Neurobiology of Disease

The Distinct Role of Medium Spiny Neurons and Cholinergic Interneurons in the D_2/A_{2A} Receptor Interaction in the Striatum: Implications for Parkinson's Disease

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A_{2A} adenosine receptor antagonists are currently under investigation as potential therapeutic agents for Parkinson's disease (PD). However, the molecular mechanisms underlying this therapeutic effect is still unclear. A functional antagonism exists between A_{2A} adenosine and D_2 dopamine (DA) receptors that are coexpressed in striatal medium spiny neurons (MSNs) of the indirect pathway. Since this interaction could also occur in other neuronal subtypes, we have analyzed the pharmacological modulation of this relationship in murine MSNs of the direct and indirect pathways as well in striatal cholinergic interneurons. Under physiological conditions, endogenous cannabinoids (eCBs) play a major role in the inhibitory effect on striatal glutamatergic transmission exerted by the concomitant activation of D_2 DA receptors and blockade of A_{2A} receptors in both D_2- and D_1-expressing striatal MSNs. In experimental models of PD, the inhibition of striatal glutamatergic activity exerted by D_2 receptor activation did not require the concomitant inhibition of A_{2A} receptors, while it was still dependent on the activation of CB_1 receptors in both D_2- and D_1-expressing MSNs. Interestingly, the antagonism of M1 muscarinic receptors blocked the effects of D_2/A_{2A} receptor modulation on MSNs. Moreover, in cholinergic interneurons we found coexpression of D_2 and A_{2A} receptors and a reduction of the firing frequency exerted by the same pharmacological agents that reduced excitatory transmission in MSNs. This evidence supports the hypothesis that striatal cholinergic interneurons, projecting to virtually all MSN subtypes, are involved in the D_2/A_{2A} and endocannabinoid-mediated effects observed on both subpopulations of MSNs in physiological conditions and in experimental PD.

Introduction

A_{2A} adenosine receptors (A_{2A}-Rs) are highly expressed in the striatum, where they are predominantly located postsynaptically in D_2 dopamine (DA) receptor (D_2-Rs)-expressing striatopallidal projecting neurons (Ferré et al., 1997; Svenningsson et al., 1999; Calon et al., 2004; Schiffmann et al., 2007). A_{2A}-R antagonists improve motor deficits in animal models of Parkinson's disease (PD) and might provide therapeutic benefit in PD patients (Xu et al., 2005; Schwarzschild et al., 2006; Morelli et al., 2007).

Concomitant activation of D_2-Rs and antagonism of A_{2A}-Rs decrease the frequency of striatal spontaneous EPSCs (Tozzi et al., 2007). Interestingly, this inhibitory effect is associated with an increased paired-pulse facilitation, suggesting a possible presynaptic mechanism of action (Fink et al., 1992; Hettinger et al., 2001).

Since A_{2A}- and D_2-Rs are mainly expressed postsynaptically in the striatum (Fuxe et al., 2007), it is possible to hypothesize that this presynaptic inhibitory effect is initiated postsynaptically, but it is expressed through a presynaptic reduction in neurotransmitter release mediated by a retrograde messenger.

Endocannabinoids (eCBs) are important retrograde messengers that mediate depression of excitatory synaptic transmission via CB_1 receptors in the striatum as well as in other brain areas (Gerdeman et al., 2002; Gubellini et al., 2002; Wilson and Nicoll, 2002; Kreitzer and Malenka, 2007), and activation of D_2-Rs leads to the production and release of these signaling molecules (Giuffrida et al., 1999; Piomelli, 2003). A_{2A} blockade facilitates D_2-R-mediated processes (Ferré et al., 1997; Strömberg et al., 2000; Tozzi et al., 2007; Kim and Palmiter, 2008), suggesting that, in physiological conditions, D_2-Rs and A_{2A}-Rs might act in concert to regulate eCB-mediated presynaptic inhibition of glutamate release in the striatum.

Profound modifications occurring in eCBs signaling have been demonstrated after DA depletion in both experimental models of PD (Gubellini et al., 2002) and patients suffering from the disease (Di Filippo et al., 2008). However, how changes in eCBs signaling are influenced by altered responses of D_2-Rs as

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well as of A_{2A}-R following DA depletion has never been addressed.

Thus, the aim of the present study is the electrophysiological characterization of the D_2/A_{2A} receptor interaction in the control of striatal glutamatergic transmission and of the possible role exerted by eCBs in mediating this interaction in both physiological and parkinsonian states.

Recent studies have demonstrated that the two main subpopulations of striatal neurons from which the direct and indirect basal ganglia pathways originate express distinct functional and synaptic features (Kreitzer and Malenka, 2007; Shen et al., 2008; Valjent et al., 2009). Nevertheless, a convergence of the role of different medium spiny neuron (MSN) subtypes in controlling major striatal functions has been suggested as in the case of the dopaminergic control of long-term depression (LTD), induction being possibly exerted by striatal large aspiny cholinergic interneurons (Wang et al., 2006). For this reason, taking advantage of bacterial artificial chromosome (BAC) transgenic mice expressing D_1 or D_2 DA receptors, we have investigated whether the observed synaptic effects induced by D_2/A_{2A} receptor modulation were segregated to one of the two basal ganglia pathways. Furthermore, we took into account the possible role of striatal cholinergic interneurons in integrating D_2 DA- and A_{2A} adenosine-mediated inputs toward both D_2- and D_1-expressing MSNs either in physiological conditions or in the parkinsonian state.

Materials and Methods

Experimental animals and procedures to induce DA depletion. All the experiments were conducted in conformity with the European Communities Council Directive of November 1986 (86/609/ECC). Two- to three-month-old male Wistar rats (Harlan) and 5- to 6-week-old male C57BL/6J-Swiss Webster mice carrying BAC that express enhanced green fluorescent protein (BAC-EGFP) under the control of D_1-R promoter (*drd1a*-EGFP) or D_2-R promoter (*drd2*-EGFP) were used for electrophysiological experiments. BAC-EGFP mice were originally generated by the GENSAT (Gene Expression Nervous System Atlas) program at the Rockefeller University (Gong et al., 2003).

Procedures for obtaining rats with 6-hydroxydopamine (6-OHDA)-induced striatal DA denervation have been previously given in detail (Picconi et al., 2003, 2008). In brief, deeply anesthetized rats were unilaterally injected with 6-OHDA (12 μg/4 μl of saline containing 0.1% ascorbic acid) into the medial forebrain bundle (Picconi et al., 2003, 2008). Sham-operated rats were injected only with vehicle at the same stereotaxic coordinates. Fifteen days later, rats were tested with 0.05 mg/kg subcutaneous administration apomorphine, and turns contralateral to the lesion were counted for 40 min. Rats with >200 contralateral turns were assigned to the group of the DA-denervated animals. Sham-operated animals did not show turning behavior. One and a half months after the lesion, the rats were used for electrophysiological experiments. The severity of the lesion was confirmed afterward by striatal and nigral immunohistochemistry tyrosine hydroxylase (Picconi et al., 2003).

In the experiments using BAC-EGFP mice, DA depletion was obtained by treating the animals with 5 mg/kg reserpine. This dose of reserpine has been previously shown to produce pronounced striatal dopamine depletion in mice (more than 90% and 95% depletion at 3 and 24 h after administration, respectively) (Starr et al., 1987). Reserpine and α-methyl-*p*-tyrosine methyl ester hydrochloride (AMPT, 300 mg/kg) were administered intraperitoneally. D_1 EGFP and D_2 EGFP mice in the reserpine/AMPT treatment received a first intraperitoneal injection of 5 mg/kg reserpine in a 0.08% glacial acetic acid vehicle (0.9% saline solution) and 5–6 h later an intraperitoneal injection of 300 mg/kg AMPT in vehicle (0.9% saline solution) for 2 successive days. The third day, 2 h before experiments, animals received a last intraperitoneal injection of reserpine (Moody and Spear, 1992).

Preparation and maintenance of corticostriatal slices. Preparation and maintenance of corticostriatal rodent slices have been previously described (Calabresi et al., 1992; Picconi et al., 2003, 2004; Costa et al., 2008). Briefly, corticostriatal coronal slices were cut from rat (thickness, 270 μm) or from BAC-EGFP mouse (thickness, 220–240 μm) brains using a vibratome. A single slice was transferred to a recording chamber and submerged in a continuously flowing Krebs' solution (34°C; 2.5–3 ml/min) bubbled with a 95% O_2–5% CO_2 gas mixture. The composition of the solution was (in mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 10 glucose, and 25 $NaHCO_3$. Drugs were bath applied by switching the solution to one containing known concentrations of drugs. Total replacement of the medium in the chamber occurred within 1 min.

Electrophysiology. Intracellular recordings of striatal MSNs were obtained by using sharp microelectrodes, pulled from borosilicate glass pipettes, backfilled with 2 M KCl (30–60 MΩ). An Axoclamp 2B amplifier (Molecular Devices) was connected in parallel to an oscilloscope (Gould) to monitor the signal in "bridge" mode and to a PC for acquisition of the traces using pClamp 10 software (Molecular Devices). After the impalement of the neuron, a small amount of current (5–20 pA) was injected via the recording electrode, when necessary. Only neurons electrophysiologically identified as spiny neurons were considered for experiments with sharp microelectrodes (Calabresi et al., 1998).

For patch-clamp recordings, neurons were visualized using differential interference contrast (DIC, Nomarski) and infrared microscopy (IR, Olympus). MSNs from slices of mice expressing BAC-EGFP under the control of D_1-R promoter (D_1-EGFP) or D_2-R promoter (D_2-EGFP) were visualized with an IR- and fluorescence-equipped microscope (Olympus). Whole-cell voltage-clamp (holding potential, −80 mV) recordings were performed with borosilicate glass pipettes (4–7 MΩ) filled with a standard internal solution (in mM): 145 K^+-gluconate, 0.1 $CaCl_2$, 2 $MgCl_2$, 0.1 EGTA, 10 HEPES, 0.3 Na-GTP, and 2 Mg-ATP, adjusted to pH 7.3 with KOH. In the BAPTA-containing internal solution, 20 mM BAPTA was added to the standard solution and K^+-gluconate was lowered to 125 mM. Signals were amplified with a Multiclamp 700B amplifier (Molecular Devices), recorded, and stored on PC using pClamp 10. Whole-cell access resistance was 5–30 MΩ, holding current ranging between 80 and −50 pA. Glutamatergic corticostriatal EPSPs and EPSCs were evoked every 10 s by means of a bipolar electrode connected to a stimulation unit (Grass Telefactor). The stimulating electrode was located in the white matter between the cortex and the striatum to activate corticostriatal fibers. The recording electrodes were placed within the dorsolateral striatum.

