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Effects of levodopa on endocannabinoid levels in rat basal ganglia: implications for the treatment of levodopa-induced dyskinesias

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Abstract

The majority of Parkinson's disease patients undergoing levodopa therapy develop disabling motor complications (dyskinesias) within 10 years of treatment. Stimulation of cannabinoid receptors, the pharmacological target of Δ^9-tetrahydrocannabinol, is emerging as a promising therapy to alleviate levodopa-associated dyskinesias. However, the mechanisms underlying this beneficial action remain elusive, as do the effects exerted by levodopa therapy on the endocannabinoid system. Although levodopa is known to cause changes in CB_1 receptor expression in animal models of Parkinson's disease, we have no information on whether this drug alters the brain concentrations of the endocannabinoids anandamide and 2-arachidonylglycerol. To address this question, we used an isotope dilution assay to measure endocannabinoid levels in the caudate–putamen, globus pallidus and substantia nigra of intact and unilaterally 6-OHDA-lesioned rats undergoing acute or chronic treatment with levodopa (50 mg/kg). In intact animals, systemic administration of levodopa increased anandamide concentrations throughout the basal ganglia via activation of dopamine D_1/D_2 receptors. In 6-OHDA-lesioned rats, anandamide levels were significantly reduced in the caudate–putamen ipsilateral to the lesion; however, neither acute nor chronic levodopa treatment affected endocannabinoid levels in these animals. In lesioned rats, chronic levodopa produced increasingly severe oro-lingual involuntary movements which were attenuated by the cannabinoid agonist R(+)-WIN55,212-2 (1 mg/kg). This effect was reversed by the CB_1 receptor antagonist rimonabant (SR141716A). These results indicate that a deficiency in endocannabinoid transmission may contribute to levodopa-induced dyskinesias and that these complications may be alleviated by activation of CB_1 receptors.

Introduction

L-3,4-dihydroxyphenylalanine (levodopa) represents the most commonly prescribed treatment for Parkinson's disease (PD). Although levodopa alleviates PD symptoms in the early stages of the disease, its long-term use results in disabling side-effects consisting of abnormal involuntary movements (dyskinesias) and psychiatric complications (Obeso *et al*., 2000; Bezard *et al*., 2001). The causes of levodopa-induced dyskinesias are poorly understood. Among the possible mechanisms, a decreased ability of nigrostriatal neurons to store dopamine, and hypersensitivity of postsynaptic dopamine receptors in the striatum, have been suggested as playing a role (Gerfen, 1992; Graybiel *et al*., 1994; Graybiel *et al*., 2000). None of these factors alone, however, seem to account for the motor complications associate with levodopa therapy, and secondary alterations in the metabolism (Brotchie, 2000; Hirsch *et al*., 2000) and neurochemistry of basal ganglia circuitry are receiving increasing attention (Brooks, 2000; Chase & Oh, 2000a; Chase & Oh, 2000b; Gerfen, 2000).

Recent studies point to the endocannabinoid system as an important modulator of dopaminergic activity in the basal ganglia. This system consists of a family of naturally occurring lipids, the endocannabinoids, of which anandamide (Devane *et al*., 1992; Di Marzo *et al*., 1994) and 2-arachidonylglycerol (2-AG) (Mechoulam *et al*., 1995; Sugiura *et al*., 1995; Stella *et al*., 1997) are the best-characterized examples, and their allied CB_1 receptors (Howlett *et al*., 2002). CB_1 receptors are highly expressed in brain areas involved in the control of motor functions, such as the basal ganglia, cerebellum and sensorimotor cortex (Herkenham *et al*., 1990; Glass *et al*., 1997; Hermann *et al*., 2002).

