In 1992 Devane and co-workers isolated an endogenous THC-like molecule from the porcine brain (Devane et al., 1992). This molecule was named anandamide (N-arachidonylethanolamine; AEA) from “ananda”, the Sanskrit word for “bliss”, and was found to be a CB1 receptor agonist able to mimic the psychotropic effects of THC. Soon after the cloning of the CB1 receptor cDNA (Matsuda et al., 1990), the molecular characterization of a type-2 (CB2) cannabinoid receptor was reported (Munro et al., 1993). In a few years a number of endogenous agonists of CB receptors were characterized and were collectively called “endocannabinoids”. Although structurally different from plant cannabinoids, these metabolites were termed endocannabinoids in analogy with the “endorphins”. The chemical structures of the most biologically active endogenous cannabimimetic compounds are represented in figure 1.
Figure 1. Chemical structures of biologically active endogenous cannabimimetic compounds.
THE ENDOCANNABINOID SYSTEM

Endocannabinoids are lipid mediators, isolated from brain and peripheral tissues, which include amides, esters and ethers of long chain polyunsaturated fatty acids. The first endocannabinoid that was discovered is anandamide, the ethanolamide of arachidonic (eicosatetraenoic) acid. Since then, other two endogenous agonists of CB receptors have been identified: 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) and 2-arachidonoylglyceryl ether or noladin ether (Hanus et al., 2001). More recently, during the development of a bioanalytical method to quantitate anandamide, a new endocannabinoid was identified, that consists of arachidonic acid and ethanolamine joined together by an ester bond, instead of the amide bond of AEA (Porter et al., 2002). For this reason, “inverted AEA” was termed “virodhamine”, after the Sanskrit word for opposition “virodha”. In human, mouse and rat brain further cannabimimetic fatty acid amides were found to be present, like N-palmitoylethanolamine (PEA), N-oleoylethanolamine (OEA) and N-stearoylethanolamine (SEA). These molecules are better considered “endocannabinoid-like” compounds (Di Marzo et al., 1998a), because they have some biological activities either cannabimimetic or non-cannabimimetic, have a potential structural role in lipid bilayers, and can have a further “entourage effect” (Lambert et al., 1999). In particular SEA has been shown to be a true cannabimimetic compound, which activates non-CB1/non-CB2 receptors within the CNS of mouse (Maccarrone et al., 2002; Terrazzino et al., 2004). The cannabimimetic activity of AEA and congeners is assessed through a tetrad of mouse behavioural assays that, when performed together, are highly indicative of a cannabinoid-like compound (Martin et al., 1991).

One of the requisites for a molecule to be considered as an endogenous modulator, is the presence of a mechanism for its synthesis and degradation in the cell. AEA and 2-AG are not stored in intracellular compartments, but are produced on demand by receptor-stimulated cleavage of lipid precursors. The non synaptic release mechanism and short life spans of AEA and 2-AG suggest that these compounds might act near their site of synthesis to regulate the effects of primary messengers, such as neurotrasmitters and hormones. The pathways of AEA degradation and, to a lesser extent, synthesis are known in details, while those of 2-AG are not clear yet.
Synthesis and Degradation

Two different pathways were proposed to explain the synthesis of AEA: the first route can occur through the direct enzymatic condensation between the free arachidonic acid and ethanolamine (Kruszka et al., 1994; Devane et al., 1994). It is now believed that this reaction is catalyzed by a reverse fatty acid amide hydrolase (FAAH) or an AEA hydrolase “working in reverse” under non-physiological \textit{in vitro} conditions (Kurahashi et al., 1997). In fact substrate concentrations in the millimolar range, much higher than those detected in cells, are required to form AEA in this way, therefore it is very likely that most of intracellular AEA is formed through a different synthetic pathway. It is now widely accepted that AEA is produced by a transacylase-phosphodiesterase-mediated synthesis (Fig. 2). In fact, the AEA precursor is \textit{N}-arachidonoylphosphatidylethanolamine (NArPE), which is believed to originate from the transfer of arachidonic acid from the \textit{sn}-1 position of 1,2-\textit{sn}-di-arachidonoylphosphatidylcholine to phosphatidylethanolamine, catalyzed by a calcium-dependent \textit{N}-acyltransferase (NAT). NArPE is then cleaved by a phosphodiesterase of the phospholipase D (PLD) type, indicated as \textit{N}-acylphosphatidylethanolamine (NAPE)-hydrolyzing PLD (NAPE-PLD), which releases AEA and phosphatidic acid. Recently, this enzyme has been purified from rat heart (Okamoto et al., 2004) and by use of the sequences of its internal peptides cloned its complementary DNAs from mouse, rat and human.
Figure 2. Mechanism of anandamide formation: the sequence of reactions is thought to include: first, the synthesis of the anandamide precursor N-arachidonoyl-phosphatidylethanolamine (PE), catalysed by the enzyme N-acyltransferase; second, the cleavage of N-arachidonoyl-PE to yield anandamide, catalysed by phospholipase D.

(Fonte: Piomelli, Nat Rev Neurosci 4: 873-884, 2004)
A similar route can be operational also for the synthesis of the other cannabimimetic NAEs, since their precursors N-acylethanolamine phospholipids are ubiquitous constituents of animal and human cells, tissues and body fluids (Schmid, 2000). This biosynthetic pathway could explain the different percentages of NAEs in various tissues, because their concentrations may reflect the amounts of arachidonic acid esterified on the sn-1 position of phospholipids. An illustration of the receptor-dependent release of AEA from membrane phospholipids has been provided by microdialysis studies, which suggest that AEA may be released in the brain striatum upon activation of D$_2$-type dopamine receptors and that such release may be involved in counterbalancing the stimulatory effects of dopamine on motor activity (Giuffrida et al., 1999; Beltramo et al., 2000).

The biological activity of AEA and 2-AG is terminated by their removal from the extracellular space, which occurs through a two-step process: i) cellular uptake, followed by ii) intracellular degradation (Fig. 3).

Figure 3. Mechanisms of endocannabinoid deactivation: anandamide (AEA) and 2-arachidonoylglycerol (2-AG) can be internalized by neurons through a high-affinity transport mechanism, the “endocannabinoid transporter”. Once inside cells, they can be hydrolysed by distinct serine hydrolases — anandamide by fatty acid amide hydrolase (FAAH) and 2-AG by monoglyceride lipase (MGL) (not shown) — to yield inactive breakdown products. (Fonte: Piomelli, Nat Rev Neurosci 4: 873-884, 2004)
AEA and 2-AG can diffuse passively through lipid membranes, but this process is accelerated by a rapid and selective AEA membrane transporter (AMT). Several lipid-carrier proteins have been molecularly cloned, inspiring optimism that, despite current controversy, endocannabinoid transporter will eventually characterized. However its activity has been demonstrated in a variety of *in vitro* systems (Hillard et al., 2000), including many cells of human origin like neuroblastoma (CHP100) and lymphoma (U937) cells (Maccarrone et al., 1998), platelets (Maccarrone et al., 1999), endothelial cells (Maccarrone et al., 2000a), mastocytes (Maccarrone et al., 2000b), and peripheral lymphocytes (Maccarrone et al., 2001). The activity of AMT has been demonstrated also in *ex vivo* systems like cortical areas and striatum of rats (Giuffrida et al., 2001), as well as synaptosomes from human, mouse and rat brain (Battista et al., 2002). Selective inhibitors of AMT are useful tools not only to study the physiological role of AEA through *in vitro* and *in vivo* systems, but also as possible therapeutic agents. In fact, several studies *in vivo* demonstrated that this compound can affect some analgesic (Beltramo et al., 1997), behavioural (Giuffrida et al., 2000a; Gonzales et al., 1999), cardiovascular (Calignano et al., 1997) and neuronal (Baker et al., 2001) effects of AEA.