Cholinergic interneurons were recorded from rat or mice slices in whole-cell current-clamp mode using an internal solution containing the following (in mM): 120 K^+-gluconate, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 0.3 Na-GTP, and 2 Mg-ATP, adjusted to pH 7.3 with KOH. Cholinergic interneurons that were not spontaneously active were injected with 10–50 pA of positive current for reaching the threshold of action potential, if necessary. The mean frequency of the firing activity was calculated in time windows of 10 s for each experimental condition. Quantitative data are expressed as a percentage of EPSP and EPSC amplitudes or firing frequency with respect to the relative control values, the latter representing the mean of responses recorded during a stable period (10–15 min). Off-line analysis was performed using Clampfit 10 (Molecular Devices) and GraphPad Prism 5.0 (GraphPad Software) software. Two-way ANOVA or Student's *t* test was used. Values given in the figures and text are mean ± SE; the number of recorded neurons (*n*) is provided for each set of experiments. The significance levels were established at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Tissue processing and triple-label immunofluorescence. Three rats were deeply anesthetized and then transcardially perfused with a saline solution containing 0.01 ml of heparin, followed by 60 ml of 4% paraformaldehyde dissolved in the saline solution. Brains were removed and postfixed overnight at +4°C in 4% paraformaldehyde in saline solution. They were then submerged for 48 h at +4°C in a cryoprotective solution whose composition was as follows: 10% sucrose and 20% glycerol dissolved in 0.1 M phosphate buffer (PB) plus 0.02% sodium azide. Brains were frozen and serially sectioned on a sliding microtome at 40 μm thickness. Thirty coronal corticostriatal sections (10 sections per animal) were mounted on gelatin-coated slides and coverslipped with GEL-MOUNT. Sections were examined using an epi-illumination fluores-

cence microscope (Zeiss Axioskop 2), and a confocal laser scanning microscope (CLSM) (Zeiss, LSM510) was subsequently used to acquire images for quantification. Controls for specificity of immunohistochemical labeling included the omission of the primary antibody and the use of preimmune normal mouse and rabbit serum.

For immunohistochemical detection, we used three commercially available antibodies: a rabbit anti-A_{2A} adenosine receptor antibody directed against full-length human recombinant A_{2A} receptor (Alexis Biochemicals, Enzo Life Sciences) and rabbit anti-D_2 dopamine receptor and mouse anti-choline acetyl transferase antibodies (Immunological Science).

A protocol for triple labeling with two primary antibodies from the same host species was used (Negoescu et al., 1994). Briefly, sections were incubated with a rabbit primary antibody against D_2 receptors (Immunological Science) at a 1:500 concentration in PBTX containing 10% normal serum for 48 h at 4°C, then rinsed three times for 15 min at room temperature (RT) and incubated with a goat anti-rabbit Fab fragment unlabeled secondary antibody (Jackson ImmunoResearch) for 2 h at RT. After this incubation, sections were rinsed and then this first antibody was visualized with anti-goat Alexa Fluor 647 IgG (1:300, Invitrogen). After this incubation, the sections were rinsed three times for 15 min in PB and then incubated with a mouse monoclonal antibody against choline acetyltransferase (ChAT) and another polyclonal antibody (anti-A_{2A} 1:200). Subsequently, sections were rinsed three times for 15 min in PB and incubated with a cocktail of labeled secondary antibodies (anti-rabbit Cy2 and anti-mouse Cy3; Jackson ImmunoResearch) at 1:100 concentration for 2 h at RT. Sections were rinsed several times in PB and subsequently mounted on gelatin-covered slides, coverslipped in GEL-MOUNT, and examined under an epi-illumination fluorescent microscope (Zeiss Axioskop 2) and CLSM (Zeiss LSM700). Digital images were acquired using the Zeiss LSM700 computer program and adjustments of brightness and contrast were made using Adobe Photoshop 10.

The triple-labeled tissue was used to determine the percentage of ChAT-positive striatal interneurons that were labeled for D_2 and A_{2A} receptors. Cells were counted in each of four 1.0-mm-square confocal microscope fields (dorsal, dorsolateral, central, medial) on each hemisphere in each of three rostrocaudally spaced sections, in each of three rats, for each triple-labeled set of sections. The total number of cells immunopositive for ChAT was counted in each field. Subsequently, the number of ChAT-immunolabeled neurons colocalizing with each of the aforementioned markers was counted. The total of the neurons in each fields was averaged across all fields to obtain an average of the number of colocalizing and non-colocalizing neurons for each subpopulation considered. A total of 750 immunohistochemically labeled cholinergic interneurons were counted.

Chemicals. Powders were dissolved in water or DMSO and then stored at −20°C in aliquots. Each aliquot was only used the day of experiment and then discarded. Drugs were applied by dissolving them to the desired final concentration in the external Krebs' solution. AMPT; AM251 (AM); pirenzepine dihydrochloride; (−)-quinpirole hydrochloride (Quin); (*R*)-baclofen; reserpine; L-sulpiride; WIN55,212-2 (WIN); and ZM241385 (ZM) were from Tocris-Cookson. 1,2-Bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA) and (−)-bicuculline methiodide were from Sigma-Aldrich. ST1535 (ST) was kindly provided by Sigma-tau. Pramipexole dihydrochloride was kindly provided by Boehringer Ingelheim.

Results

Concomitant D_2 dopamine receptor activation and A_{2A} adenosine receptor blockade decrease striatal glutamatergic transmission in physiological conditions

Intracellular recordings with sharp microelectrodes and whole-cell patch-clamp recordings were obtained from electrophysiologically identified MSNs from dorsolateral striata of control rats. The main characteristics of these cells have been described in detail previously (Calabresi et al., 1998; Costa et al., 2008). Single stimulations of corticostriatal afferents, delivered every 10 s in the presence of the $GABA_A$-R antagonist bicuculline (10 μM), evoked EPSPs and EPSCs during intracellular and patch-clamp recordings, respectively (Fig. 1). A stable EPSP (Fig. 1*A*,*B*) or EPSC (Fig. 1*C*) was recorded for 10–15 min to obtain a baseline control. In this condition, neither 10 μM quinpirole, a D_2 receptor agonist, nor 1 μM ZM241385 or 10 μM ST1535 (Minetti et al., 2005; Stasi et al., 2006), two A_{2A} receptor antagonists, bath applied alone, affected the EPSP or EPSC amplitude (Fig. 1*A*–*C*). Conversely, the coapplication of 10 μM quinpirole and 1 μM ZM241385, reduced the EPSP or EPSC amplitudes with respect to the baseline [EPSP: quinpirole, 95 ± 1.6%, $n = 9$; quinpirole plus ZM, 62.5 ± 4.3%, $n = 9$ (Fig. 1*A*); EPSC: quinpirole, 95 ± 2.3%, $n = 8$; quinpirole plus ZM, 73 ± 5.1%, $n = 6$ (Fig. 1*C*)]. The coapplication of quinpirole and ST1535 also produced a reduction of the amplitude of the postsynaptic response (EPSP: quinpirole plus ST, 51 ± 3.8%, $n = 8$) (Fig. 1*B*). The effects of quinpirole in the presence of either ZM241385 ($n = 6$) or ST1535 ($n = 6$) were dose dependent (Fig. 1*D*,*E*). When the slices were preincubated with the D_2 receptor antagonist L-sulpiride (10 μM), no significant reduction of the EPSP amplitude was obtained during the coapplication of quinpirole with each of the two A_{2A} antagonists tested ($n = 6$ for each condition) (Fig. 1*D*,*E*), confirming the pivotal role of the D_2 DA receptor in this interaction.

It is worth noting that in our experiments, the majority of neurons recorded from rat slices, either with intracellular sharp microelectrodes or in whole-cell patch clamp, responded to D_2/A_{2A} modulation. In fact, in 85.3% of intracellularly recorded neurons (29 of 34 cells) and in 72.7% of patch-clamped neurons (16 of 22 cells), the EPSP/EPSC amplitudes were reduced (at least by 10%) in the presence of quinpirole (1–10 μM) plus ZM (1 μM) or quinpirole plus ST (10 μM).

CB_1 receptor antagonism reduces the effects of D_2/A_{2A} receptor modulation on striatal glutamatergic transmission

The modulation of striatal excitatory postsynaptic responses is known to involve a retrograde signaling mediated by eCBs, mainly acting on presynaptic CB_1 receptors and reducing glutamate release (Ferré et al., 2009). For this reason, we investigated the possible role played by eCB-mediated transmission in the inhibition of striatal glutamatergic transmission induced by the concomitant modulation of D_2 and A_{2A} receptors. The incubation of slices with a 3 μM concentration of the selective CB_1 receptor antagonist AM251 for 10–15 min did not alter per se the evoked postsynaptic responses ($n = 11$) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

The application of 3 μM AM251 significantly reduced the inhibitory effect on striatal glutamatergic transmission induced by the coapplication of quinpirole plus ZM241385 both in experiments with sharp electrodes ($n = 8$) and in patch-clamp recordings ($n = 6$) (Fig. 1*A*,*C*).

We also investigated the effect of CB_1 receptor blockade on the EPSP reduction obtained by coapplying quinpirole and ST1535 (Fig. 1*B*). Also in this case, the reduction of the EPSP amplitude mediated by D_2 receptor activation and A_{2A} receptor inhibition, recorded in the presence of 3 μM AM251 ($n = 7$), was significantly smaller than the one recorded in the presence of quinpirole and ST1535 and in the absence of CB_1 receptor blockade (Fig. 1*C*). Cumulative dose–response curves for quinpirole were also obtained in the presence of ZM plus AM ($n = 6$) (Fig. 1*D*) and in the presence of ST plus AM ($n = 6$) (Fig. 1*E*).