In rodents, pharmacological and neurochemical evidence indicates that stimulation of dopamine D_2 receptors is accompanied by an increase in anandamide, but not 2-AG, levels in dorsal striatum (Giuffrida *et al*., 1999); such selective release may serve as an inhibitory feedback signal countering dopamine activation of motor behaviour (Giuffrida *et al*., 1999; Masserano *et al*., 1999). Abnormalities in dopamine signalling, as reported after levodopa administration in animal models of PD (Abercrombie *et al*., 1990; Gerfen *et al*., 1990), may compromise this inhibitory feedback, resulting in a functional state characterized by motor disturbances. In keeping with this hypothesis, chronic levodopa has been shown to increase CB_1 mRNA levels in the striatum of rats unilaterally lesioned with the neurotoxin 6-hydroxydopamine (6-OHDA) (Zeng *et al*., 1999). However, there is no information on whether levodopa may also affect endocannabinoid levels in the brain. Here, we show that levodopa selectively elevates anandamide in different areas of the basal ganglia of normal rats via activation of

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dopamine D_1/D_2 receptors. This response is disrupted after 6-OHDA lesion of the nigro-striatal pathway. The implications of endocannabinoid disturbances on levodopa-induced oro-lingual involuntary movements are also discussed.

Materials and methods

Drugs

Fatty acyl chlorides (5,8,11,14-eicosatetraenoylchloride, hexadecanoylchloride and 9-cis-octadecenoylchloride) were from Nu-Check Prep (Elysian, MN, USA). [$^2H_4$]-labelled ethanolamine (isotopic atom enrichment = 98%) was from Cambridge Isotope Laboratories (Andover, MA, USA); [$^2H_8$]-labelled 2-AG was from Cayman Chemical (Ann Arbor, MI, USA). L-3,4-dihydroxyphenylalanine methyl ester (methyl levodopa), S-[-]-α-Hydrazino-3,4-dihydroxy-2-methylbenzeneпropanoic acid (carbidopa), apomorhine hydrochloride, R(+)-WIN55,212-2 (WIN) and S(−)-WIN55,212-3 were from Sigma-Aldrich (St Louis, MO, USA). S(−)-raclopride L-tartrate and R(+)-SCH-23390 hydrochloride were from RBI (Natick, MA, USA). All solvents were from Burdick and Jackson (Muskegon, MI, USA).

Animals

Animal care and experiments were conducted in accordance with the NIH Guide for care and use of laboratory animals and approved by the UCI animal use committee.

Male Wistar rats (200–250 g; Charles Rivers Laboratories, Wilmington, MA, USA) were acclimatized to the laboratory conditions (12-h light–dark cycle, 22 ± 1 °C) 1 week before experimental use. Food and water were available *ad libitum*. Unilaterally 6-OHDA-lesioned and sham-operated rats were anaesthetized with equithensin (30 mL/kg, i.p.) and stereotaxically injected with 6-OHDA hydrochloride (or saline, respectively) in the left substantia nigra at the following coordinates: from bregma, AP, −1.5; ML, +1.8; DV, −7.5 mm (incisor bar set at +2.5 mm). At 3 weeks after 6-OHDA injections, lesioned rats were screened for apomorphine-induced (0.5 mg/kg, one injection, s.c.) contralateral rotation (O'Dell & Marshall, 1996) to assess the efficacy of the lesions. The number of full turns contralateral to the lesioned hemisphere were counted using a rotometer (Raturn System, BAS; West Lafayette, IN, USA). Net contralateral turns were calculated by subtracting the number of ipsilateral from controlateral rotations. Only rats displaying >300 rotations per 30 min were included in the study. These rotation scores were found to correspond to >90% depletion of tyrosine hydroxylase (TH) staining in the substantia nigra, using a mouse monoclonal anti-TH antibody (Sigma; data not shown). Either acute or chronic levodopa treatments were started 48 h after apomorphine administration.

Brain tissue preparation and HPLC/MS analyses

Animals were killed with halothane (Halocarbon laboratories; River Edge, NJ, USA) and their brains were rapidly collected 1 h after the last injection of drugs or vehicle, snap-frozen in cold 2-methylbutane (−50 °C), placed on an ice-cold stainless steel mould and cut into 1-mm coronal slices using razor blades. Tissue punches were excised from the caudate–putamen (S), lateral globus pallidus (GP), substantia nigra (SN) and motor cortex (C). The punches were thawed in 1 mL of methanol containing 25 pmol of [$^2H_4$]-anandamide and [$^2H_8$]-2-AG, and homogenized. Endocannabinoids were extracted with methanol–chloroform (1 : 2, v/v) and quantified using high performance liquid chromatography/mass spectrometry (HPLC/MS) using an isotope dilution assay (Giuffrida *et al.*, 2000). MS analyses were performed with an electro-spray ion source set in the positive ionization mode.