Once taken up by cells, AEA is cleaved by a fatty acid amide hydrolase (arachidonoylethanolamide amidohydrolase, EC 3.5.1.4; FAAH), which breaks the amide bond and releases arachidonic acid and ethanolamine. In 1996 this protein (Cravatt et al., 1996) was isolated, purified and cloned; its primary sequence suggests the presence of: *i*) a highly hydrophobic transmembrane domain at the N terminus, which does not affect enzyme activity but directs protein oligomerization; *ii*) a serine- and glycine-rich domain, which contains a typical “amidase signature” sequence between amino acids 215-257 in mammalian FAAH; and *iii*) a proline-rich domain, which is homologous to the class II SH3-binding domain. This amide hydrolase recognizes a broad spectrum of fatty acid amides and esters, such as oleamide and 2-AG. FAAH is a membrane-bound enzyme found mainly in microsomal and mitochondrial fractions of rat brain and liver (Deutsch et al., 1993; Desarnaud et al., 1995; Hillard et al., 1995), and of porcine brain (Ueda et al., 1995). The enzyme exhibits a molecular weight of 64 kDa, works optimally at pH values around 9 (Ueda et al., 2000), and has been recently crystallized and analyzed at 2.8Å resolution (Bracey et al., 2002). Interestingly, within the CNS the distribution of FAAH correlates well with the presence of CB1 receptors, though there seems to be no direct correlation between the levels of expression of the genes coding for these two proteins.
(Thomas et al., 1997). Recent histochemical data show that in mouse brain FAAH is mainly localized in neuronal somata and their dendrites (Egertová et al., 2003). Moreover, AEA was shown to serve as a substrate for oxidative processes by a number of fatty acid oxygenases. These reactions are catalysed by enzymes of the arachidonic acid cascade such as cyclooxygenase-2 (COX-2), lipoxygenase (LOX) and cytochrome P450 (Burstein et al., 2000). Conformational analysis of these AEA-derivatives revealed clear changes in their interaction with CB1 or CB2 receptors and FAAH, but not with AEA transporter (Van der Stelt et al., 2002). Accordingly, it was demonstrated that in U937 cells the hydroperoxides generated from AEA by LOX are potent inhibitors of FAAH (Maccarrone et al., 1998; Van der Stelt et al., 2002). These data suggest that the oxygenated metabolites of AEA may well influence the endocannabinoid system, with an obvious impact on its biological activities.

**Molecular Targets**

Once released by cells, endocannabinoids act primarily at cannabinoid receptors. The receptor family includes type-1 (CB1) cannabinoid receptors, that are present mainly on the central and peripheral neurons, type-2 (CB2) cannabinoid receptors, expressed predominantly by peripheral cells, non-CB1/non-CB2 cannabinoid receptors, non-cannabinoid receptors and vanilloid receptors (Reggio et al., 2000; Khanolkar et al., 2000; Howlett et al., 2000, 2001). CB1 and CB2 receptors belong to the family of the seven trans-membrane spanning receptors coupled to G proteins, particularly those of the G\textsubscript{i/o} family (Howlett et al., 2000). The binding of endocannabinoids to these receptors induces some biological actions such as the inhibition of adenylate cyclase (with its consequent decrease in cytosolic cAMP concentrations), the regulation of ionic currents (inhibition of voltage-gated L, N and P/Q-type Ca\textsuperscript{2+} channels, activation of K\textsuperscript{+} channels), the activation of focal adhesion kinase, of mitogen-activated protein kinase, of cytosolic phospholipase A\textsubscript{2} and of nitric oxide synthetase (NOS) (Table 1).

A new molecular target of AEA which is attracting great interest is the type-1 vanilloid receptor (TRPV1), a six trans-membrane spanning protein with intracellular N- and C-terminals and a pore-loop between the fifth and sixth transmembrane helices (Jung et al., 1999). TRPV1 is a ligand-gated and non-selective cationic channel, activated by molecules derived from plants, such
as capsaicin, the pungent component of “hot” red peppers, and resiniferatoxin, and also by stimuli like heat and protons (Szallasi et al., 1999). In the last three years, a number of studies suggested a physiological role for AEA as TRPV1 agonist, leading to the concept that AEA, besides being an endocannabinoid, is also a true “endovanilloid” (De Petrocellis et al., 2001). The interaction of AEA with TRPV1 occurs at a cytosolic binding side (De Petrocellis et al., 2001; Jordt et al., 2002), and triggers the following intracellular responses: activation of non-selective ion channels, activation of protein kinases, increase in intracellular Ca\(^{2+}\) concentration, and interestingly mitochondrial uncoupling (Maccarrone et al., 2000c; Yamaji et al., 2003). TRPV1 is expressed in peripheral sensory fibres (Szallasi, 1999) and also in several nuclei of the CNS (Mezey et al., 2000), thus suggesting the existence of brain endogenous agonists for this receptor. SAR studies and the finding that some synthetic \(N\)-acyldopamines (Bisogno et al., 2000) are ligands for CB1 receptor has led to the identification of \(N\)-arachidonoyldopamine (NADA) (Fig. 1), as an endogenous capsaicin-like substance with high potency (Huang et al., 2002). In fact, this molecule can activate TRPV1, but it is also a potent cannabimimetic compound. Altogether, these reports expand the overlap between the endogenous cannabinoid system and the vanilloid system.
<table>
<thead>
<tr>
<th>Molecular target</th>
<th>Biological actions</th>
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<tbody>
<tr>
<td>“Classical” cannabinoid (CB1 or CB2) receptors</td>
<td>Inhibition of adenyl cyclase (i.e., of forskolin-induced cAMP formation)</td>
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<td></td>
<td>Inhibition of L-type, N-type and P/Q-type Ca\textsuperscript{2+} channels</td>
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<td>Activation of inwardly rectifying K\textsuperscript{+} channels</td>
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<td>Activation of the mitogen-activated protein kinase (MAPK) pathway</td>
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<td>Activation of cytosolic phospholipase A\textsubscript{2}</td>
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<td></td>
<td>Activation of neuronal focal adhesion kinase (FAK)</td>
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<td></td>
<td>Activation of nitric oxide synthase (NOS)</td>
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<td></td>
<td>Suppression of prolactin receptors</td>
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<td></td>
<td>Decline in systemic and mesenteric vascular resistance</td>
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<td></td>
<td>Protection against apoptosis</td>
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<td>“Non-classical” (non-CB1/non CB2) cannabinoid receptors (CBn)</td>
<td>Release of arachidonic acid</td>
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<td></td>
<td>Activation of the mitogen-activated protein kinase (MAPK) pathway</td>
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<td></td>
<td>Inhibition of gap junction activity</td>
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<td>Inhibition of gap junction-mediated and glutamate-triggered Ca\textsuperscript{2+} waves</td>
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<td>Activation of platelets</td>
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<td>Hypotension and mesenteric vasodilation</td>
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<td>Stimulation of cytokine-dependent proliferation of lymphoid cells</td>
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<tr>
<td>Non-cannabinoid receptors and/or non-receptor-mediated actions</td>
<td>Inhibition of L-type Ca\textsuperscript{2+} channels</td>
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<td>Inhibition of Shaker-related voltage-gated K\textsuperscript{+} channels</td>
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<td>Inhibition of serotonin 5-HT\textsubscript{3} receptor-mediated currents</td>
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<td></td>
<td>Activation of N-methyl-D-aspartate (NMDA)-mediated Ca\textsuperscript{2+} currents</td>
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<td>Release of arachidonic acid</td>
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<td></td>
<td>Increase in synaptosomal membrane lipid order</td>
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<td></td>
<td>Activation of protein kinase C</td>
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<td></td>
<td>Activation of platelets</td>
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<tr>
<td>Vanilloid (TRPV1) receptors</td>
<td>Vascular relaxation</td>
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<td></td>
<td>Release of the vasoactive calcitonin gene-related peptide (CGRP)</td>
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<td>Rise in intracellular Ca\textsuperscript{2+}</td>
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<td>Mitochondrial uncoupling</td>
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<td>Induction of apoptosis</td>
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Table 1. Effects of AEA, 2-AG or both at different molecular targets.
THE DOPAMINERGIC SYSTEM

Dopamine (DA) is the most abundant catecholamine in the brain and it plays an important role as neurotransmitter in the central nervous system (CNS). DA is synthesized by two steps: the aminoacid tyrosine is transformed in L-3,4-dihydroxyphenylalanine (L-DOPA) and sequentially it is decarboxylated to produce DA.

![Dopamine Synthesis Diagram]

Projections originating from brain areas that synthesize this neurotransmitter give rise to four axonal pathways: (1) nigro-striatal; (2) mesolimbic; (3) mesocortical; and (4) tuberoinfundibular. Each pathway is responsible for the regulation of different physiological functions in CNS.

Dopamine exerts its actions by binding to specific membrane receptors, which belongs to the family of seven transmembrane domain (7TM) G-protein coupled receptors. Dopamine receptors are divided into two subfamilies, D1- and D2-like, on the basis of their biochemical and pharmacological properties. The D1 and D5 receptors are classified as “D1-like” because they share high sequence homology and have classical D1 pharmacology. They are located exclusively on postsynaptic neurons, while the “D2-like” (D2-, D3- and D4-R) has a presynaptic location. The binding to one of DA-R activates numerous signal trasduction pathways (Fig. 4) such as the activation or inhibition of the cAMP and modulation of Ca^{2+} signaling, which are the best-described effects. D1- and D2-R are also involved in the cascade of arachidonic acid (AA). It has been demonstrated that in rat striatal cells D1- and D2-R have opposing effects; indeed, while D2-R stimulates AA synthesis, D1-R inhibits it, thus demonstrating a cell specific effect of D1- and D2-R activation.