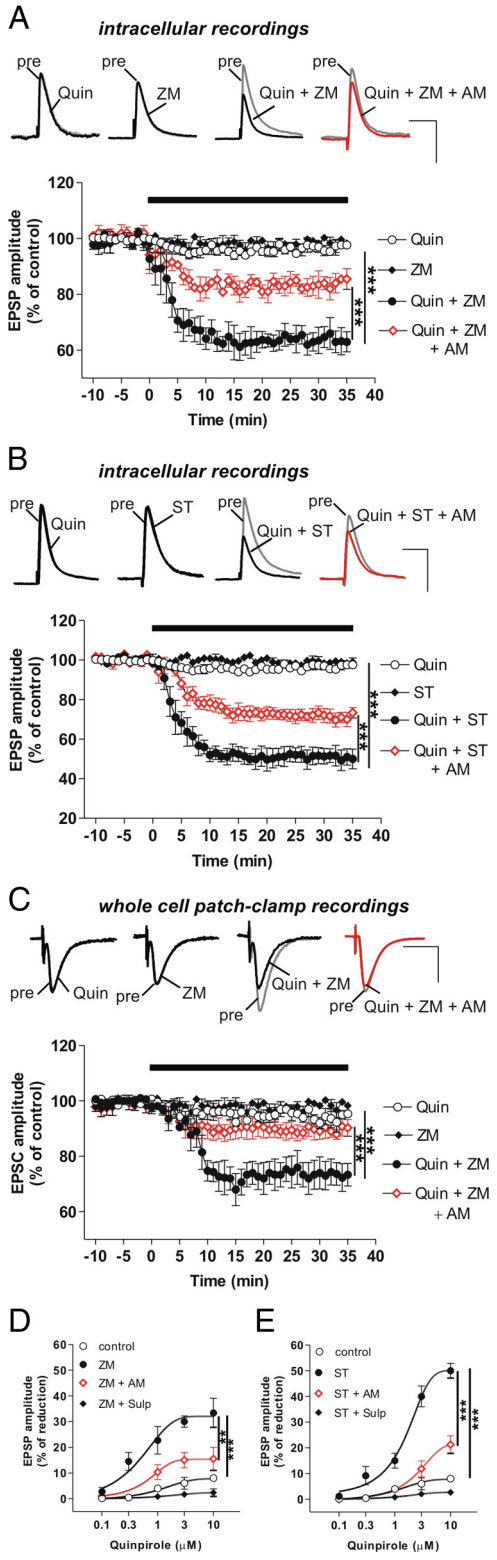

Figure 1. Depression of evoked EPSPs and EPSCs induced by concomitant D_2 DA-R activation and A_{2A}-R inhibition in striatal MSNs is partially reversed by CB_1-R blockade. ***A***, Graph of the time course of EPSP amplitude recorded with sharp microelectrodes in the presence of Quin (10 μM) or ZM (1 μM) given in isolation and Quin plus ZM bath applied together with or without AM (3 μM). Quin versus Quin plus ZM ($n = 9$ for each condition), $F_{(45,720)} = 42.0$, $^{***}p < 0.001$; AM plus Quin plus ZM ($n = 8$) versus Quin plus ZM ($n = 9$), $F_{(45,675)} = 10.9$, $^{***}p < 0.001$. Top traces, Example of four superimposed EPSP pairs before and 30–35 min after the application of Quin, ZM, Quin plus ZM, or Quin plus ZM plus AM. ***B***, Time course of EPSP amplitude and example EPSP superimposed pairs in the presence of Quin, ST (10 μM), or Quin plus ST coapplied with or without AM. Quin ($n = 9$) versus Quin plus ST ($n = 8$), $F_{(45,675)} = 119.4$, $^{***}p < 0.001$; AM plus Quin plus ST ($n = 7$) versus Quin plus ST ($n = 8$), $F_{(45,585)} = 19.6$, $^{***}p < 0.001$. ***C***, Time course of the EPSC amplitude recorded in whole-cell patch clamp and example EPSC superimposed pairs in the presence of Quin or ZM in isolation and Quin plus ZM applied with or without AM. Quin ($n = 8$) versus Quin plus ZM ($n = 6$), $F_{(45,540)} = 40.5$, $^{***}p < 0.001$; AM plus Quin plus ZM versus Quin plus ZM ($n = 6$ for each condition), $F_{(45,450)} = 16.6$, $^{***}p < 0.001$. ***D***, Dose–response curves of the reduction of the EPSP amplitudes induced by Quin in control conditions and in the presence of ZM, ZM plus AM, or ZM plus L-sulpiride (Sulp, 10 μM). Quin versus Quin plus ZM ($n = 6$ for each condition), $F_{(4,25)} = 10.2$, $^{***}p < 0.001$; Quin plus ZM versus AM plus Quin plus ZM ($n = 6$ for each condition), $F_{(3,20)} = 6.3$, $^{**}p < 0.01$. ***E***, Dose–response curves of the reduction of the EPSP amplitudes induced by Quin in control conditions and in the presence of ST, ST plus AM, or ST plus Sulp. Quin versus Quin plus ST ($n = 6$ for each condition), $F_{(45,25)} = 42.9$, $^{***}p < 0.001$; Quin plus ST versus AM plus Quin plus ST ($n = 6$ for each condition), $F_{(1,15)} = 244.9$, $^{***}p < 0.001$. Calibration: ***A***, ***B***, 50 ms, 10 mV; ***C***, 20 ms, 50 pA.

The D_2/A_{2A} pharmacological modulation of excitatory synaptic transmission is present in both D_1 receptor- and D_2 receptor-expressing striatal MSNs

Striatal spiny neurons from slices obtained from mice expressing BAC-EGFP under the control of D_1-R promoter (D_1-EGFP) or D_2-R promoter (D_2-EGFP) were visualized with an infrared and fluorescence-equipped microscope (Olympus) (Fig. 2*A*). Only neurons that displayed a marked fluorescence were approached for patch-clamp recordings and underwent subsequent electrophysiological characterization. As presented in Figure 2*B*, the current–voltage relationships from D_1-EGFP ($n = 20$) and D_2-EGFP ($n = 21$) MSNs showed no major differences.

To characterize the D_2/A_{2A} receptor-mediated modulation of the EPSC in these neurons, we bath applied 10 μM quinpirole plus 1 μM ZM, in the continuous presence of 10 μM bicuculline, after having obtained a stable EPSC baseline. In these conditions, quinpirole plus ZM241385 application produced a significant reduction of the EPSC amplitude in 70% of D_1-EGFP neurons (14 of 20 cells) and in 71.4% of D_2-EGFP neurons (15 of 21 cells). As presented in Figure 2, *C* and *D*, after 20 min of quinpirole and ZM241385 application, the EPSC amplitudes were reduced to 81.7 ± 4% of control in D_1-EGFP MSNs ($n = 14$) and to 87.0 ± 5% of control in D_2-EGFP MSNs ($n = 15$).

The CB_1-dependent inhibitory effect induced by concomitant modulation of D_2 and A_{2A} receptors is associated with an increased paired-pulse facilitation and is occluded by a CB_1 receptor agonist

Paired-pulse modification of neurotransmission has been attributed to a presynaptic change in release probability (Manabe et al., 1993). We have previously demonstrated that the decrease of the corticostriatal EPSP induced by a D_2-DA receptor agonist plus A_{2A} receptor antagonists is coupled to an increase of paired-pulse ratio (PPR) (Tozzi et al., 2007). Striatal eCBs are known to induce a depression of synaptic transmission by activating presynaptic CB_1 receptors. To better characterize the role of the CB_1 receptor activation in the D_2/A_{2A}-mediated response, we measured PPR of EPSCs during the coadministration of quinpirole plus either ZM241385 or ST1535, with and without the application of the CB_1 receptor antagonist AM251. We observed an increase of the PPR during the coadministration of quinpirole plus either ZM241385 ($n = 5$) or ST1535 ($n = 5$) but not when quinpirole was given alone ($n = 6$) (Fig. 3*A*), suggesting a significant decrease in release probability during concomitant modulation of D_2 and A_{2A} receptors.

PPR augmentation, obtained in the presence of the D_2 agonist plus A_{2A} antagonists, was prevented either by the D_2-R antagonist

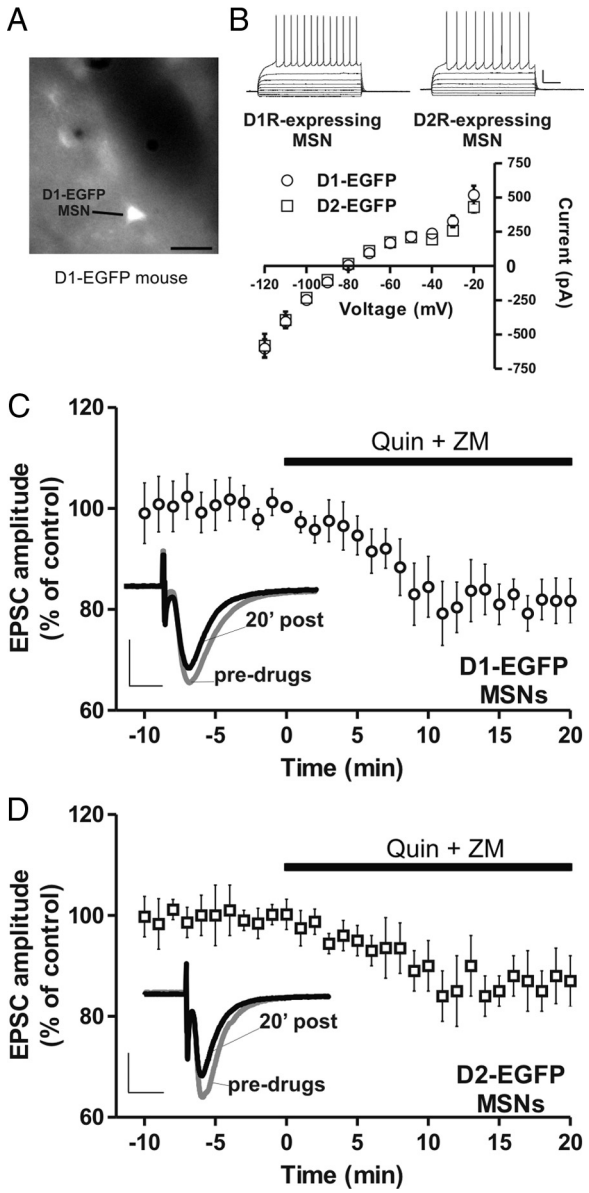

Figure 2. D_2/A_{2A} pharmacological modulation affects excitatory synaptic transmission in both D_2 and D_1 receptor-expressing striatal MSNs. ***A***, Image of a corticostriatal slice preparation from a D_1-EGFP mouse showing a representative neuron expressing D_1-EGFP receptors. ***B***, Voltage traces of both a D_1-EGFP- and a D_2-EGFP-striatal MSN recorded during hyperpolarizing and depolarizing current steps. The graph shows similar current–voltage plots of several striatal spiny neurons pooled together from D_2-EGFP ($n = 21$) and D_1-EGFP ($n = 20$) mice, respectively ($F_{(10,390)} = 0.94$, $p > 0.05$). ***C***, ***D***, Time course plots of the EPSC amplitude and EPSC superimposed pairs recorded in whole-cell patch clamp in the presence of Quin plus ZM in D_1-EGFP MSNs ($n = 14$) (***C***) and in D_2-EGFP MSNs ($n = 15$) (***D***). Scale bar: ***A***, 40 μm. Calibration: ***B***, 20 ms, 20 mV; ***C***, ***D***, 10 ms, 100 pA.