Fatty acid amidohydrolase (FAAH) assay

Microsome fractions from pooled striatal punches were collected from 6-OHDA-lesioned and sham-operated rats ($n = 5$) and processed as described (Désarnaud *et al.*, 1995). FAAH assays were performed under linear conditions (Désarnaud *et al.*, 1995) except that [^{3}H]anandamide (arachidonyl-[1–^{3}H]ethanolamide; 60 Ci/mmol) was included as a substrate and radioactivity was measured in the aqueous phase after chloroform extraction.

Behavioural studies

Oral involuntary movements (vacuous chewing movements and tongue protrusions) were induced in 6-OHDA-lesioned animals ($n = 11$) by daily intraperitoneal (i.p.) injections of levodopa (50 mg/kg) plus carbidopa (12.5 mg/kg) for up to 11 days. One and two hours after administration of levodopa or vehicle, rats were individually placed in plexiglas cages and observed for 10 min by an investigator blind to the study. Oral dyskinesias were scored on a severity scale from 0 to 4 (modified from Lundblad *et al.*, 2002): 0, absent; 1, chewing movements present for less than half of the observation time; 2, chewing movements present for more than half of the observation time; 3, chewing movements present for more than half of the observation time and accompanied by tongue protrusions; 4, oral movements present all the time and accompanied by self-biting. To investigate the effects on levodopa-induced oral dyskinesias, the cannabinoid agonist WIN (1 mg/kg, i.p.), its inactive enantiomer S-(−)WIN55,212-3 mesylate (1 mg/kg, i.p.), or vehicle (5% Tween 80, 5% polyethylene glycol (PEG) in saline; 1 mL/kg, i.p.) were administered daily (one injection/day, from day 6 to day 11) 20 min before levodopa treatment. The CB_1 antagonist rimonabant (0.3 mg/kg, i.p.) was coadministered with WIN on day 11 only. All behavioural analyses were carried out in independent groups of lesioned rats that were distinct from those used for the quantitative analyses of endocannabinoids.

Statistical analyses

Data were analysed by using one-way ANOVA followed by Student's *t*-test with Bonferroni's correction, or by using Student's *t*-test where appropriate. Behavioural data were analysed using the Kruskal–Wallis test followed by Dunn's multiple comparison test.

Results

Effects of levodopa on endocannabinoid levels in intact rats

Tissue levels of endocannabinoids were measured by monitoring $[M + Na]^+$ quasi-molecular ions in the selected ion monitoring (SIM) mode (*m/z* 370.3 and 374.3 for endogenous and [$^2H_4$]-labelled anandamide, respectively; *m/z* 401 and 409 for unlabelled and [$^2H_8$]-labelled 2-AG, respectively) (Fig. 1A and B). Due to the nonenzymatic isomerization of 2-AG into 1-AG, this lipid eluted from the HPLC column as two distinct peaks (at 15.7 and 16.4 min, respectively; Fig. 1B). Thus, both peaks were taken in account for 2-AG quantification. In intact rats ($n = 14$), we found the following endocannabinoid levels: anandamide, 18.9 ± 4.4 pmol/g in caudate–putamen (S), 18.2 ± 4.3 pmol/g in lateral globus pallidus (GP), 21.6 ± 6.0 pmol/g in substantia nigra (SN) and 15.6 ± 4.6 pmol/g in motor cortex (C) (Fig. 1C); 2-AG, 7.52 ± 0.8 nmol/g in S, 11.01 ± 1.67 nmol/g in GP, 18.8 ± 4.51 nmol/g in SN and 4.23 ± 0.71 nmol/g in C (Fig. 1D).