The importance of DA in the control of movements is demonstrated in pathological conditions, such as Parkinson’s disease (PD). In fact, it has
been shown that the administration of low doses of D2-R agonists causes a reduction of locomotor functions, probably by stimulating pre-synaptic receptors, and this should lead to a reduction of dopaminergic cell firing and DA release. The injection of D2-R antagonists, as well as D1-R antagonists, decreases motor activity. The generation of genetically modified mice for different components of the dopaminergic pathway could be an useful tool for studying the roles of these proteins in dopamine-related neuropsychiatric disorders.

Instead, the mesolimbic dopaminergic system is implicated in the control of reward mechanism and in the psychomotor effects generated by drugs of abuse, including cocaine. Addiction results from changes in brain function in response to repeated exposure to one or more drugs. Activation of mesolimbic dopamine projections underlies the reinforcing properties of virtually all drugs of abuse. Psychostimulants increase extracellular dopamine levels in limbic nuclei by inhibiting the dopamine transporter (DAT), which is responsible for removing dopamine from synapse, and by reversing DA transport via DAT.

DA plays a critical role in habit formation and in the effects of cocaine in the striatum (Jog et al. 1999; Canales et al., 2000; Everitt et al., 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 et al., 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 et al., 1995; Johanson et al., 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 et al., 2000).

Using animal models and pharmacological approaches, it has been possible to demonstrate that the blockade of DA receptors by D1- or D2-like receptor
antagonists, attenuate hyperlocomotion and the reward effects caused by amphetamine, cocaine and morphine in mice or rats. On the contrary, the administration of D2-like receptor agonists, such as quinpirole, mimic the effect of cocaine or enhance these effects when administered in combination with cocaine.

Figure 4. Schematic representation of dopaminergic system and dopamine neurotransmission.
ANANDAMIDE, DOPAMINE AND PD

Anandamide exhibits a wide range of brain-mediated effects in mice, rats and humans. Several experimental data point towards a role for AEA in the control of critical neuronal functions, like pain initiation (Calignano et al., 1998, 2000; Walker et al., 1999), psychomotor behaviour (Giuffrida et al., 2000b), memory and sleep (Fride et al., 2002), and vision (Yazulla et al., 1999). The use of cannabis in folklore medicine both to alleviate the symptoms of neurodegenerative diseases and as appetite-stimulant in AIDS and cancer patients, prompted the researchers to compare the pharmacological effects of AEA to those of THC (Grundy, 2002). Unfortunately, the rapid in vivo degradation of AEA by FAAH (Di Marzo et al., 1998b) prevents this endogenous compound from displaying the same activity as the plant-derived and synthetic cannabinoids.

Each behavioural effect of endocannabinoids can be assigned to a specific brain region, which can be shown to contain also CB1 receptors. The density of the latter proteins is highest in brain areas controlling movement disorders (substantia nigra, globus pallidus, basal ganglia), while low levels of CB1 receptors are found in cortex, diencephalon and cerebellum. Yet, the concentrations of AEA in these areas do not correlate with the distribution of CB1 receptors, for instance the brainstem is one of the regions with the highest levels of endocannabinoids and the lowest density of CB1 receptors (Bisogno et al., 1999; Di Marzo et al., 2000). This lack of association seems to suggest that the molecular targets of AEA and congeners go beyond the cannabinoid receptors.

The importance of the endocannabinoid system is also due to its ability to interfere with the release, uptake and actions of excitatory and inhibitory transmitters such as dopamine, γ-aminobutyric acid (GABA) and glutamate. The proposed model to explain the neuromodulatory actions of AEA involves the release of endocannabinoids from a postsynaptic neuron upon stimulation, then the back diffusion to presynaptic terminals, where AEA activates G-protein-coupled CB1 receptors, thus modulating neuronal membrane permeability to Ca\(^{2+}\) and K\(^+\) ions and the activity of adenylyl cyclase (Fig. 5). The final outcome is a modified action of neurotransmitters (Pertwee, 1997).

The first indication to a possible interactions between different receptors is given, when both receptors are expressed in the same brain region and within the same neuron. The wide CB1 receptor distribution in the striatum, a brain region involved in motor processes, cognition and motivation (Herkenham et
and the high coexpression rate of CB1 and D1-, D2-R in basal ganglia (Hermann et al., 2002), provide the opportunity for functional interactions of endogenous cannabinoids, such as AEA and 2-AG, with the dopaminergic nigrostriatal pathway. Interestingly, D2 and CB1 receptors share the same signal transduction pathway, and closely cooperate in the negative regulation of striatal excitatory transmission (Meschler et al., 2001). In fact, a recent evidence in freely moving rats has shown that AEA release is enhanced by quinpirole, a D2-like DA receptor agonist (Giuffrida et al., 1999), and that AEA transport inhibition reverses DA D2-like receptor responses (Beltramino et al., 2000). Moreover, chronic treatment with D2 antagonists results in an increased expression of CB1 receptor mRNA in the striatum (Mailleux et al., 1993). Finally, activation of both D2 and CB1 receptors decreases adenyl cyclase activity through the same G protein (Meschler et al., 2001), which in turn stimulates mitogen-activated protein kinases (Boulaboula et al., 1995). This pathway is involved in cell survival and synaptic plasticity (Coogan et al., 1999; Otani et al., 1999; Calabresi et al., 2001; Sweatt, 2001), supporting the idea of a close interaction between DA and (endo)cannabinoids in these critical functions.
Figure 5. The endocannabinoid system at the pre- and post-synaptic nerve terminal (AA, arachidonic acid; AC, adenylate cyclase; AEA, anandamide; AMT, anandamide membrane transporter; cAMP, cyclic AMP; CB1R, CB1 receptor; EtNH2, ethanolamine; FAAH, fatty acid amide hydrolase).
The actions and interactions of these neurotransmitters and receptors have great relevance for the physiology and the potential pharmacological treatment of several movement disorders, of which PD is a well-documented example.

PD is a chronic, progressive disorder characterized by resting tremors, dyskinesia and postural instability (Soubrouillard et al., 1997; Blandini et al., 2000). The clinical syndrome appears when approximately 80% of striatal dopamine has been lost. It has been reported that permanent loss of dopaminergic terminals in the striatum results in an abnormal activity of corticostriatal glutamatergic transmission (Calabresi et al., 1993, 2000). In addition the critical role of D1 dopamine receptors in dyskinetic animals has been shown, along with its involvement in the loss of depotentiation observed in PD patients treated with L-DOPA (Picconi et al., 2003). Only recently it was demonstrated that the endocannabinoid system is involved in PD.

Antagonists of endocannabinoid receptors were used to reduce dyskinesia, which is the major side-effect elicited by long-term dopamine replacement therapy (Segovia et al., 2003). In the same line, CB1 receptor antagonist SR141716A has been tested on rats treated with reserpine, and was found to be effective in restoring locomotion when injected with a D2 receptor agonist (Di Marzo et al., 2000). Recent data indicate that the systemic administration of AM404, a modulator of AEA function, induce significant antiparkinsonian effects, as revealed by improvement of akinesia and sensorimotor orientation, as well as a reduction of drug-induced turning (Fernandez-Espejo et al., 2004). However, conflicting results were reported in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)-treated non-human primates (Meschler et al., 2001), suggesting that species-specific constrains might exist. In particular, modulation of the expression of CB1 receptors could be critical for the development of Parkinson’s disease, because the level of CB1 receptor mRNA falls in the striatum of reserpine-treated rats (Silverdale et al., 2001). Furthermore, a pilot clinical study performed in a group of PD patients demonstrated that nabilone, a synthetic cannabinoid receptor agonist commercially available in United States and in England, significantly alleviated L-DOPA-induced dyskinesia (Sieradzan et al., 2001).
OBJECTIVES OF THE RESEARCH

The etiology of PD is still not fully understood, but genetic analysis, neuropathologic investigations, and new experimental models of PD are providing important new insights into this pathogenesis. The aim of this study is to evidence the involvement of the endocannabinoid system in an animal model of PD, to suggest the potential therapeutic use of its tools for the treatment of this disease and to confirm the functional relationship between cannabinoid and dopaminergic systems not only in basal ganglia-related movement disorders, but also in drug abuse problems. In a preliminary study, we have characterized, in a rat model of PD, the activity of enzymes responsible for the synthesis and degradation of AEA and we analyzed the effects of some inhibitors of these proteins on corticostriatal glutamatergic transmission. On the ground of these first results, we studied the endogenous cannabinoid system after chronic treatment by levodopa to investigate if the abnormalities in the endocannabinoid system could be correlated to pharmacological therapy used in PD treatment. In the last study, we focused on the role of endocannabinoids in abuse drugs problem and we highlighted that cocaine alters the endocannabinoid system by a mechanism which involves CB1 receptors.
PARKINSON’S DISEASE RESULTS

In our first study, we show that experimental parkinsonism, induced by 6-OHDA treatment, causes complex plastic changes of the endocannabinoid system. In particular, we have obtained four novel findings in the striatum of 6-OHDA-denervated rats: (i) the levels of endogenous AEA are increased; (ii) the activity of the AEA membrane transporter, as well as that of the AEA hydrolase FAAH, is reduced; (iii) the level of CB1 receptor and the binding of AEA to this receptor is not affected; (iv) the pharmacological inhibition of FAAH produces a much stronger depression of striatal glutamatergic activity compared to naïve rats.