L-sulpiride ($n = 4$) or by the CB_1 receptor antagonist AM251 ($n = 4$) (Fig. 3*A*).

These results suggest that the inhibitory effect induced by concomitant D_2 receptor stimulation and A_{2A} receptor blockade is mediated by a presynaptic mechanism involving CB_1 receptors, possibly located on glutamatergic terminals.

As previously reported (Gerdeman et al., 2002; Gubellini et al., 2002; Yin and Lovinger, 2006), the pharmacological stimulation of CB_1 receptors induces a reduction of the corticostriatal postsynaptic response. To confirm the role of the CB_1 receptor in mediating the reduction of striatal glutamatergic transmission obtained by D_2-R stimulation and A_{2A}-R inhibition, we performed occlusion experiments using the CB_1 receptor agonist WIN. The pretreatment of corticostriatal slices with 3 μM WIN occluded the D_2/A_{2A}-induced effect on striatal glutamatergic transmission. In particular, bath application of 3 μM WIN alone produced after 15 min a reduction of the EPSC amplitude by $40.9 \pm 6.6\%$ ($n = 11$). However, the subsequent application of 10 μM quinpirole plus 1 μM ZM241385 did not alter the EPSC amplitude any further ($n = 11$) (Fig. 3*B*).

The effect caused by concomitant D_2/A_{2A} receptor modulation is prevented by buffering postsynaptic calcium

Since eCB release depends on the elevation of intracellular Ca^{2+} concentration (Piomelli, 2003), we buffered intracellular Ca^{2+} to try to prevent eCB release and retrograde diffusion. For this reason, we recorded a group of neurons while adding 20 mM BAPTA into the patch pipette solution. Five to ten minutes after obtaining the whole-cell configuration, EPSCs were evoked and monitored until a stable baseline was reached. In these conditions, bath application of 10 μM quinpirole plus 1 μM ZM241385 had no effect on the EPSC amplitude ($n = 6$) (Fig. 3*C*,*D*).

It is interesting to note that intracellular BAPTA was not able to alter the presynaptic inhibitory effect induced by the application of the CB_1 agonist WIN (3 μM, $42.8 \pm 4.7\%$ reduction of the EPSC amplitude, $n = 6$) or of the $GABA_B$ agonist baclofen (0.5 μM, $40.7 \pm 6.4\%$ reduction of the EPSC amplitude, $n = 6$).

These experiments reveal that, in contrast to the presynaptically mediated action of WIN and baclofen, the D_2/A_{2A} modulation of the EPSC requires an increase of intracellular Ca^{2+} levels at the postsynaptic site. Thus, the reduction of striatal glutamatergic transmission obtained by D_2-R stimulation and A_{2A}-R inhibition seems to be induced postsynaptically but expressed through a presynaptic mechanism involving eCB release and activation of presynaptic CB_1 receptors.

Role of endocannabinoids on the D_2-mediated modulation of striatal glutamatergic transmission in an experimental model of PD

Evoked corticostriatal postsynaptic responses of MSNs from 6-OHDA-denervated rats are known to be depressed by a selective activation of D_2 DA receptor (Calabresi et al., 1993; Picconi et al., 2004). However, whether eCBs play a role in the D_2 receptor-mediated inhibition of glutamatergic corticostriatal transmission in this pathogenetic model of PD is still unknown. Thus, we first compared the effect of two D_2 receptor agonists, quinpirole and pramipexole, on corticostriatal EPSPs in a group of 6-OHDA-denervated rats with respect to a group of sham-operated animals. In agreement with previous studies (Calabresi et al., 1993; Picconi et al., 2004), in none of the recorded neurons was an effect of these agonists observed in slices obtained from sham-operated rats ($n = 6$ for each drug and each concentration) (Fig. 4). Conversely, in DA-denervated rats, both quinpirole ($n = 12$ for each concentration) (Fig. 4*A*) and pramipexole ($n = 11$ for each concentration) (Fig. 4*B*) significantly reduced the EPSP amplitude in a dose-dependent manner.

Interestingly, the incubation of the slices obtained from 6-OHDA rats with 3 μM AM251 partially prevented the D_2-mediated reduction of the EPSP amplitude produced either by quinpirole ($n = 15$) or by pramipexole ($n = 14$) (Fig. 4*A*,*B*; supplemental Fig. S2, available at www.jneurosci.org as supplemental material), revealing a critical role of eCBs in the D_2-mediated inhibition of glutamatergic transmission in the parkinsonian state. Similarly to the effects observed in physiolog-

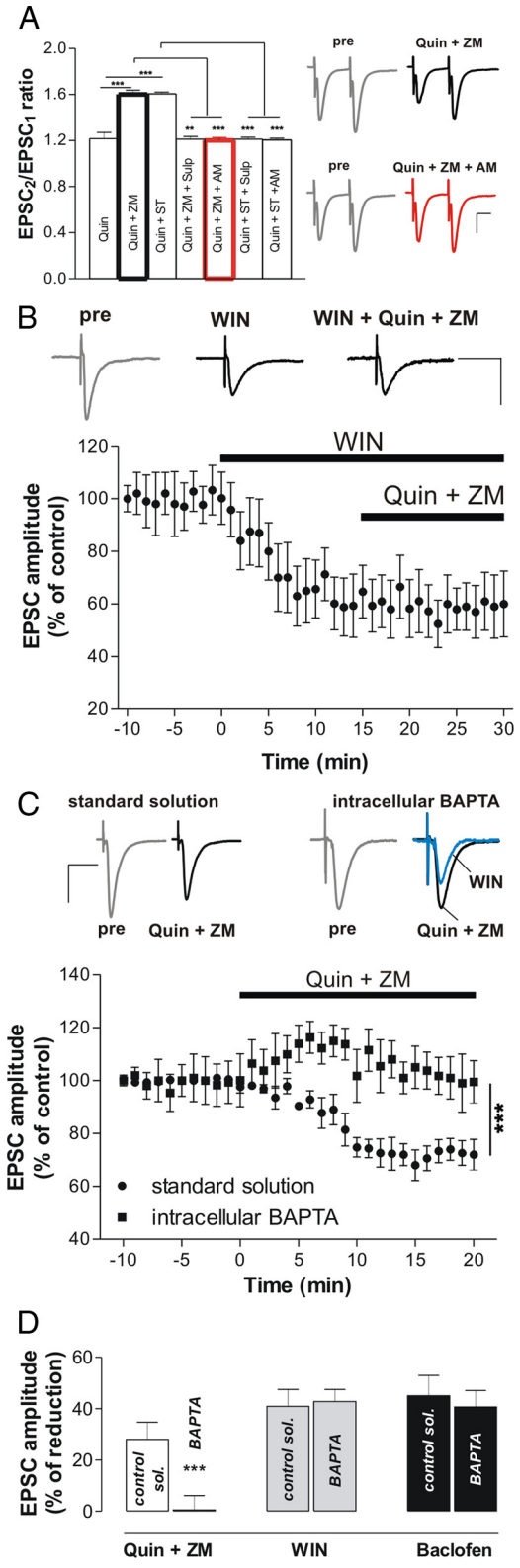

Figure 3. The CB_1-dependent inhibitory effect induced by concomitant D_2/A_{2A}-R modulation is associated with an increased paired-pulse facilitation and is prevented by buffering postsynaptic calcium. ***A***, Histogram shows pooled ratios of EPSC amplitudes ($EPSC_2/EPSC_1$) in different pharmacological conditions. Note that the significant effect of the concomitant application of Quin (10 μM) plus either ZM (1 μM) or ST (10 μM) [Quin ($n = 6$) vs Quin plus ZM, $n = 5$, $t_{(9)} = 5.8$, ***$p < 0.001$; Quin vs Quin plus ST ($n = 5$) $t_{(9)} = 6.2$, ***$p < 0.001$] is reversed by either the D_2 DA-R antagonist Sulp (10 μM) [Quin plus ZM ($n = 5$) vs Quin plus ZM plus 10 μM Sulp ($n = 4$), $t_{(7)} = 4.3$, **$p < 0.01$; Quin plus ST ($n = 5$) vs Quin plus ST plus 10 μM Sulp ($n = 4$), $t_{(7)} = 7.2$, ***$p < 0.001$] or the CB_1-R antagonist AM (3 μM) [Quin plus ZM vs Quin plus ZM plus AM251 ($n = 4$), $t_{(7)} = 5.8$, ***$p < 0.001$; Quin plus ST vs Quin plus ST plus AM251 ($n = 4$), $t_{(7)} = 6.7$, ***$p < 0.001$]. Shown are paired EPSC traces (50 ms paired-pulse stimulation interval) acquired before and 15 min after the application of Quin plus ZM (upper traces) and before and 15 min after Quin plus ZM in the presence of AM (bottom traces). ***B***, Time course showing the averaged EPSC amplitudes in control conditions and after the subsequent application of the CB_1-R agonist WIN (3 μM) and WIN plus Quin plus ZM ($n = 11$). EPSC traces recorded from a single striatal MSN in control conditions and after the application of the CB_1-R agonist WIN and WIN plus Quin plus ZM. Note that the effect of Quin plus ZM is occluded in the presence of WIN. ***C***, Time courses of the EPSC amplitudes during concomitant application of Quin and ZM in the presence of a BAPTA-containing internal solution compared to the standard internal solution [Quin plus ZM vs Quin plus ZM in BAPTA ($n = 6$ for each condition), $F_{(30,155)} = 3.2$, ***$p < 0.001$]. Upper traces show the effect of Quin plus ZM on the EPSCs of a striatal MSN recorded with the standard internal solution (left traces) and an MSN recorded with intracellular BAPTA (right traces). Note the large reduction of the EPSC obtained in the presence of 3 μM WIN in the MSN recorded with intracellular BAPTA, while the effect of Quin plus ZM is blocked in these experimental conditions. ***D***, Histograms showing the reduction of the EPSC amplitudes of MSNs recorded with the standard internal solution, with a BAPTA-containing internal solution in the presence of Quin plus ZM (at 20 min), 3 μM WIN, and 0.5 μM $GABA_B$ agonist baclofen ($n = 6$ for the experimental conditions). Quin plus ZM versus Quin plus ZM in BAPTA ($n = 6$), $t_{(10)} = 3.2$, ***$p < 0.001$. Calibration: 20 ms, 100 pA.

ical conditions for concomitant application of quinpirole and ZM, also in the parkinsonian state intracellular BAPTA was able to block the pharmacological action of quinpirole (EPSC reduction for quinpirole in BAPTA, 0.8 ± 7.8% of baseline, $n = 6$) (Fig. 4*C*) but not the presynaptic action of WIN (EPSC reduction for WIN in BAPTA, 41 ± 8.5% of baseline, $n = 4$) (Fig. 4*C*).