A single injection of levodopa (50 mg/kg, i.p.) caused a significant elevation of anandamide in S, GP and SN 1 h after drug administration (Fig. 1C) (one-way ANOVA, followed by *t*-test with Bonferroni's correction, $P < 0.001$), whereas it had no effect on 2-AG levels (Fig. 1D).

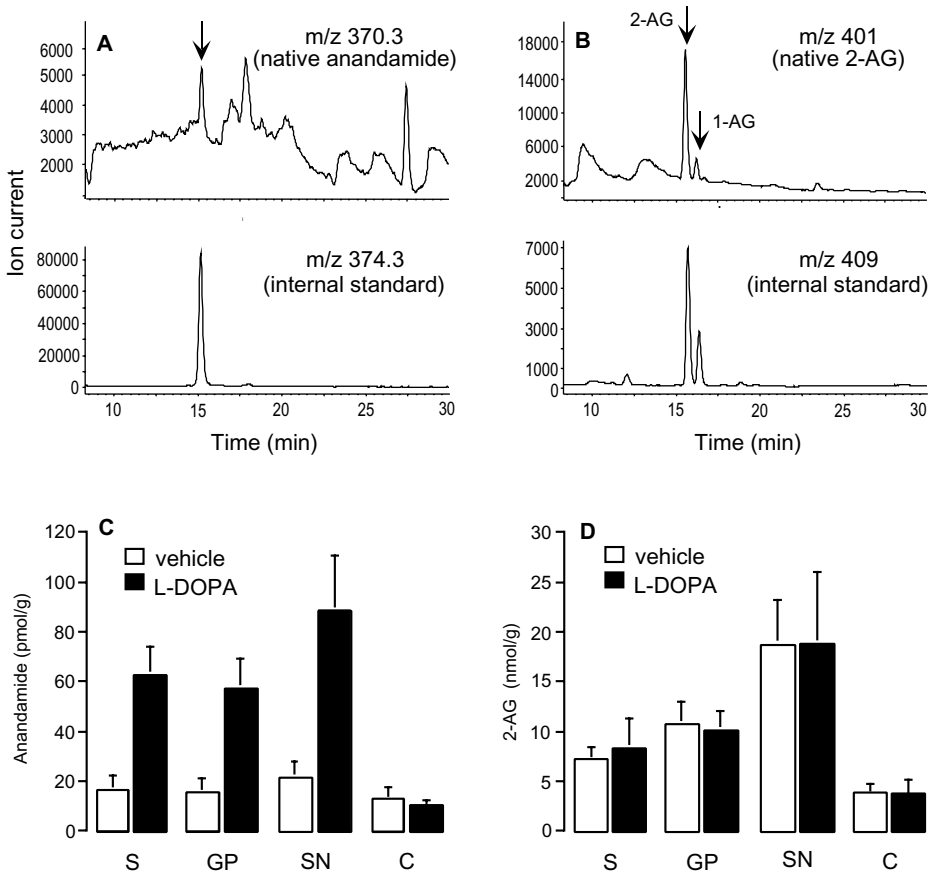

FIG. 1. Effects of levodopa on anandamide and 2-AG levels in the basal ganglia of intact rats. (A and B) Representative selected ion tracings of a striatal tissue punch before levodopa administration, showing (A) endogenous anandamide and synthetic [$^2H_4$]-anandamide, and (B) endogenous 2-AG and synthetic [$^2H_8$]-2-AG. Arrows indicate the retention times of the deuterated standards. (C and D) Effects of acute levodopa (50 mg/kg, i.p) on (C) anandamide and (D) 2-AG levels in caudate–putamen (S), globus pallidus (GP), substantia nigra (SN) and cortex (C). Values are mean ± SEM ($n = 14$). $^*P < 0.05$, $^{***}P < 0.001$ compared to vehicle (ANOVA followed by *t*-test with Bonferroni's correction).