Rearrangement of striatal cannabinoid system in parkinsonian rats

The analysis of the level of endogenous cannabinoid showed a content of anandamide 3-fold higher in the striatum of 6-OHDA-lesioned rats, compared to naïve animals, whereas endogenous 2-AG was unaffected (Table 2). We found that the activity of both AEA membrane transporter and AEA-hydrolyzing enzyme FAAH decreased in parkinsonian animals (Table 2 and Fig. 6A-B). The affinity of AMT for AEA was identical in naïve and parkinsonian rats (K_m = 383 ± 45 and 351 ± 49 nM, respectively; n = 4, p > 0.05, Student’s t (ST) test), while the apparent V_max (181 ± 9 and 81 ± 4 pmol/min per mg protein, respectively) was significantly lower in the latter group (n = 4, p < 0.01, ST test). Remarkably, the affinity of AMT for AEA was very close to that reported for the transporter in several cellular models and analogously, the V_max of FAAH decreased in parkinsonian rats (580 ± 48 and 1687 ± 165 pmol/min per mg protein, respectively; n = 4, p < 0.01, ST test), while the K_m for AEA was unaffected (10.5 ± 1.7 and 12.4 ± 2.2 µM, respectively; n = 4, p > 0.05, ST test). Thus, the finding that endogenous levels of AEA are higher in parkinsonian rats may reflect a compensatory mechanism to control the cortical glutamatergic drive to the striatum. However, this mechanism seems not to be sufficient in 6-OHDA-lesioned rats, since spontaneous excitatory activity is still higher in these animals. In fact, only the further increase of endogenous AEA tone, achieved by AMT or FAAH blockade, restores the normal corticostriatal function. The binding of the synthetic cannabinoid [^3^H]CP55,940 to CB1 receptors was not affected after 6-OHDA denervation (Table 2 and Fig. 6C), showing...
a $K_d = 358 \pm 80$ and $315 \pm 73$ pM, and a $B_{max} = 350 \pm 35$ and $346 \pm 35$ fmol/mg protein, in naïve and parkinsonian rats, respectively ($n = 4$; $p > 0.05$ for both, ST test). These $K_d$ values are close those previously reported for the binding of $[^3H]CP55,940$ to rat striatal membranes. Interestingly, also the binding of $[^3H]AEA$ was not affected by 6-OHDA denervation (Table 2), neither was the activity of phospholipase D (PLD), which remained almost identical in naïve and 6-OHDA-lesioned striata (Table 2). This is noteworthy, because NAPE-hydrolyzing PLD activity is considered the check-point in AEA synthesis, though the lack of specific inhibitors for this enzyme makes it difficult to assess conclusively its contribution to AEA metabolism (Hansen et al., 2000).
### Table 2. Endogenous levels of anandamide (AEA) and 2-arachidonylglycerol (2-AG), activity of phospholipase D (PLD), of the AEA membrane transporter (AMT) and of the fatty acid amide hydrolase (FAAH), and binding of AEA and CP55,940 in the striatum of rats.

<table>
<thead>
<tr>
<th></th>
<th>Endogenous levels of AEA&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Endogenous levels of 2-AG&lt;sup&gt;1&lt;/sup&gt;</th>
<th>NAPE-PLD activity&lt;sup&gt;2&lt;/sup&gt;</th>
<th>AMT activity&lt;sup&gt;3&lt;/sup&gt;</th>
<th>FAAH activity&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Binding of AEA&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Binding of CP55,940&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>naïve</td>
<td>250 ± 20</td>
<td>1250 ± 120</td>
<td>320 ± 32</td>
<td>90 ± 10</td>
<td>470 ± 50</td>
<td>150 ± 15</td>
<td>114 ± 11</td>
</tr>
<tr>
<td>6-OHDA lesioned</td>
<td>750 ± 80 *</td>
<td>1500 ± 125</td>
<td>350 ± 35</td>
<td>35 ± 4 *</td>
<td>180 ± 20 *</td>
<td>167 ± 16</td>
<td>130 ± 12</td>
</tr>
</tbody>
</table>

<sup>1</sup>Expressed as pmol per mg protein.

<sup>2</sup>Expressed as pmol/min per mg protein (substrate was 10 µM 1,2-dioleoyl-3-phosphatidyl-[2-<sup>14</sup>C]ethanolamine).

<sup>3</sup>Expressed as pmol/min per mg protein (substrate was 400 nM [<sup>3</sup>H]AEA).

<sup>4</sup>Expressed as pmol/min per mg protein (substrate was 5 µM [<sup>3</sup>H]AEA).

<sup>5</sup>Expressed as fmol per mg protein (ligand was 200 pM [<sup>3</sup>H]AEA).

<sup>6</sup>Expressed as fmol per mg protein (ligand was 200 pM [<sup>3</sup>H]CP55,940).

*<sup>P < 0.01 compared to control (n = 4).</sup>
Figure 6. Biochemical changes of the cannabinoid system in experimental parkinsonism. A. The activity of the anandamide (AEA) membrane transporter (AMT) is reduced in 6-OHDA-lesioned animals compared to naïve. B. The activity of the AEA hydrolase FAAH is lower in parkinsonian animals. C. The binding of \([^3H]CP55,940\) is not changed after the treatment with 6-OHDA.
Spontaneous glutamatergic activity in naïve and 6-OHDA-lesioned rats

Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from cortico-striatal rat slices by means of whole-cell patch-clamp techniques. Spiny neurons from 6-OHDA-lesioned rats showed in average a higher sEPSCs frequency and amplitude compared to naïve rats (1.9 ± 0.8 vs. 6.2 ± 1.2 Hz, n = 30, p < 0.01, ST test, and 13.7 ± 2.1 vs. 22.3 ± 5.5 pA, n = 28, p < 0.01, ST test, respectively), while the intrinsic membrane properties of these neurons were not affected (Calabresi et al., 1993). This synaptic activity was suppressed by 10 µM CNQX, an AMPA receptor blocker, but it was not affected by 3 µM bicuculline, a GABA_A receptor antagonist (not shown).

Activation of CB1 cannabinoid receptor and endogenous anandamide reduce striatal glutamatergic spontaneous activity

Activation of CB1 cannabinoid receptors has been reported to reduce glutamate release in the striatum through a presynaptic mechanism (Gerdeman et al., 2001; Huang et al., 2001).

In our preparation, 10 min application of HU-210, a CB1 receptor agonist (Pertwee, 1997), reduced sEPSCs frequency in a dose-dependent manner in both 6-OHDA-lesioned and naïve rats. Fig. 7A shows single experiments where this effect was evident, while all the experiments in naïve and 6-OHDA-lesioned animals are pooled in Fig. 8A. Interestingly, the average sEPSCs amplitude was not affected. Fig. 9A shows the normalized effect of HU-201 on sEPSCs frequency that was similar in the two groups.

Blockade of AEA uptake by 10 min application of AM-404 (Piomelli et al., 1999; Beltramo et al., 2000) significantly reduced the frequency of glutamatergic sEPSCs in a dose-dependent manner in both naïve and 6-OHDA-lesioned rats. Single experiments are shown in Figure 7B, while all data are pooled in figure 8B. Also AM-404 did not significantly affect sEPSCs amplitude. The normalized effect of AM-404 on sEPSCs frequency was similar in the two groups (Fig. 9B).