Effect of the concomitant activation of D_2 receptors and antagonism of A_{2A} receptors in an experimental model of PD

We then aimed at investigating whether in striatal MSNs from 6-OHDA slices the eCB- and D_2 receptor-mediated depression of corticostriatal synaptic transmission was also affected by the blockade of A_{2A} receptors. Thus, we tested whether the functional antagonism of D_2 stimulation and A_{2A} blockade also occurred in 6-OHDA animals.

In neurons from 6-OHDA animals, the reduction of the glutamatergic EPSPs by the activation of D_2 receptors following bath application of a low dose of quinpirole (0.3 μM) was significantly increased when this concentration of quinpirole was applied in combination with either 1 μM ZM241385 or 10 μM ST1535 (quinpirole plus ZM, $n = 8$; quinpirole plus ST, $n = 7$) (supplemental Fig. S2*A*, available at www.jneurosci.org as supplemental material). Conversely, the combined effect of a high dose of quinpirole (3 μM) with either 1 μM ZM241385 or 10 μM ST1535 produced an effect on the EPSP amplitude that was not significantly different from the one of 3 μM quinpirole applied in isolation (quinpirole plus ZM, $n = 8$; quinpirole plus ST, $n = 7$) (supplemental Fig. S2*A*, available at www.jneurosci.org as supplemental material).

Moreover, similarly to quinpirole, a low dose of pramipexole (0.3 μM) applied in conjunction with either 1 μM ZM241385 or 10 μM ST1535 produced a significant reduction of the EPSP with respect to the application of pramipexole in isolation (pramipexole vs pramipexole plus ZM, $n = 6$; pramipexole vs pramipexole plus ST, $n = 6$), while the inhibition produced by a high dose (3 μM) of pramipexole was not further increased when this receptor agonist was applied in conjunction with A_{2A} receptor antagonists ($n = 6$) (supplemental Fig. S2*B*, available at www.jneurosci.org as supplemental material).

We then explored, in 6-OHDA animals, the contribution of CB_1 receptors to the modulation of the EPSP amplitude of MSNs

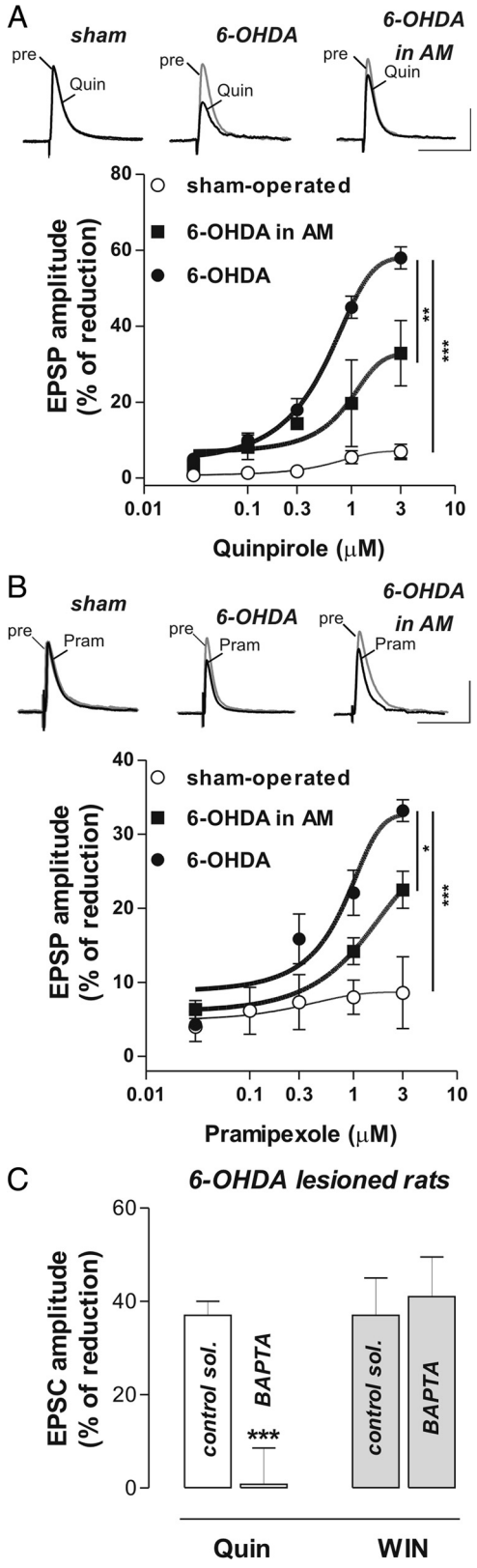

Figure 4. In the parkinsonian state, activation of D_2 DA-R reduces glutamatergic transmission by an endocannabinoid-dependent mechanism. ***A***, ***B***, Superimposed traces of EPSPs of MSNs recorded from sham-operated and 6-OHDA-denervated rats, before and 15 min after bath application of 3 μM Quin (***A***) and 3 μM pramipexole (Pram) (***B***) either in the presence or in the absence of 3 μM AM. Dose–response curves show the effects on EPSP amplitudes exerted by Quin (***A***) or by Pram (***B***) in sham-operated rats and in 6-OHDA-denervated rats either in the presence or in the absence of AM. Quin, 6-OHDA ($n = 12$) versus sham ($n = 6$), $F_{(4,76)} = 73.4$, $^{***}p < 0.001$; Pram, 6-OHDA ($n = 11$) versus sham ($n = 6$), $F_{(3,60)} = 16.4$, $^{***}p < 0.001$. Note that the effects of D_2 DA-R agonists in 6-OHDA animals is partially reversed by the CB_1-R antagonist AM. Quin versus Quin plus AM ($n = 15$), $F_{(4,121)} = 8.4$, $^{**}p < 0.01$; Pram versus Pram plus AM ($n = 14$), $F_{(2,69)} = 8.4$, $^{*}p < 0.05$. ***C***, Histogram showing the reduction of the EPSC amplitudes of MSNs recorded with the standard internal solution, with a BAPTA-containing internal solution in the presence of either Quin (at 20 min) or 3 μM WIN. Quin versus Quin in BAPTA ($n = 6$), $t_{(10)} = 5.2$, $^{***}p < 0.001$. Calibration: 100 ms, 10 mV.

recorded in the presence of the D_2 receptor agonists and A_{2A} antagonists. As shown in the histogram of supplemental Figure S2 (available at www.jneurosci.org as supplemental material), the effect on the corticostriatal EPSP of the concomitant application of D_2 receptor agonists and A_{2A} receptor antagonists was significantly reduced in the presence of the CB_1 receptor antagonist AM251 (3 μM, $n = 9$, for each experimental condition).

Pharmacological activation of D_2 receptor produces similar effects in both D_1 and D_2 receptor-expressing striatal MSNs following DA depletion

We found that in physiological conditions, the effects of concomitant modulation of D_2 and A_{2A} receptors were observed in both D_1 and D_2 receptor-expressing striatal MSNs. Thus, we investigated whether, after DA depletion, also the inhibitory action of D_2 receptor activation was equally expressed in both these subclasses of striatal neurons.

Interestingly, we found that in slices obtained from reserpine-treated D_1-EGFP and D_2-EGFP mice, 3 μM quinpirole was able to reduce the EPSC amplitude in both D_1-EGFP-positive ($n = 8$) and D_2-EGFP-positive ($n = 8$) MSNs to 77.2 ± 2% and to 79.3 ± 3% of control, respectively (Fig. 5). Experiments performed with BAPTA-containing electrodes showed that buffering of postsynaptic Ca^{2+} was able to prevent the effect of quinpirole in both D_1- and D_2-expressing MSNs in all the neurons recorded under this experimental condition ($n = 5$, for both groups) (Fig. 5).

Adenosine A_{2A} and dopamine D_2 receptor coexpression in striatal ChAT-positive interneurons

It has been recently suggested that D_2 receptors might indirectly boost synaptic Ca^{2+} influx by decreasing acetylcholine release from striatal cholinergic interneurons (Wang et al., 2006). This effect opens the possibility that D_2 receptors and A_{2A} receptors might be coexpressed in cholinergic interneurons. To explore this possibility, we performed immunofluorescence analysis of corticostriatal sections obtained from three transcardially perfused mice.

Our triple-label immunofluorescence study indicates that D_2 dopamine receptors are localized on the cell somata of the cholinergic interneurons. Such observation is consistent with the data of Alcantara et al. (2001, 2003). As shown in the triple immunofluorescence images (Fig. 6), A_{2A}-Rs and D_2-Rs colocalize in ChAT-positive neurons. Interestingly, our quantitative analysis showed that all 750 ChAT-positive neurons were labeled for D_2 and A_{2A} receptors (Fig. 6), providing for the first time strong immunohistochemical evidence for the presence of both A_{2A} and D_2 receptors in striatal cholinergic interneurons.

A_{2A} and D_2 receptor-mediated regulation of firing activity in striatal cholinergic interneurons

The pharmacological stimulation of D_2 DA receptor and concomitant inhibition of A_{2A} adenosine receptor produced similar effects on the glutamatergic synaptic transmission in D_2 and D_1 receptor-expressing MSNs, thus even in MSNs in which D_2 or

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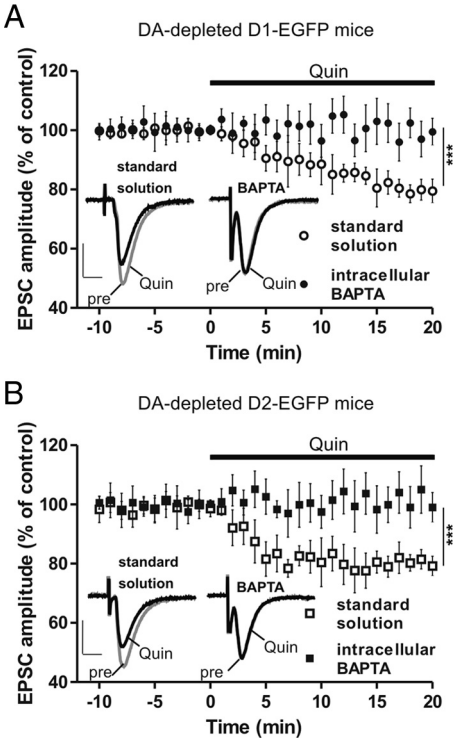

Figure 5. Activation of D_2 receptor produces similar effects in both D_1 and D_2 receptor-expressing striatal MSNs following DA depletion. ***A***, ***B***, Time course plots of the EPSC amplitude recorded in whole-cell patch clamp in the presence of 3 μM Quin in D_1-EGFP MSNs (***A***) and in D_2-EGFP MSNs (***B***) with the standard internal solution and with a BAPTA-containing internal solution. D_1-EGFP MSNs: Quin ($n = 8$) versus Quin in BAPTA ($n = 5$), $F_{(30,330)} = 11.8$, ***$p < 0.001$; D_2-EGFP MSNs: Quin ($n = 8$) versus Quin in BAPTA ($n = 5$), $F_{(30,330)} = 6.8$, ***$p < 0.001$. The insets show EPSC superimposed pairs recorded before and 20 min after Quin application in a D_1-EGFP MSN (***A***) and in a D_2-EGFP MSN (***B***) in the presence either of the standard internal solution or of the BAPTA-containing solution. Calibration: 10 ms, 100 pA.