To investigate the pharmacological mechanisms of levodopa-induced anandamide elevation, we studied the effects of systemic administration of D_1-like (SCH-23390, 0.3 mg/kg, i.p., $n = 8$) or D_2-like (raclopride, 2 mg/kg, i.p., $n = 8$) receptor antagonists on this response. When applied alone, neither SCH-23390 nor raclopride affected anandamide brain concentrations (Fig. 2A and B). However, SCH-23390 completely blocked the stimulatory effect of levodopa on anandamide throughout the basal ganglia (Fig. 2A), whereas raclopride prevented the levodopa-induced anandamide increase in S and GP but not in SN (Fig. 2B). These results indicate that levodopa exerts its stimulatory action on anandamide levels via activation of both D_1- and D_2-like receptors in S and GP and of D_1-like receptors in SN. None of these treatments affected 2-AG levels (data not shown). On the other hand, chronic levodopa administration in intact animals (50 mg/kg, one injection/day for 11 days) produced only a modest, although not significant, increase in anandamide in the basal ganglia (data not shown).

Together, these results show that levodopa acting on dopamine receptors selectively changes the levels of anandamide, and raise the possibility that disturbances of dopamine transmission might affect endocannabinoid signalling.

Effects of levodopa on endocannabinoid levels in 6-OHDA-lesioned rats

Loss of dopamine innervation in the striatum has been shown to induce both quantitative and qualitative changes in the response of basal ganglia neurons to acute application of dopamine agonists (Waszczak *et al*., 1984; Huang & Walters, 1994). To test whether striatal deafferentation may affect endocannabinoid levels, we measured anandamide and 2-AG in the basal ganglia of unilaterally 6-OHDA-lesioned rats. Sham-operated rats of similar age were used as controls.

Quantitative analyses of endocannabinoids in the lesioned (left) hemisphere showed significant lower levels of anandamide (one-way ANOVA, followed by *t*-test with Bonferroni's correction, $P < 0.05$) in S ($n = 8$) (Fig. 3A); no changes were observed in anandamide or 2-AG levels in any of the other areas examined, including those in the right (intact) hemisphere (Fig. 3 and data not shown). The decrease in striatal anandamide levels in lesioned rats was not accompanied by changes in the activity of FAAH, the enzyme responsible for intracellular anandamide hydrolysis (Schmid *et al*., 1985; Cravatt *et al*., 1996), which was identical to that observed in control animals (in pmol per min per mg of protein: sham-operated rats, 417 ± 69; lesioned rats, 403 ± 76, $n = 5$).

Acute levodopa administration (50 mg/kg, i.p., $n = 8$) caused a significant elevation of anandamide in S and GP of sham-operated animals ($P < 0.05$, Student's *t*-test; Fig. 3A), an elevation which was similar to that observed in intact rats. No significant changes, however, were observed in the left SN, most probably as consequence of the tissue damage caused by the insertion of the infusion cannula (Fig. 3A). By contrast, neither acute nor chronic levodopa elevated anandamide in 6-OHDA-lesioned rats (Fig. 3 and data not shown).