Figure 9D shows the time-course of 10 µM AM-404 effect. This figure also shows that AM-404 occludes a further reduction of synaptic activity by 1 µM HU-210. These effects, when normalized, were similar in naïve and 6-OHDA-lesioned animals. Similar results were obtained with 10 µM VDM11.
Figure 7. Cannabinoids reduce the frequency of glutamatergic sEPSCs in striatal spiny neurons of naïve and parkinsonian (6-OHDA) rats. A-D. Cumulative probability plots of glutamatergic sEPSCs recorded from single striatal neurons of naïve (left) and a 6-OHDA-lesioned (right) rats. Electrophysiological traces show spontaneous striatal glutamatergic activity before (upper) and after (lower) drug administration. Application of 1 µM HU-210 (A) reduces significantly sEPSCs frequency (expressed as inter-event interval) in both neurons (p < 0.01 for both, KS test). A similar effect is obtained with 10 µM AM-404 (B) in both naïve (p < 0.05, KS test) and 6-OHDA-lesioned (p < 0.01, KS test) animals. Conversely, 10 µM PMSF (C) is ineffective on the naïve neuron (p > 0.05, KS test) but reduces sEPSCs frequency (p < 0.01, KS test) in the 6-OHDA-lesioned cell. Similarly, 25 nM MAFP was effective in decreasing sEPSCs frequency only in the cell from a parkinsonian rat (p < 0.01, KS test) but not in the naïve one (p > 0.05, KS test).
Figure 8. Effect of cannabinoids on glutamatergic sEPSCs frequency and amplitude. Histograms in A-D summarize the effect of the different concentrations of HU-210 (A), AM 404 (B), PMSF (C), and MAFP (D) on sEPSCs frequency (left) and amplitude (right) of all experiments. Note that the average control frequency (expressed in Hz) and amplitude is higher in 6-OHDA-lesioned rats. Both HU-210 and AM-404 significantly reduce sEPSCs frequency at all doses, without affecting their amplitude. Conversely, PMSF is effective in naïve animals only at the higher dose (100 µM), while parkinsonian rats are more sensitive to low doses (3 µM) of this compound. Similar results were obtained with MAFP, which is active at 100 nM on naïve animals, while at 25 nM is active on 6-OHDA-lesioned ones. Also PMSF and MAFP do not affect sEPSCs amplitude. (*p < 0.01 compared to pre-drug control; †p < 0.01 compared to pre-drug control, and p > 0.05 between groups; ‡p < 0.05 compared to pre-drug control; all values on graphs A-C, except †, are p < 0.01 between groups; ST test; n = 4 for each condition).
Figure 9. Pharmacology of cannabinoid-mediated reduction of striatal glutamatergic activity. A-C. Normalized dose-response curves of the sEPSCs frequency inhibition by HU-210 (A), AM-404 (B), and PMSF (C). Only the effect of PMSF is significantly different in the two experimental groups (*p < 0.01 compared to pre-drug control, and p > 0.05 between groups; §p > 0.05 compared to pre-drug control; ^p < 0.05 compared to pre-drug control, and p > 0.01 between groups; ST test; n = 4 for each point). D. The inhibition of sEPSCs frequency by 10 µM AM-404 (p < 0.01 compared to control, ST test) shows that this effect was long-lasting and occluded further depression by 1 µM HU-210. This effect was similar in naïve and parkinsonian rats (p > 0.05 between groups, ST test; n = 3 for each group).
(n = 4; not shown), another selective inhibitor of AMT (De Petrocellis et al., 2001). Application in naïve animals of phenylmethylsulfonyl fluoride (PMSF), an inhibitor of FAAH (Ueda et al., 2000; Wiley et al., 2000), caused a significant reduction of sEPSCs frequency only at high doses (100 µM), whereas methyl-arachidonoyl fluorophosphonate (MAFP), a potent inhibitor of FAAH (Deutsch et al., 1997; Ueda et al., 2000), was effective at 100 nM (Fig. 8D). Surprisingly, 6-OHDA treatment revealed a potent inhibitory effect of PMSF even at very low concentrations (3-30 µM), and MAFP was effective even at 25 nM (see Fig. 8C-D for single experiments and Fig. 8C-D for average data on both compounds). Again, neither PMSF nor MAFP had significant effects on sEPSCs amplitude either in naïve or in 6-OHDA-lesioned rats. Figure 9C shows the normalized effect of PMSF on sEPSCs frequency in both groups.

Pharmacology of the endocannabinoid effect

We also investigated the possibility that the effect of AEA on synaptic spontaneous activity was specifically mediated by CB1 receptors. Since AEA is quickly hydrolyzed by FAAH, in these experiments we co-administered 1 µM AEA and a low dose of PMSF (10 µM). Slices were pre-incubated in PMSF for 10 minutes before their co-application with AEA. While 3-30 µM PMSF per se was not able to affect sEPSCs frequency in naïve rats (Figure 2C, 8C, 9C and 10A), AEA plus PMSF was effective in reducing glutamatergic activity in both naïve and 6-OHDA-lesioned animals (Fig. 10A).

We also used SR-141716, a specific CB1 receptor antagonist (Pertwee, 1997), to further investigate the pharmacological site of action of endogenous cannabinoids. While SR-141716 per se did not alter glutamatergic spontaneous activity, 10 minutes of pre-treatment with this drug blocked the effect of 10 µM AM-404 or 10 µM PMSF in both naïve and 6-OHDA-lesioned animals (Fig. 10B).

It has been reported that AEA can also activate other receptors rather than CB1, for example the vanilloid TRPV1 receptor (Zygmunt et al., 1999; De Petrocellis et al., 2001). Thus, we tested whether the pre-incubation of the slices for 10 min in 1 µM capsazepine, a vanilloid receptor antagonist (Zygmunt et al., 1999), could prevent the effect of AM-404 and PMSF. Capsazepine alone did not alter glutamatergic spontaneous activity.
Figure 10. Specific activation of CB1 receptor reduces sEPSCs frequency. A. Activation of CB1 receptor by AEA reduced glutamatergic spontaneous activity in spiny neurons from both naïve and 6-OHDA-lesioned rats (*p < 0.01, ST test; n = 4 for each condition). A low dose of PMSF was added to anandamide to avoid the rapid degradation by endogenous FAAH (see text for details). B. The histogram shows that blockade of CB1 receptor by 1 µM SR-141716 prevents the inhibitory action of AM-404 or PMSF, while blockade of TRPV1 vanilloid receptor has no effect (*p < 0.01, ST test; n = 4 for each condition).
Moreover, it failed to alter the effect of AM-404 and PMSF on glutamatergic spontaneous activity, both in naïve and 6-OHDA animals (Fig. 10B). All the compounds that we utilized did not significantly alter per se the membrane properties (resting membrane potential, RMP, and input resistance, IR) of the recorded neurons.

Presynaptic effect of CB1 receptor activation

We have performed a set of experiments in order to address the specific presynaptic effect of CB1 receptor activation. Evoked EPSCs were elicited by cortical fibers stimulation. Paired pulses (40-60 ms interval) were delivered at 0.1 Hz, and EPSCs were recorded throughout the whole experiment. Application of 1 µM HU-210 for 10 min reduced evoked EPSCs amplitude, increasing paired-pulse facilitation (Fig. 11A).

We also analyzed the action potential-independent miniature EPSPs (mEPSPs) recorded in the presence of 1 µM TTX. While sEPSCs result from neurotransmitter release elicited by action potentials, mEPSCs are supposed to arise from the spontaneous fusion of neurotransmitter-containing vesicles to the presynaptic terminal membrane. This latter phenomenon is independent of the activation of presynaptic voltage-dependent ion channels. The efficacy of TTX was assessed by observing the disappearance of evoked EPSCs. In these recording conditions, amplitude and frequency of mEPSCs were lower compared to sEPSCs (Fig. 11B, see histograms). This effect was similar in naïve and 6-OHDA-lesioned animals. Application of 1 µM HU-210 did not affect either the frequency or the amplitude of mEPSCs (Fig. 11B, traces and histograms). These results suggest that activation of CB1 receptors by HU-210 specifically reduces glutamate release from presynaptic terminals mediated by action potentials, while it does not affect action potential-independent release of this neurotransmitter.

In good agreement with previous studies, all the electrophysiological experiments we have performed indicate that CB1 receptor activation reduces glutamate release through a presynaptic mechanism (Gerdeman et al., 2001; Huang et al., 2001). First, endogenous AEA, as well as HU-210, reduces sEPSCs frequency without affecting their amplitude. Second, evoked EPSCs amplitude is reduced by HU-210 increasing paired-pulse facilitation. Third, mEPSCs are not affected by this drug in terms of both frequency and amplitude. Taken together, these data suggest that presynaptic
action potentials are required to observe the inhibitory effect of CB1 receptor activation, according to Huang et al. (2001).

**Figure 11.** Endocannabinoid CB1 receptor activation modulates glutamatergic activity with a presynaptic mechanism in both naïve and parkinsonian rats. A. The CB1 agonist HU-210 (1 µM) reduces evoked postsynaptic currents. Traces are from single neurons of a naïve (left) and a 6-OHDA-lesioned (right) animal, before (upper traces) and after (lower traces) the application of 1 µM HU-210. Note that the inhibition of evoked EPSCs by this compound increases paired-pulse facilitation, as shown also in the histogram (*p < 0.01, ST test; n = 4 for each group). B. In the presence of 1 µM TTX, action potential-independent release of glutamate generates mEPSCs, whose frequency and amplitude are both reduced compared to sEPSCs. Electrophysiological traces show spontaneous striatal glutamatergic activity recorded from single striatal spiny neurons of a naïve (left) and a 6-OHDA-lesioned (right) rat, before (upper) and after (lower) the administration of 1 µM HU-210. Application of this compound did not further reduce mEPSCs frequency (left histogram) nor amplitude (right histogram) in both naïve and parkinsonian animals (p > 0.05, ST test; n = 4 for each group).
Biochemical analysis of the endocannabinoid system after levodopa treatment

Levodopa is the main drug used during PD therapy, so in the second phase of study we performed a set of experiments to observe the effect of this compound on ES in our PD model. We demonstrated that the complex plastic changes of the endocannabinoid system caused by experimental parkinsonism are restricted to the striatum and are completely reversed by chronic L-DOPA treatment. The level of endogenous AEA was 3-fold higher in the striatum of 6-OHDA-lesioned rats, compared to sham-operated animals, and was reported to normal values after levodopa treatment, whereas endogenous 2-AG was unaffected (Table 3).