A_{2A} receptors may not be expressed. Striatal cholinergic interneurons represent a major intrastriatal source of acetylcholine, projecting to virtually all MSN subtypes. For this reason, the cholinergic interneuron may represent a pivotal player in regulating glutamatergic synaptic transmission in D_2-R and even in D_1-R-expressing neurons.

Cholinergic interneurons were first localized under IR-DIC visualization by their large soma and second identified by their electrophysiological properties recorded in whole-cell patch-clamp mode (Bennett and Wilson, 1998, 1999). These cells presented a pronounced h-current (I_h) and a typical sag potential in response to hyperpolarizing steps of current (Fig. 7*A*). Their resting membrane potential ranged from −54 to −60 mV, and the majority of them (>80%) were firing spontaneously (Kawaguchi, 1993; Bennett and Wilson, 1999).

Whole-cell recordings were obtained from 35 cholinergic interneurons displaying a firing rate that ranged from 0.4 to 4 Hz. As shown in Figure 7, *B* and *C*, 0.3 μM quinpirole ($n = 6$) did not affect either the resting membrane potential or the firing rate of the recorded neurons. Conversely, in agreement with a previous study (Maurice et al., 2004), a higher dose of quinpirole (3 μM) reduced the firing rate of these neurons of 30.5 ± 7.7% ($n = 4$) (Fig. 7*C*).

The expression of adenosine A_{2A} receptor that we found in ChAT/D_2-positive cholinergic interneurons from striatal slices (Fig. 6) raises the possibility that D_2 DA and A_{2A} adenosine signaling might interact to functionally converge in the fine regulation of firing discharge of cholinergic interneurons and hence modulating acetylcholine (Ach) release at the synaptic sites of MSNs. The altered Ach release could, in turn, influence the properties of MSNs favoring the release of eCBs as previous postulated for the induction of striatal LTD (Wang et al., 2006).

Interestingly, while bath application of a 1 μM concentration of the A_{2A} receptor antagonist ZM for 15 min did not affect the spontaneous firing rate of the recorded neurons (data not shown), the coapplication of 1 μM ZM and quinpirole for 4 min significantly reduced the firing rate in a dose-dependent manner (0.1 and 3 μM Quin, $n = 4$; 0.3 μM Quin, $n = 6$) (Fig. 7*C*,*D*), providing the first functional evidence of a D_2 DA and A_{2A} adenosine receptor interaction in these striatal interneurons.

Inhibition of M1 muscarinic receptor prevents the reduction of EPSC obtained by D_2-R stimulation and A_{2A}-R inhibition in D_1 and D_2 receptor-expressing striatal MSNs

Corticostriatal synapses are particularly enriched in M1 muscarinic receptors, whose main targets in MSNs are L-type voltage-operated Ca^{2+} channels, which are also abundantly and strategically expressed at the postsynaptic density of glutamatergic synapses (Olson et al., 2005). In striatum, the modulation of the cholinergic drive to MSNs, as in the case of a reduced firing rate of the spontaneously active cholinergic interneurons, may lead to a decreased release of acetylcholine at MSNs synaptic sites, and in turn to a depressed corticostriatal glutamatergic transmission through the regulation of intracellular calcium levels and eCB release via L-type Ca^{2+} channels.

To explore the possible contribution of M1 receptor activity to the D_2/A_{2A}-mediated modulation of corticostriatal excitatory postsynaptic response, we performed whole-cell recordings in both D_2 and D_1 receptor-expressing MSNs from D_2- and D_1-BAC mice in the presence of the selective M1 muscarinic receptor inhibitor pirenzepine. Bath application of 2 μM pirenzepine slightly reduced glutamatergic synaptic transmission in both D_2- and D_1-expressing MSNs of 13.4 ± 2.0% ($n = 6$) and 11.57 ± 6.5% ($n = 6$), respectively, most likely unmasking a cholinergic tonic effect possibly on presynaptic and postsynaptic muscarinic receptors (Fig. 8). After obtaining a stable baseline for 10 min in the presence of 2 μM pirenzepine, 10 μM quinpirole plus 1 μM ZM was bath applied for 20 min. In these conditions, the EPSC amplitude was not altered either in D_2-EGFP- or in D_1-EGFP-expressing MSNs ($n = 6$, for both groups), whereas in the same groups of neurons, the CB_1 agonist WIN (3 μM) reduced the EPSC amplitude of 44.3 ± 6% in D_2-EGFP MSNs and 42.2 ± 5% in D_1-EGFP MSNs ($n = 6$, in both groups) (Fig. 8*A*).

D_2 DA receptor inhibition is sufficient to reduce firing rate of cholinergic interneurons in a model of PD

We have shown that in experimental models of PD (Figs. 4, 5), D_2 DA receptor stimulation is sufficient to produce a reduction of excitatory postsynaptic response in striatal MSNs, whereas in physiological conditions, the D_2/A_{2A} receptor interaction is required to recruit a depression of the postsynaptic response. To test whether a possibly enhanced level of D_2 DA receptors on cholinergic interneurons could mediate a modulation of the firing rate of these neurons, we performed whole-cell current-clamp recordings of cholinergic interneurons from DA-depleted mice subchronically treated with reserpine (see Materials and Methods). Under these experimental conditions, even low concentrations of quinpirole (0.1–0.3 μM) applied for 4 min significantly reduced the firing frequency of the recorded neurons in a

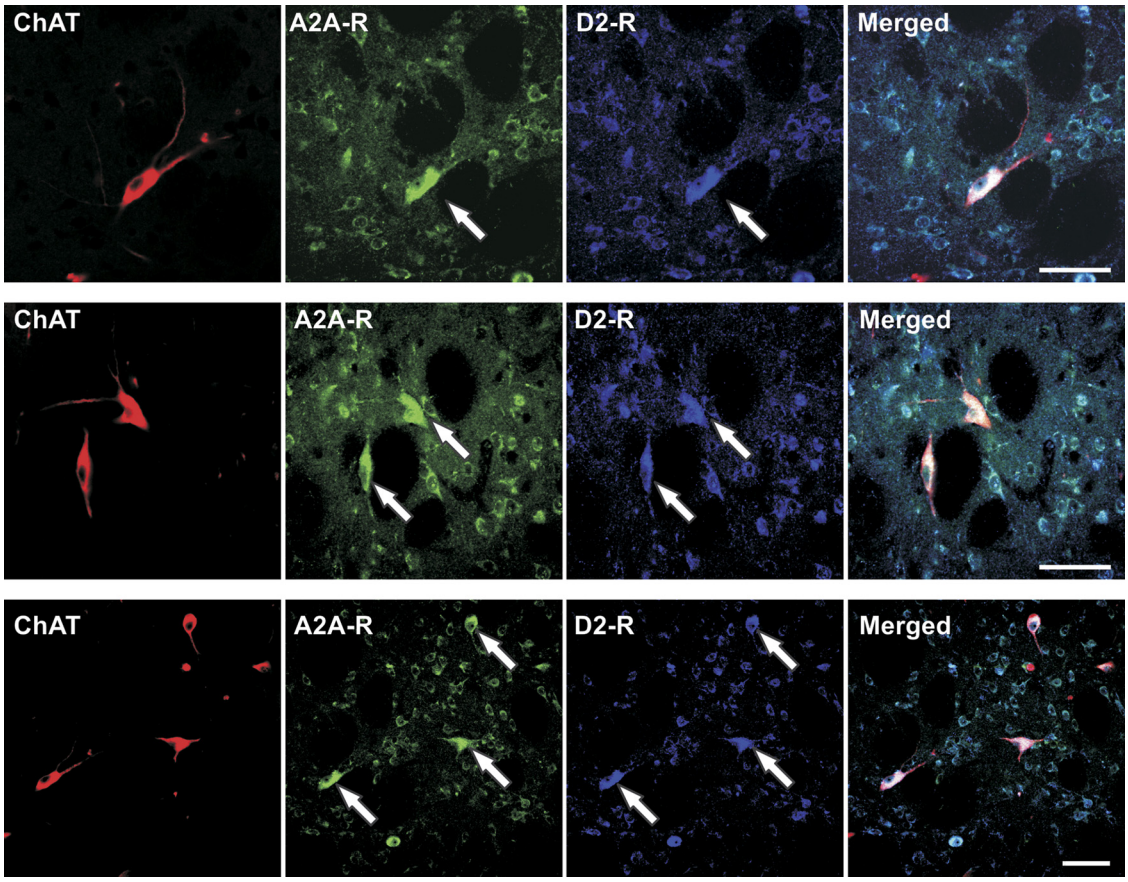

Figure 6. Coimmunolocalization of A_{2A} adenosine and D_2 dopamine receptors in cholinergic striatal interneurons. Representative confocal laser scanning microscopy images of triple-labeled immunofluorescence for A_{2A} adenosine and D_2 dopamine receptors in cholinergic striatal interneurons labeled with anti-ChAT. A_{2A} is visualized in green-cy2 fluorescence, ChAT in red-cy3 fluorescence, and D_2 immunolabeling in blue Alexa 647 fluorescence. The arrows show cholinergic interneurons colocalizing A_{2A} and D_2 receptors. Scale bar, 50 μm.

dose-dependent manner (0.1 and 3 μM Quin, $n = 4$; 0.3 μM Quin, $n = 6$) (Fig. 7*C*,*E*), suggesting an increased sensitivity and an altered function of this class of interneurons in this experimental model of PD.