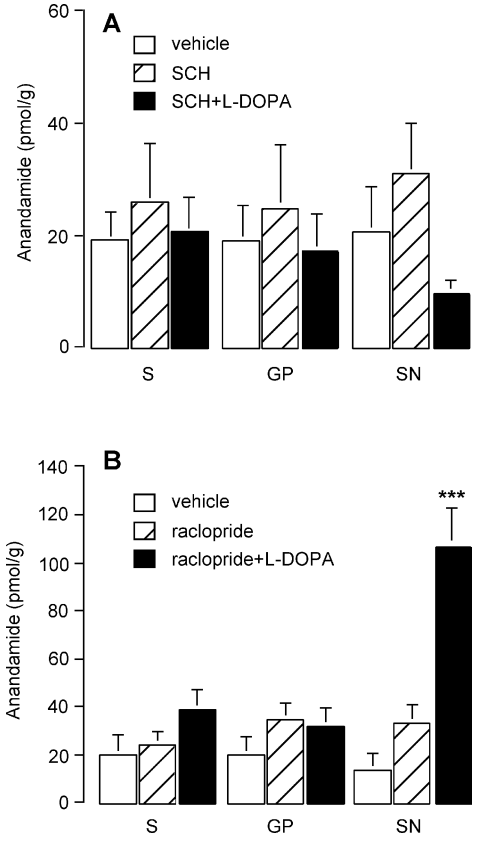

FIG. 2. Effects of selective (A) D_1 and (B) D_2 dopamine receptor antagonists on levodopa-induced anandamide elevation in normal rats. Animals were killed 1 h after administration of vehicle (open bars), SCH-23390 (SCH, 0.3 mg/kg, i.p) or raclopride (2 mg/kg, i.p) alone (hatched bars) or SCH + levodopa or raclopride + levodopa (filled bars). Either SCH or raclopride were administered 30 min before levodopa (50 mg/kg, i.p.) Anandamide concentrations were quantified in caudate–putamen (S), globus pallidus (GP) and substantia nigra (SN) by isotope dilution HPLC/MS. Values are mean ± SEM ($n = 8$). $^{***}P < 0.001$ compared to vehicle (ANOVA followed by *t*-test with Bonferroni's correction).

Taken together, these results show that dopamine depletion induced by 6-OHDA lesions prevents levodopa from exerting its stimulatory effect on anandamide levels.

Effects of apomorphine on anandamide production in 6-OHDA-lesioned rats

To evaluate whether the inability of levodopa to increase anandamide levels in lesioned animals was related to the extensive degeneration of dopaminergic nigro-striatal neurons (which may represent a possible source of anandamide) or to altered dopamine receptor stimulation, we used the nonselective dopamine agonist apomorphine. This drug does not share transport or metabolic pathways with levodopa and directly stimulates dopamine D_1/D_2 receptors. As shown in Fig. 3B, apomorphine (0.5 mg/kg, s.c., $n = 8$) produced a significant elevation of anandamide in S (Student's *t*-test, $P < 0.01$) and GP (Student's *t*-test, $P < 0.05$) but not in SN, suggesting that anandamide elevation in S and GP is triggered by dopamine receptor stimulation independently from the integrity of the nigro-striatal pathway. Similar results were obtained in the intact hemisphere ($n = 8$): S, controls 10.7 ± 1.1 pmol/g, apomorphine 18.7 ± 1.7 pmol/g (Student's *t*-test, $P < 0.05$); GP, controls 8.6 ± 2.6 pmol/g, apomorphine 22.7 ± 8.8 pmol/g ($P < 0.05$); SN, controls 37.6 ± 19.2 pmol/g, apomorphine 37.9 ± 13.3 pmol/g ($P > 0.05$).

Effects of CB_1 receptor stimulation on levodopa-induced oro-lingual movements

In 6-OHDA-lesioned rats, chronic administration of levodopa (50 mg/kg, one injection/day for 11 days) elicited a time-dependent sensitization of vacuous chewing movements and tongue protrusions which reached a plateau between days 7 and 11 (Fig. 4). By contrast, no behavioural sensitization was observed in sham-operated or intact animals (data not shown). Application of the cannabinoid agonist WIN from day 6 to day 11 (1 mg/kg, i.p., one injection/day, 20 min before levodopa) significantly reduced levodopa-induced involuntary oral movements (Fig. 4, arrow) (Kruskal–Wallis test followed by Dunn's multiple comparison test, $P < 0.001$). By contrast, administration of its inactive enantiomer S(−)-WIN55,212-3 (1 mg/kg i.p. 20 min before levodopa) had no antidyskinetic effect (Fig. 4). Finally, administration of the CB_1 antagonist rimonabant (0.2 mg/kg, i.p., 20 min before WIN) on the last day of levodopa + WIN treatment completely reversed the antidyskinetic effects of WIN (Fig. 4, arrowhead).

Discussion

Understanding the motor complications associated with levodopa use largely depends on our knowledge of how this drug affects the interplay of activities among neurotransmitter systems in the basal ganglia.

In this study, we show that acute levodopa administration causes a significant and selective elevation of anandamide in the basal ganglia of normal rats. This elevation is prevented by pharmacological blockade of either D_1- or D_2-like receptors in S and GP, and by D_1 antagonists only in SN.