<table>
<thead>
<tr>
<th>Brain area</th>
<th>AEA (nmol/mg protein)</th>
<th>2-AG (nmol/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>Striatum (sham-operated)</td>
<td>0.25 ± 0.02</td>
<td>1.25 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Striatum (6-OHDA)</td>
<td>0.75 ± 0.08</td>
<td>1.30 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>(300%)*</td>
<td>(104%)</td>
</tr>
<tr>
<td>Striatum (6-OHDA+L-DOPA)</td>
<td>0.30 ± 0.03</td>
<td>1.15 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>(120%)</td>
<td>(92%)</td>
</tr>
<tr>
<td>Cerebellum (sham-operated)</td>
<td>0.15 ± 0.02</td>
<td>2.40 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Cerebellum (6-OHDA)</td>
<td>0.17 ± 0.02</td>
<td>2.25 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>(113%)</td>
<td>(94%)</td>
</tr>
<tr>
<td>Cerebellum (6-OHDA+L-DOPA)</td>
<td>0.16 ± 0.02</td>
<td>2.30 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>(107%)</td>
<td>(96%)</td>
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</table>

* p < 0.01 compared to striatum (sham-operated); † p < 0.01 compared to striatum (6-OHDA); p>0.05 in all other cases (n=4; Student’s t-test).

Table 3. Endogenous levels of AEA and 2-AG in parkinsonian rats
The activity of PLD was almost identical (~330 ± 35 pmol.min\(^{-1}\).mg protein\(^{-1}\)) in striata from sham-operated, 6-OHDA-lesioned and 6-OHDA + L-DOPA rats (Fig. 12A), AMT activity decreased in the striatum of parkinsonian animals (35 ± 4 vs 90 ± 10 pmol.min\(^{-1}\).mg protein\(^{-1}\); p < 0.01; ST test), as did the activity of FAAH, which was 180 ± 20 vs 470 ± 50 pmol.min\(^{-1}\).mg protein\(^{-1}\) (p < 0.01; ST test) (Fig. 12 B-C). Conversely, we found that the down-regulation of striatal AMT and FAAH by 6-OHDA denervation was completely reversed after the treatment (AMT activity = 100 ± 10 pmol.min\(^{-1}\).mg protein\(^{-1}\); FAAH activity = 400 ± 40 pmol.min\(^{-1}\).mg protein\(^{-1}\), p < 0.01 vs 6-OHDA-lesioned rats in both cases; ST test), as were the \(V_{\text{max}}\) values, but not apparent \(K_{\text{m}}\), of both proteins (Table 4). On the other hand, the binding of \([^{3}\text{H}]\)AEA was ~160 ± 15 fmol.mg protein\(^{-1}\) in all animals (Fig. 13A). Rat striatal membranes were also able to bind \([^{3}\text{H}]\)CP55,940 according to saturation curves, which yielded apparent \(K_{d}\) of 358 ± 80 pM and \(B_{\text{max}}\) of 350 ± 35 fmol.mg protein\(^{-1}\) (Fig. 13B). These \(K_{d}\) values are close to those previously reported for the binding of \([^{3}\text{H}]\)CP55,940 to rat striatal membranes (Pertwee, 1997). Treatment with 6-OHDA or L-DOPA did not change \([^{3}\text{H}]\)CP55,940 binding by striatal membranes (Fig. 13B), corroborating the hypothesis that CB1 receptor function was not affected by experimental parkinsonism.

Finally, it is noteworthy that the cerebellum of 6-OHDA-lesioned and L-DOPA-treated lesioned animals did not show any significant alteration in endogenous levels of AEA and 2-AG (Table 3), nor in the activity of PLD, AMT or FAAH, nor in \([^{3}\text{H}]\)AEA or \([^{3}\text{H}]\)CP55,940 (not shown) binding (Figs 12 and 13), compared to sham-operated rats (for a statistical analysis with 2-way ANOVA test see the legends of Figs 12 and 13). This observation suggests that perturbation of the endocannabinoid system in parkinsonian animals was restricted to the striatum.
<table>
<thead>
<tr>
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<th>AMT</th>
<th>FAAH</th>
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<tr>
<td></td>
<td>$K_m^a$</td>
<td>$V_{max}^b$</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>383±45</td>
<td>181±9</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>351±49</td>
<td>81±4*</td>
</tr>
<tr>
<td>6-OHDA+L-DOPA</td>
<td>365±46</td>
<td>195±8*</td>
</tr>
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</table>

$^a$Expressed as nM.

$^b$Expressed as pmol.min$^{-1}$.mg protein$^{-1}$.

$^c$Expressed as µM.

* $p < 0.01$ compared to sham-operated; $^*$ $p < 0.01$ compared to 6-OHDA; $p > 0.05$ in all other cases (n = 4; Student's t-test).

Table 4. Kinetic constants of AMT and FAAH in parkinsonian rats
Figure 12. Changes in the endocannabinoid system in the striatum and in the cerebellum of parkinsonian rats. A. The activity of PLD is affected neither by 6-OHDA lesion nor chronic L-DOPA treatment in the striatum and the cerebellum (p = 0.22; F(2,18) = 1.65). B. Conversely, the activity of AMT is reduced in the striatum, but not in the cerebellum, of parkinsonian animals, and returns to the values observed in sham-operated rats upon chronic L-DOPA treatment (p < 0.01; F(2,18) = 19.75). C. Similarly, the activity of FAAH is reduced in the striatum of parkinsonian rats, and L-DOPA restores it to normal values, while the cerebellum is not affected (p < 0.01; F(2,18) = 8.38).
Figure 13. Binding to cannabinoid receptors in the striatum and in the cerebellum of parkinsonian rats. A. The binding of \([^{3}H]\)AEA is not affected by 6-OHDA lesion in both striatum and cerebellum. Chronic L-DOPA treatment of parkinsonian animals also does not affect \([^{3}H]\)AEA binding \((p = 0.44; F_{2,18} = 0.87)\). B. Saturation curves of the binding of \([^{3}H]\)CP55,940 to striatal membranes of sham-operated (filled circles), 6-OHDA-lesioned (open squares) or L-DOPA treated (open triangles) animals.
Electrophysiological analysis of spontaneous glutamatergic activity

sEPSCs were recorded from striatal spiny neurons by means of whole-cell patch-clamp techniques. According to previous reports, the neurons recorded from the three experimental groups had similar intrinsic membrane properties (Kita et al., 1984; Jiang et al., 1991; Calabresi et al., 1993; Gubellini et al., 2002; Picconi et al., 2002). As shown in figure 14A-C, the frequency and amplitude of sEPSCs recorded from spiny neurons of 6-OHDA-lesioned rats was significantly higher compared to sham-operated, but interestingly, chronic treatment of parkinsonian rats with L-DOPA restored both sEPSC frequency and amplitude to the basal levels (Fig. 14 A-C) observed in sham-operated animals (2.3 ± 0.3 Hz, n = 20, and 16.0 ± 2.1 pA, n = 20, p > 0.05 compared to sham-operated; ST test). The sEPSCs, recorded in the presence of 3 µM bicuculline, a GABA_A receptor antagonist, were suppressed by the application of 10 µM CNQX, an AMPA receptor blocker (not shown). Also in this case, the pharmacological treatment with HU-210, a CB1 receptor agonist (Pertwee, 1997), revealed a dose-dependent inhibition of sEPSC frequency, that was comparable in the three experimental groups. Figure 15A shows the normalized effect of this drug on the frequency of sEPSCs. Similarly, the blockade of AMT by VDM11 (De Petrocellis et al., 2001) caused a dose-dependent inhibition of sEPSC frequency in the three experimental groups (Fig. 15B). Conversely, the FAAH blocker MAFP (De Petrocellis et al., 2001) had a much stronger inhibitory effect in 6-OHDA-lesioned animals than in sham-operated and in L-DOPA-treated lesioned rats (Fig. 15C). These three compounds did not significantly alter sEPSC amplitude in the three experimental groups (Fig. 15D-F), supporting a presynaptic action of CB1 receptor activation (Gerdeman et al., 2001; Huang et al., 2001; Gubellini et al., 2002). Moreover, according to previous findings (Huang et al., 2001; Gubellini et al., 2002), CB1 receptor stimulation affected neither the frequency nor the amplitude of miniature EPSPs.
Figure 14. Electrophysiology of striatal spiny neurons of control, parkinsonian, and L-DOPA-treated parkinsonian rats. The frequency of glutamatergic sEPSCs is increased in 6-OHDA-lesioned animals (A), as well as their amplitude (B). L-DOPA treatment in parkinsonian rats restores both sEPSC frequency (A) and amplitude (B) to sham-operated levels. Electrophysiological traces (C) show glutamatergic sEPSCs from single cells in the three different experimental conditions (all these neurons were clamped at –80 mV). * p < 0.01 compared to sham-operated;  # p < 0.01 compared to 6-OHDA-lesioned, and p > 0.05 compared to sham-operated (n = 6 for each condition; Student's t-test).
Figure 15. Different pharmacological modulation of the cannabinoid system in sham-operated, 6-OHDA-lesioned and parkinsonian rats chronically treated with L-DOPA. A. The CB1 receptor agonist HU-210 reduces sEPSC frequency in a dose-dependent manner. This effect, when normalized, is similar in sham-operated, 6-OHDA-lesioned, and 6-OHDA-lesioned rats chronically treated with L-DOPA. B. The AMT blocker VDM11 shows effects comparable to those obtained with HU-210. C. Conversely, inhibition of FAAH activity by MAFP is much more effective on 6-OHDA-lesioned animals compared to sham-operated and L-DOPA-treated parkinsonian rats. The amplitude of sEPSCs is not significantly affected by these drugs in the three experimental models (graphs in D, E and F). (* p < 0.01 compared with pre-drug control; n = 6 for each condition; Student's t-test).
PARKINSON’S DISEASE DISCUSSION