Role of M1 muscarinic receptor on MSNs in a dopamine-depleted model of PD

We recorded D_2-EGFP and D_1-EGFP MSNs from mice subchronically treated with reserpine in the presence of 2 μM pirenzepine to prevent M1 muscarinic receptor activation. In this condition, while pirenzepine produced a mild reduction of glutamatergic synaptic transmission, 3 μM quinpirole failed to reduce the EPSC amplitude in both D_2- and D_1-EGFP MSNs ($n = 6$) (Fig. 8*B*), suggesting that also in corticostriatal slices from DA-depleted mice M1 muscarinic receptors and cholinergic interneurons have a major role in mediating the D_2 receptor-dependent reduction of the EPSC amplitude in D_2 and in D_1 receptor-expressing MSNs.

Discussion

Major findings

In the present study, we obtained four new major findings having both physiological and clinical relevance:

1. Under physiological conditions, the concomitant activation of D_2 DA receptors and the blockade of A_{2A} adenosine receptors decreased striatal glutamatergic transmission by a presynaptic mechanism. This presynaptic action was mainly mediated by a retrograde action of eCBs released by postsynaptic spiny neurons and acting on CB_1 cannabinoid receptors located on glutamatergic terminals. Since this inhibitory effect was not achieved when either D_2 receptor agonists or A_{2A} receptor antagonists were given in isolation, we can argue that the convergence of these two neurotransmitter systems on the endocannabinoid pathway may represent a potent feedback mechanism to control glutamatergic transmission in the striatum (Fig. 9).

2. In DA-depleted animals, even D_2 receptor agonists alone were able to reduce glutamatergic transmission via an endocannabinoid-dependent mechanism. Thus, increased response of these receptors after denervation (Calabresi et al., 1993; Picconi et al., 2004) is sufficient to amplify the dopaminergic control on glutamate release via a retrograde mechanism acting on CB_1 receptors. Interestingly, in the DA-denervated striatum, we also found that A_{2A} receptor antagonists were able to enhance the inhibitory effect exerted by low doses of D_2 receptor agonists. This latter evidence might have profound implications for a novel rationale in the clinical pharmacology of PD supporting the use of a combination of D_2 receptor agonists and A_{2A} receptor antagonists.

3. The observed D_2-dependent pharmacological effects were not segregated to D_2 receptor-expressing MSNs but were also observed in D_1 receptor-expressing neurons. Interestingly, this effect implicates a postsynaptic site of action in both these neuronal subtypes, since it was reduced by buffering of postsynaptic intracellular Ca^{2+}.

4. Finally, we found that cholinergic interneurons, coexpressing D_2 and A_{2A} receptors, are implicated in this pharmacological

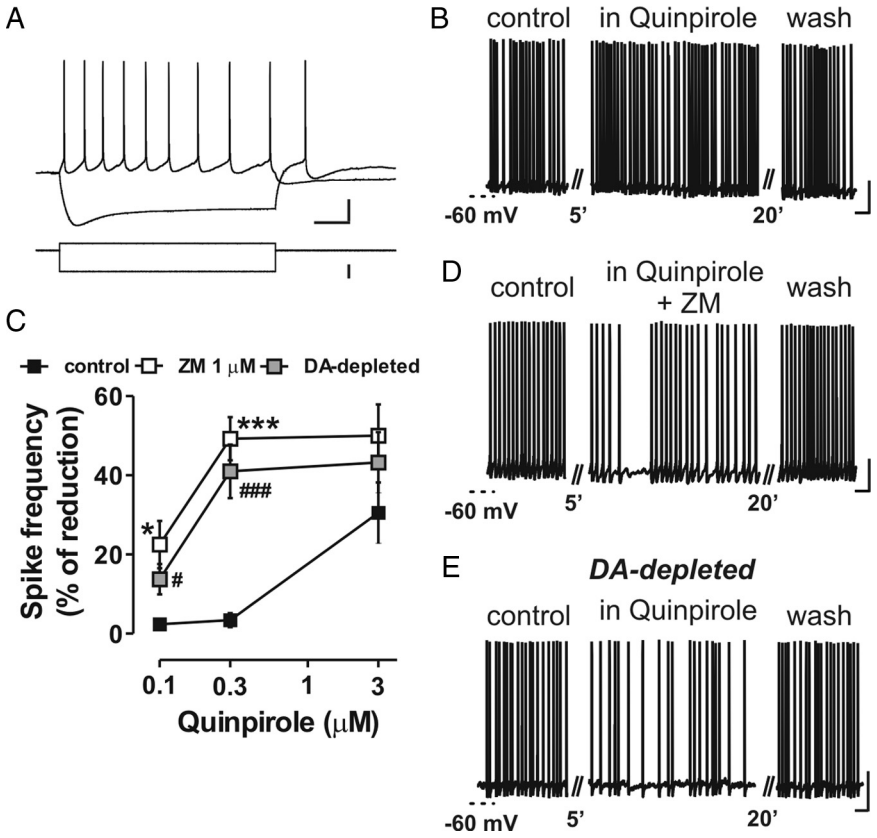

Figure 7. Stimulation of D_2 receptors and inhibition of A_{2A} receptors reduce the firing discharge rate in striatal cholinergic interneurons. ***A***, Representative voltage responses of a striatal large cholinergic interneuron (resting membrane potential = −58 mV) after depolarizing and hyperpolarizing current steps. ***B***, ***D***, Example traces of two whole-cell recordings of cholinergic interneurons in control conditions and after the application of 0.3 μM Quin (***B***) or 0.3 μM Quin plus 1 μM ZM (***D***). ***C***, Dose–response curves of spike frequency, as percentage of reduction, from cholinergic interneurons measured in physiological and DA-depleted conditions. Quin was bath applied alone or together with 1 μM ZM. For 0.1 μM Quin, Quin versus Quin plus ZM ($n = 4$ for each condition), $t_{(6)} = 3.4$, $^{*}p < 0.05$; Quin versus Quin in DA-depleted conditions ($n = 4$ for each condition), $t_{(6)} = 2.9$, $^{\#}p < 0.05$. For 0.3 μM Quin, Quin versus Quin plus ZM, $n = 6$ for each condition, $t_{(10)} = 8.0$, $^{***}p < 0.001$; Quin versus Quin in DA-depleted condition ($n = 6$ for each condition), $t_{(10)} = 5.4$, $^{\#\#\#}p < 0.001$. The higher dose of quinpirole (3 μM) reduced the firing rate of these neurons in a similar way in all the three experimental groups ($n = 4$ for each condition). ***E***, Representative trace of a cholinergic interneuron recorded from a corticostriatal slice of a DA-depleted mouse in control conditions and after the application of 0.3 μM Quin. Calibration: ***A***, top, 200 ms, 20 mV; bottom, 100 pA; ***B***, ***D***, ***E***, 1 s, 20 mV.

modulation, since concomitant activation of D_2 DA receptors and blockade of A_{2A} receptors reduces the firing rate of these interneurons and M1 receptor antagonism blocks the D_2/A_{2A} receptor-mediated modulation of excitatory transmission in both D_2- and D_1-expressing MSNs (Fig. 9).

The D_2/A_{2A} receptor interaction in the control of striatal glutamatergic transmission is expressed at a postsynaptic site, but it requires presynaptic inhibition via a retrograde endocannabinoid signal

Both types of reciprocal antagonistic A_{2A}–D_2 receptor interactions coexist in the same cells. In fact, under normal conditions, there is a strong tonic activation of D_2 receptors that blocks the ability of A_{2A} receptors to signal through the cAMP–PKA pathway. Conversely, the antagonistic A_{2A}–D_2 receptor interaction determines the ability of A_{2A} receptors to control the inhibitory role of D_2 receptors in neuronal excitability and neurotransmitter release (Ferré et al., 2008).

In line with our previous studies (Calabresi et al., 1993; Picconi et al., 2004; Tozzi et al., 2007), we found that the application of D_2 receptor agonists alone did not affect glutamate-mediated synaptic potentials/currents in striatal slices under physiological conditions. Conversely, simultaneous A_{2A} receptor antagonism and D_2 receptor activation resulted in a reduction of excitatory glutamatergic transmission. In our model, electrical stimulation of the slice mainly activates glutamatergic projections to the striatum. However, this stimulation most likely also affects dopaminergic terminals projecting to striatal neurons, thus leading to occlusion phenomena produced by the local release of DA (Higley and Sabatini, 2010). Thus, in our experiments conducted in physiological conditions, the activation of intrastriatal DA fibers during repeated electrical stimulation may increase the levels of endogenous DA, making it more difficult to observe pharmacological effects of D_2 agonists in reducing corticostriatal synaptic transmission. Conversely, in DA-depleted slices, the virtual absence of endogenous DA together with the increased sensitivity to DA agonists better reveals the pharmacological effects of these drugs. The inhibition of the evoked EPSCs following the concomitant application A_{2A} receptor antagonists and D_2 receptor agonists was associated with an increased paired-pulse facilitation, indicating a decrease in the probability of striatal glutamate release. This presynaptic mechanism was blocked by a CB_1 receptor antagonist, suggesting the critical involvement of retrograde action eCBs targeting this receptor subtype. Accordingly, activation of CB_1 receptors on corticostriatal glutamatergic terminals reduces the release of this excitatory neurotransmitter (Gerdeman et al., 2002; Gubellini et al., 2002; Kreitzer and Malenka, 2005). We also found that the inhibitory action on the glutamatergic transmission exerted by the concomitant modulation of A_{2A} and D_2 receptors was occluded by a selective CB_1 receptor agonist, further supporting the hypothesis of a specific involvement of a presynaptic eCB-mediated mechanism in the synergistic action of A_{2A} receptor antagonists and D_2 receptor agonists.

Nevertheless, we observed that the intracellular application of a calcium chelating agent such as BAPTA is able to prevent the inhibitory effects on glutamatergic transmission obtained by D_2-R stimulation and A_{2A}-R inhibition, providing strong evidence in favor of a postsynaptic site for the interaction between A_{2A} and D_2 receptors.