The participation of both D_1- and D_2-like receptors in the regulation of endocannabinoid levels confirms our previous microdialysis study showing that activation of D_2 receptors is accompanied by anandamide release in rat striatum (Giuffrida *et al.*, 1999). In the same study we found that SKF38393, which acts as partial agonist on D_1 receptors, was not able to increase anandamide output. This apparent incongruity is probably attributable to the use of a low dose of SKF38393, which may have not been sufficient to lead to an increase in extracellular anandamide (Giuffrida *et al.*, 1999). The D_1/D_2 receptor-mediated anandamide elevation is also consistent with: (i) the high degree of colocalization of CB_1 and D_1/D_2-like receptors in the basal ganglia (Aizman *et al.*, 2000; Hohmann & Herkenham, 2000; Meschler & Howlett, 2001; Hermann *et al.*, 2002); (ii) the existence of functional interactions between CB_1 receptor stimulation and D_1 (Anderson *et al.*, 1995; Miyamoto *et al.*, 1996), and D_2 (Sañudo-Peña *et al.*, 1998; Beltramo *et al.*, 2000; Rodríguez de Fonseca *et al.*, 1995 Glass & Felder, 1997) and D_1/D_2 (Meschler *et al.*, 2000; Meschler & Howlett, 2001) receptor-mediated responses in rodents.

In contrast to the responses observed in S and GP, levodopa-induced anandamide elevation in SN was dependent on activation of D_1 but not D_2 receptors. The higher expression of D_1 vs. D_2 receptors in SN (Savasta *et al.*, 1986; Gerfen *et al.*, 1990) may account for the predominant role of D_1 receptors in this region. Nonetheless, these data indicate that the mechanisms responsible for endocannabinoid production may differ throughout distinct areas of the brain.

Although the physiological significance of levodopa-induced anandamide elevation remains to be elucidated, we hypothesize that this increase represents a negative feedback to enhanced dopaminergic transmission following levodopa administration. In keeping with this hypothesis, anandamide has been shown to counteract dopamine-induced motor activation (Giuffrida *et al.*, 1999; Romero *et al.*, 1995). Furthermore, unilateral intrapallidal and intranigral injections of the cannabinoid agonist CP55940 produce ipsilateral and contralateral turning behaviour in rodents (Sañudo-Peña & Walker, 1998;