In these studies we have reported, for the first time to our knowledge, that alterations in the endocannabinoid system, associated to a neurological disorder, are restricted to the brain area responsible for this disorder, and are reversed by a treatment which corrects the symptoms of the disease. These observations speak in favor of a genuine cause-effect relationship. In particular, we found that in the striatum – but not in the cerebellum – of 6-OHDA-denervated rats: (i) the increased levels of endogenous AEA are due to down-regulation of its degradation, rather than to up-regulation of its synthesis; (ii) the binding of AEA to CB1 receptors does not change; (iii) the pharmacological inhibition of FAAH, but not that of AMT, produces a much stronger depression of striatal glutamatergic activity compared to sham-operated and L-DOPA-treated lesioned rats. This latter finding, taken together with the observation that denervated striata express a dramatic overactivity of glutamatergic transmission, suggests that targeting FAAH might be beneficial in experimental parkinsonism by reducing this abnormal synaptic transmission. Accordingly, ionotropic glutamate receptor antagonists improve experimental PD symptoms (Chase et al., 2000). These results suggest that the higher level of AEA in the striatum of parkinsonian rats might be due to a decreased cleavage rather than an increased synthesis. On the other hand, the synthesis of 2-AG by phospholipases A\textsubscript{1} and C, and/or its degradation by monoacylglycerol lipase (Sugiura et al., 2000), do not seem to be affected by 6-OHDA denervation. It is not clear from our data why parkinsonian rats are more sensitive to FAAH inhibition than control and L-DOPA-treated lesioned animals, while the other pharmacological tools acting on the endocannabinoid system have the same effects in the three groups. We can speculate that, since FAAH is less active in 6-OHDA-lesioned rats, this enzyme is more vulnerable to inhibition by MAFP. However, also AMT is reduced in these animals, thus we should also expect an increased sensitivity to VDM11, which is not the case. It is clear, therefore, that further studies are necessary to address these issues. Presumably, FAAH activity plays a major role in determining AEA levels in the striatum of 6-OHDA-lesioned animals, in accordance with a recent report showing that mice lacking FAAH have a 15-fold augmented level of AEA in the brain (Cravatt et al., 2001). Since FAAH seems to be the “check point” in AEA degradation (Cravatt et al., 2001), we propose that drugs modulating its activity might represent a novel pharmacological approach in the therapy of Parkinson’s disease. This
seems noteworthy, because therapeutic options for managing L-DOPA-induced dyskinesia in PD are still limited (Sieradzan et al., 2001).
DRUG ABUSE RESULTS

In the present study, we provide evidence that cocaine significantly perturbs endocannabinoid system in the striatum, and we identify a possible mechanism by which this perturbation modulates the cellular mechanisms of drug addiction. In fact, we have demonstrated that cocaine, through the stimulation of DA D2-like receptors, increases 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. Moreover, we also show that increased levels of AEA significantly contributed to the cocaine-induced presynaptic inhibition of GABA transmission in the striatum, presumably through the stimulation of cannabinoid CB1 receptors.

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 (Fig. 16). 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). 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 (Fig. 16). Accordingly, the D2-like receptor agonist quinpirole, but not the D1-like receptor agonist SKF38393, increased AEA levels by stimulating NAPE-PLD and inhibiting FAAH, an effect prevented by L-sulpiride but not by SCH23390 (Fig. 17).
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 (Fig. 18). Analysis of the binding of \(^{3}\)H]CP55,940 to striatal membranes, untreated or treated for 10 min with 10 \(\mu\)M cocaine, showed saturation curves superimposable to those recently reported in the same brain area (Maccarrone et al., 2003). From these saturation curves, apparent K\(_d\) values of 340 ± 70 pM and 310 ± 91 pM (n = 4; p > 0.05), and B\(_{\text{max}}\) values of 330 ± 40 fmol/mg protein and 499 ± 57 fmol/mg protein (n = 4; p < 0.05) were calculated in controls and cocaine-treated samples, respectively.
Figure 16. 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-like 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 non significant compared to the control value.
Figure 17. 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 of 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 non significant compared to the control value.
Figure 18. 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-like 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 of 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 figure 16 and 17.
Spontaneous GABAergic activity in striatal neurons

Electrophysiological recordings with cesium chloride-containing pipettes allowed detecting, at –80 mV holding 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, decay time constants, and half widths) significantly different from glutamate-mediated excitatory post-synaptic 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) (Fig. 19). As expected for a presynaptic site of action, neither cocaine nor quinpirole altered IPSC mean amplitude, rise time, decay time, and half width (Fig. 20A-D).

Cocaine has been reported to modulate striatal neuron firing activity through a direct interaction with voltage-dependent sodium channels (Kiyatkin et al., 2000). In a further set of experiment, 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 anaesthetic-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 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 fully restore spontaneous IPSC frequency to control values (n=4) (Fig. 19).

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), both 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 (Figs 19 and 20). 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 than 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 et al., 2002) and that also HU 210 depressed sIPSCs recorded from striatal neurons (Fig. 19) seem to rule out the involvement of non-CB1 receptors.
Figure 19. 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, D, E, and 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 16.
Figure 20. 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 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 non significant compared to the control value.
DRUG ABUSE DISCUSSION

From behavioural 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 behaviour, 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 et al., 2002; Gerdeman et al., 2003). 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 pre-treatment with the cannabinoid antagonist SR141716A enhanced the stimulation of motor behaviour 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 et al., 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.

Finally, we have found that cocaine-stimulated AEA significantly contributes to inhibit GABA-mediated synaptic transmission in the striatum, thereby favouring 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 et al., 1998). Accordingly, in vivo, blockade of ongoing GABAergic inhibition of striatal neurons has been found to significantly elevate basal activity of these cells (Nisenbaum et al., 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 et al., 1998; Koos et al., 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 et al., 1998; Koos et al., 1999). Since these GABAergic interneurons are a preferential location of cannabinoid CB1 receptors in the striatum (Hohmann et al., 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. 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 et al., 1993; Berke et al., 2000). More recently, synaptic changes in the striatum have been claimed to be involved in the advanced stages of drug addiction, which are characterised by compulsive drug-seeking and drug-taking behaviours despite serious negative consequences (Gerdeman et al., 2003). 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 et al., 2000; Hyman et al., 2001; Gerdeman et al., 2003).
CONCLUSION AND FUTURE PERSPECTIVES

The data obtained from our studies evidence that endocannabinoid signaling might be seen as an adaptive response to stimuli or conditions that pose a threat to the organism, and to brain in particular. Endocannabinoids are able to compensate, both at neurochemical and behavioral level, for the abnormal neurotransmission caused by several pathologies. The endogenous “anti-stress” response can cause other effects, which can contribute to some of the symptoms of the disorders observed during their progression, as shown in animal models of Parkinson’s disease or during the relapse of drug abuse (van der Steelt et al., 2003). Moreover, we underlined that FAAH can be a critical point in AEA degradation and we propose that drugs modulating its activity might represent a novel pharmacological approach in the therapy of Parkinson’s disease and might offer an innovative target for the treatment of some features of marijuana withdrawal, i.e. anxiety (Piomelli, 2004).