The fact that in our experiments we were able to detect significant electrophysiological effects in both D_1 and D_2 receptor-expressing MSNs suggests that, at least from a functional point of view, the concomitant modulation of A_{2A} and D_2 receptors not only affects neurons of the so-called "indirect pathway," but also seems to involve striatal spiny neurons in a larger scale. Similar conclusions could be also drawn according to a previous seminal study of Lovinger's group (Yin and Lovinger, 2006). In this study, Lovinger's group has shown that in most of the recordings from

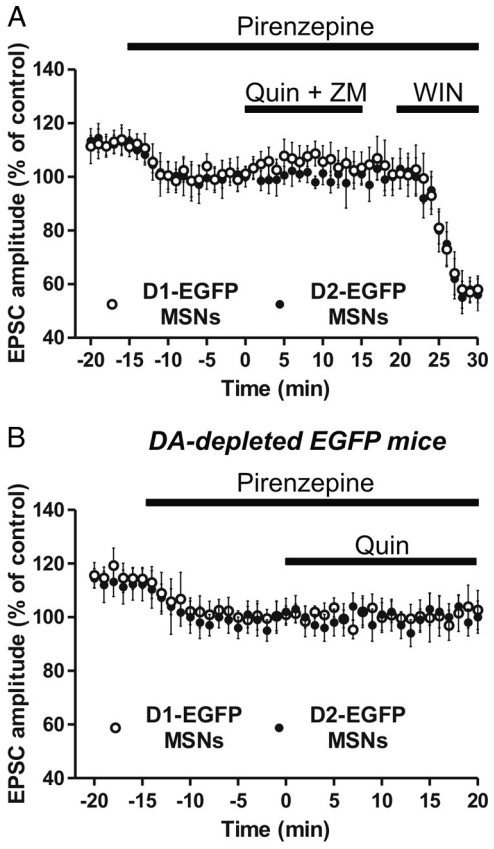

Figure 8. M1 muscarinic receptor inhibition prevents the combined effect of D_2-R stimulation and A_{2A}-R inhibition in D_1- and D_2-R-expressing MSNs. ***A***, Time course of the pooled EPSC amplitudes recorded from either D_2- or D_1-MSNs in control conditions and in the presence of 2 μM pirenzepine. Quin plus ZM or WIN (3 μM) was bath applied to activate the D_2/A_{2A}-mediated response or to stimulate the CB_1 receptor function in the presence of pirenzepine ($n = 6$ for each neuronal population). ***B***, In the DA-depleted model of PD, 2 μM pirenzepine prevents the effect of 3 μM Quin in D_1- and in D_2-expressing MSNs ($n = 6$ for each neuronal population).

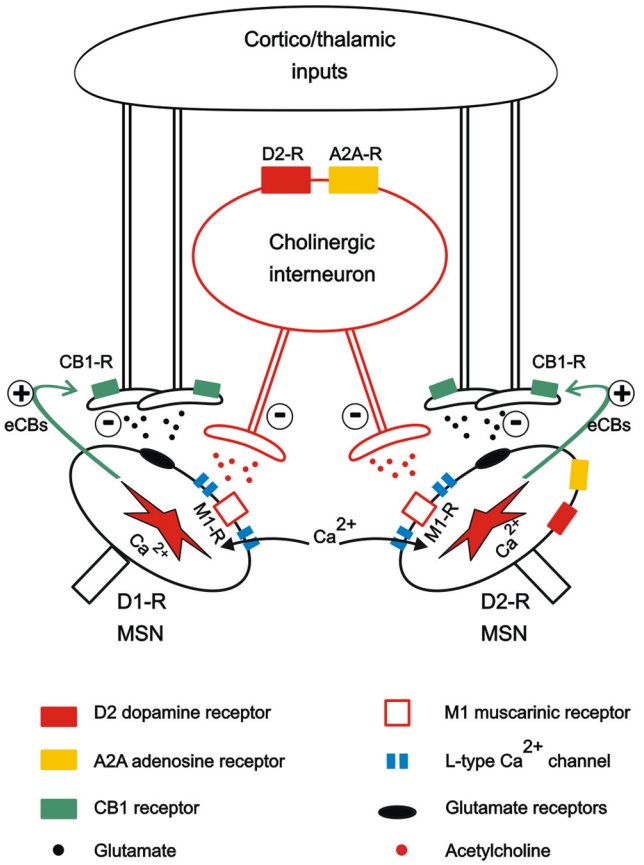

Figure 9. Model of a cholinergic interneuron projecting to either D_1 or D_2 receptor-expressing striatal MSNs. The combined activation of both A_{2A} and D_2 receptors on cholinergic interneurons may decrease the release of acetylcholine at the synaptic sites of D_1- and D_2-R-expressing MSNs and trigger a Ca^{2+} inflow by relieving the cholinergic inhibition of L-type Ca^{2+} channels mediated by M1 muscarinic receptors. The increase in intracellular Ca^{2+} concentration might in turn trigger endocannabinoid release at the postsynaptic sites of both D_1- and D_2-R-expressing MSNs and hence depress glutamatergic synaptic transmission of both the direct and indirect pathways.

striatal MSNs, activation of D_2 receptors reduces the release of glutamate in the striatum by a retrograde endocannabinoid signaling during stimulation at high frequencies. This effect, however, was not detected at low frequencies (or in the absence) of synaptic stimulation (Yin and Lovinger, 2006). In the present study, we demonstrate that, when A_{2A} receptors are antagonized, activation of D_2 receptors is able to trigger a retrograde signaling even in the absence of high-frequency stimulation.

Accordingly, similar evidence confirming the interesting effect of D_2 agonist on striatal neurons of the direct pathway comes from an *in vivo* study in which in striatonigral neurons from 6-OHDA-treated rats, a physiological responsiveness could be restored by administering the D_2 receptor agonist quinpirole (Ballion et al., 2009).

In the parkinsonian state, activation of D_2 receptor per se is sufficient to reduce glutamatergic transmission by an endocannabinoid-dependent mechanism

In previous studies, we have observed that a reduction of glutamate transmission by D_2 receptor activation was achieved only if animals were subjected to interventions designed to drive DA receptor signaling into a supersensitive state, as after the overexpression of the short isoform of D_2 receptor (D_{2S}) or after nigrostriatal DA denervation in PD animal models (Calabresi et al., 1993; Centonze et al., 2004; Picconi et al., 2004). In the striatum of mice lacking D_2 receptors, increase of spontaneous glutamate events has been convincingly correlated with the loss of the presynaptic inhibition of glutamate release by endogenous DA (Cepeda et al., 2001). Our present findings show that hypersensitivity of D_2 receptors after the DA denervation is sufficient to widen the dopaminergic control on glutamate release via a retrograde mechanism acting on CB_1 receptors.

It is also possible that DA denervation, in addition to the hypersensitivity of D_2 receptors, also triggers adaptive effects on the striatal eCB signaling that could well explain the increased response to D_2 agonists in the PD model. In fact, in this model we found increased striatal levels of anandamide coupled with a decreased activity of the anandamide membrane transporter (AMT) and of the anandamide hydrolase [fatty acid amide hydrolase (FAAH)] (Gubellini et al., 2002).

The critical role of cholinergic interneurons

Striatal Ach supplied by an intrinsic neural network of large-sized cholinergic interneurons can possibly have a critical integrative role in the basal ganglia circuit by modulating both striatonigral and striatopallidal neurons (Maurice et al., 2004; Calabresi et al., 2006; Wang et al., 2006). The role of striatal cholinergic interneurons might be even more relevant in PD, where the reduced dopaminergic input to the striatum causes a relative cholinergic

overactivity (Calabresi et al., 2006). Accordingly, in our DA-depleted model of experimental PD, we found a significant effect of low doses of quinpirole on the cholinergic firing frequency, whereas low doses of the D_2 receptor agonist failed to affect the firing rate in physiological conditions.

Here we show for the first time a synergistic action of DA D_2 and adenosine A_{2A} receptors in inhibiting the firing rate of cholinergic interneurons in physiological conditions. One of the final effects of this inhibition would be a reduction of the release of endogenous Ach and the consequent reduced activation of M1 muscarinic receptors on MSNs. The established effect of M1 receptor inhibition would be the opening of L-type Ca^{2+} channels (Wang et al., 2006). This latter event might, in turn, trigger postsynaptic effects on MSNs, leading to eCB release and reduction of glutamatergic transmission by the activation of presynaptic CB_1 receptors (Fig. 9). Accordingly, we found that in the presence of pirenzepine, a M1 receptor inhibitor, the effects of D_2/A_{2A} modulation on glutamatergic transmission were fully prevented. Similarly, the inhibition of M1 receptor also prevented the D_2 receptor-mediated modulation of the excitatory response in D_2- and D_1-expressing MSNs in experimental PD. Muscarinic receptors may represent a viable target for treatment of disorders involving impaired cognitive function (Calabresi et al., 2006). However, a major limitation in using M1 receptor agonists has been a lack of highly selective ligands for individual muscarinic ACh receptor subtypes. However, it is intriguing to speculate on the possible role that M1 agonists might have in the normalization of D_1 and D_2 receptor-expressing MSNs in the therapy of neurodegenerative dysfunction involving cognitive impairments such as PD.

Clinical implications and conclusions

Overactivity of striatal glutamatergic transmission has been observed in experimental models of PD (Calabresi et al., 1993; Tang et al., 2001; Gubellini et al., 2002; Picconi et al., 2004). The eCB-mediated inhibitory effect on the glutamatergic transmission induced by D_2 receptor agonists may represent a critical mechanism to counteract this overactivity. In fact, this inhibition might increase the signal-to-noise ratio within the striatum, allowing only relevant signals to impinge on striatal spiny neurons and to induce long-term changes in synaptic transmission (either long-term potentiation or LTD) (Calabresi et al., 2007).

Hypersensitivity of D_2 receptors, and possibly adaptive changes in the eCB signaling, following DA denervation seems to play a major role in this filtering mechanism. However, it is interesting to note that both quinpirole and pramipexole, a DA receptor agonist that is widely used in clinical practice (Reichmann et al., 2003), bind not only D_2 receptors but also D_3 DA receptors (Matsukawa et al., 2007). Pramipexole has a preferential affinity for DA D_3 receptors versus DA D_2 receptors (Mierau et al., 1995). It is interesting to note that the maximal effect of pramipexole is lower than the maximal effect achieved with quinpirole. Although both quinpirole and pramipexole act on D_2 and D_3 receptors, it is possible that the different pharmacological interactions with these two distinct DA receptors explain the differential efficacy of these compounds on glutamatergic transmission.

The DA D_3 receptor subtype not only has been involved in motor control, but it also influences cognitive and behavioral aspects in the parkinsonian state (Boileau et al., 2009; Costa et al., 2009). Thus, the eCB-mediated striatal electrophysiological effects of pramipexole might be involved in both motor and behavioral responses to this agonist in PD patients. Moreover, while preclinical studies have supported a clear amelioration in animal models of PD using A_{2A} antagonists (Schwarzschild et al., 2006; Morelli et al., 2007), the real clinical impact of this class of drugs remains to be further explored in clinical studies (Xu et al., 2005).

Our pharmacological data might suggest the combined use of low doses of DA agents and A_{2A} receptor antagonists in PD in the attempt to delay the induction of dyskinesias. However, it is possible that this therapeutic strategy may not be as effective as higher doses of DA agents in isolation in counteracting PD motor symptoms. Further, *in vivo* studies are required to test this hypothesis.

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