In conclusion, we are performing novel experiments, using mice genetically modified in the D2 receptor gene, to test the effect of the endocannabinoid system on dopaminergic transmission and to try to identify the molecular logic of these endogenous compounds.
EXPERIMENTAL PROCEDURES

Materials and Methods

Drugs were applied by dissolving them to the desired final concentration in the saline solution perfusing the slice. AM-404 (N-(4-hydroxyphenyl) arachidonoyl amide), anandamide (N-arachidonoylethanolamine) and phenylmethanesulfonyl fluoride (PMSF) were from Sigma (Milano, Italy); bicuculline, tetrodotoxin (TTX), VDM11 and HU-210 from Tocris-Cookson (Bristol, UK); capsazepine (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide) was from Calbiochem; methyl-arachidonoyl fluorophosphonate (MAFP) was from Cayman Chemicals (Ann Arbor, USA); N-piperedino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxyamide (SR-141716) was a kind gift from Sanofi Recherche (Montpellier, France); [3H]AEA (223 Ci/mmol) and [3H]CP55,940 (126 Ci/mmol) were from Perkin Elmer (Monza, Italy); [3H]NArPE (200 Ci/mmol) was from ARC (St. Louis, MO, U.S.A.).

6-OHDA lesions and L-DOPA treatment

All the experiments were conducted in conformity with the European Communities Council Directive of November 1986 (86/609/EEC). Wistar rats were injected unilaterally with 6-OHDA (8 µg/4 µl of saline containing 0.1% ascorbic acid) rostral to the substantia nigra under stereotaxic coordinates (Paxinos et al., 1986): A = 3.7 mm anterior to the interaural line, V = 2.2 mm dorsal to the interaural line; L = 2.2 mm from the midline. Twenty days later, the rats were tested with 0.05 mg/kg (s.c.) apomorphine and the controlateral turns were recorded with automatic rotometers for 3 hours (Ungerstet et al., 1970). Only those rats consistently making at least 200 controlateral turns were used for our studies. After brain dissection, we confirmed that the nigrostriatal pathway was lesioned. This was established by noting a > 95% loss of DA neurons in the substantia nigra compacta and the almost complete absence of DA terminals in the striatum. This was detected by the immunoperoxidase technique utilizing a monoclonal antibody for tyrosine hydroxylase. Rats were utilized 2-3 months after the 6-OHDA lesion. As control animals we utilized sham-operated rats of similar
ages injected with saline not containing 6-OHDA. Chronic treatment with L-DOPA was performed by i.p. injections (25 mg/kg L-DOPA plus 6.5 mg/kg benserazide) twice per day, for 3 weeks. As control animals we used sham-operated rats of similar ages injected with saline. From each animal, striatum and cerebellum were removed and were immediately subjected to biochemical analysis.

**Determination of anandamide levels**

The endogenous levels of AEA and 2-AG in the striatum and in the cerebellum were determined by gas chromatography-electron impact mass spectrometry (GC/MS) as described (Maccarrone et al., 2001). Immediately after decapitation, rat brains were washed in phosphate-buffered saline, pre-cooled at 4°C, were dissected and frozen in liquid nitrogen, and were kept at -70°C until processed. A maximum of 8 min elapsed between rat decapitation and freezing of dissected tissues, a time not sufficient to cause artifactual rises in endocannabinoid levels. 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 x 0.25 mm i.d.) from SGE (Milan, Italy), and interfaced with a VG Micromass model QUATTRO spectrometer (Manchester, United Kingdom). Analyses were performed in “splitless” mode at temperatures rising from 70°C to 250°C, at a rate of 30°C/min. The identity of AEA and 2-AG 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, whereas 2-AG was quantitated by the internal standard method with AEAd4.

**Determination of anadamide uptake**

The uptake of [3H]AEA by the anandamide membrane transporter (AMT) was assayed in synaptosomes prepared from the striatum or the cerebellum as reported (Maccarrone et al., 2001). 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 1000xg for 5 min, at 4°C, then supernatants were centrifuged again at 17000xg for 15 min, at 4°C. The final
pellets were resuspended in 136 mM NaCl, 5 mM KCl, 0.16 mM CaCl₂, 0.1 mM EGTA, 1.3 mM MgCl₂, 10 mM glucose, 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 [³H]AEA per test. Q₁₀ value was calculated as the ratio of AEA uptake at 30°C and 20°C (Hillard et al., 2000). Incubations (15 min) were also carried out with different concentrations of [³H]AEA, in the range 0-800 nM, in order to determine apparent Michaelis-Menten constant (Kₘ) and maximum velocity (Vₘₐₓ) of AMT.

**Fatty Acid Amide Hydrolase (FAAH) Activity**

Anandamide hydrolase (arachidonoylethanolamide amidohydrolase, E.C. 3.5.1.4; fatty acid amide hydrolase, FAAH) activity was assayed in rat brain homogenates by reversed phase high performance liquid chromatography, using 5 µM [³H]AEA as substrate (Maccarrone et al., 1998). FAAH activity was expressed as pmol arachidonate released per min per mg protein.

**NAPE-PLD Activity**

The activity of N-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (phosphatidylecholine phosphatidohydrolase, EC 3.1.4.4; NAPE-PLD) was assayed in rat brain homogenates (100 µg/test) using 100 µM N-[³H]arachidonoylphosphatidylethanolamine([³H]NArPE) as substrate (Okamoto et al., 2004), unless indicated otherwise. NAPE-PLD activity was expressed as pmol of [³H]AEA released per min per mg protein.

**CB Binding Assay**

The binding of [³H]AEA or of the synthetic cannabinoid [³H]CP55.940 to striatal or cerebellar membranes was determined by using rat membrane fractions prepared, quickly frozen in liquid nitrogen and stored at -80°C for no longer than one week as reported (Maccarrone et al., 2000c). These membrane fractions were used in rapid filtration assays with the synthetic cannabinoid [³H]CP55.940 (used at 400 pM). Unspecific binding was
determined in the presence of 100 nM “cold” agonist. Receptor binding was expressed as fmol ligand bound per mg protein.

Electrophysiology

Corticostriatal coronal slices (190-200 µm) were prepared 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 an artificial cerebrospinal fluid (ACSF), whose composition was (in mM): (126) NaCl, (2.5) KCl, (1.2) MgCl₂, (1.2) NaH₂PO₄, (2.4) CaCl₂, (11) glucose, (25) NaHCO₃. The temperature of ACSF was maintained at 35°C and it was gassed with O₂/CO₂ (95/5 %). The striatum could be readily identified under low power magnification, whereas individual neurons were visualized in situ using a differential interference contrast (Nomarski) optical system. This employed an Olympus BX50WI (Japan) non-inverted microscope with x40 water immersion objective combined with an infra-red filter, a monochrome CCD camera (COHU 4912), and a PC compatible system for analysis of images and contrast enhancement (WinVision 2000, Delta Sistemi, Italy). For whole-cell patch-clamp recordings, electrodes (4-5 MΩ) were filled with a solution containing (mM): (125) K⁺-gluconate, (10) NaCl, (1.0) CaCl₂, (2.0) MgCl₂, (0.5) BAPTA, (19) HEPES, (0.3) GTP, (1.0) Mg-adenosine triphosphate, adjusted to pH 7.3 with KOH. Striatal spiny neurons were clamped at -80 to -85 mV, close to their resting membrane potential. All recordings were performed in the presence of 3 µM bicuculline to avoid the contamination of sEPSCs by a GABAₐ-mediated component. To study spontaneous GABAₐ-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), CaCl₂ (0.1), Mg-ATP (4), Na-GTP (0.3). MK-801 and CNQX were added to the external solution to block, respectively, NMDA and nonNMDA glutamate receptors. Spontaneous glutamatergic activity and spontaneous GABAₐ-mediated IPSCs were monitored utilizing Axopatch 200B and 1D amplifiers, and pClamp 8.1 software (Axon Instruments, Union City, USA). Afterwards, the currents were analyzed offline by MiniAnalysis 5.4.1 software (Synaptosoft, Leonia, USA).
Statistical analysis

Apparent $K_m$ and $V_{max}$ values of the hydrolysis of $[^3H]$AEA (in the range 0 – 15 µM) by FAAH and of AMT (in the range 0 – 800 nM) and, also, apparent dissociation constant ($K_d$) and maximum binding ($B_{max}$) values of $[^3H]$CP55,940 (in the range 0-800 pM) were calculated by nonlinear regression analysis, performed though the Prism 3 program (GraphPAD Software for Science, San Diego, CA, USA) (Maccarrone et al., 1998). For data presented as the mean ± SEM, statistical analysis was performed using the Student’s $t$-test (between two groups) and by the 2-way ANOVA test (between the three groups). The significance level was established at $p < 0.05$.

For electrophysiology 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 $p < 0.05$. 

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REFERENCES


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SHORT CURRICULUM VITAE

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