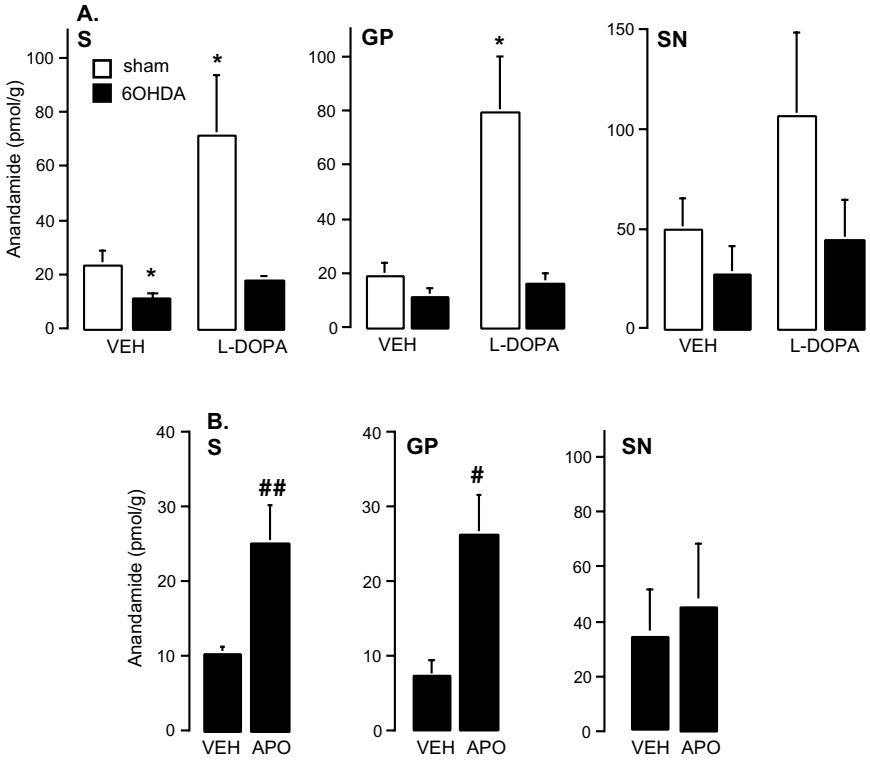

FIG. 3. Effects of (A) levodopa and (B) apomorphine administration on anandamide levels in 6-OHDA-lesioned rats. Measurements refer to basal ganglia areas in the lesioned hemisphere ($n = 8$). Anandamide levels were monitored in the caudate–putamen (S), globus pallidus (GP) and substantia nigra (SN) of sham-operated (open bars) and 6-OHDA-lesioned rats (filled bars) 1 h after injection of vehicle (saline), levodopa (50 mg/kg, i.p.) or apomorphine (0.5 mg/kg, s.c.) Values are expressed as mean ± SEM. $^{*}P < 0.05$ compared to sham-operated controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to 6-OHDA-lesioned controls (Student's *t*-test). VEH, vehicle; L-DOPA, levodopa; APO, apomorphine.

Sañudo-Peña *et al*., 1998); these behaviours are attenuated by the D_2 agonist quinpirole, thus suggesting reciprocal interactions between dopamine and cannabinoid receptor systems in the basal ganglia (Sañudo-Peña & Walker, 1998; Sañudo-Peña *et al*., 1998). Within this area, endocannabinoid signalling may also modulate responses mediated by other neurotransmitter systems. As an example, dopamine participates in the control of synaptic excitation in S through a complex interplay with NMDA receptors (Carlsson & Carlsson, 1990; Starr, 1995; Calabresi *et al*., 2000), and by favouring neuronal communication via gap junctions (Moore & Grace, 2002; Onn & Grace, 1994). Enhanced anandamide levels may play a role in these processes, as indicated by the ability of CB_1 receptor stimulation to reduce glutamate release in dorsal striatum (Gerdeman & Lovinger, 2001; Gubellini *et al*., 2002), and by the observation that anandamide modulates neuron–glial interactions by regulating gap-junction permeability (Venance *et al*., 1995).

Unlike acute administration, chronic exposure to levodopa did not significantly affect anandamide levels in intact rats, most probably as consequence of an agonist-induced desensitization of dopamine receptor function (Gardner *et al*., 2001; Kim *et al*., 2001).

Our data show that extensive degeneration of dopaminergic nigrostriatal neurons causes a significant decrease in anandamide levels in the S ipsilateral to the lesion. This decrease is most probably due to a deficiency in anandamide production, as no changes in the activity of FAAH (the enzyme responsible for anandamide inactivation; Cravatt *et al*., 1996) were observed in the same region. These results differ from previous observations showing enhanced anandamide levels in the S of 6-OHDA-lesioned rats as consequence of decreased FAAH activity (Gubellini *et al*., 2002). Differences in the experimental setting, such as extension of the lesion and/or analytical procedures used, may explain this inconsistency. Indeed, in their paper, Gubellini *et al*. (2002) report that the basal levels of striatal anandamide in intact animals are in the range of 250 pmol/mg of protein. This value is extremely high if compared with previous studies carried out in the same area (Felder *et al*., 1996; Yang *et al*., 1999; Lastres-Becker *et al*., 2001; Gonzalez *et al*., 2002), which find basal levels of striatal anandamide ranging between 20 and 96 pmol/g. This range is consistent with our own data (18.9 ± 4.4 pmol/g) which corresponds, when converted from pmol/g of tissue into pmol/mg of protein, to 0.55 ± 0.85 pmol/mg of protein. Therefore, the high endocannabinoid levels measured by Gubellini *et al*. (2002) may be due to ischemic insult of brain tissue during postmortem manipulation of samples, which is known to cause endocannabinoid release (Hansen *et al*., 2001; Schmid *et al*., 1995; Panikashvili *et al*., 2001). On the other hand, the decreased endocannabinoid tone observed by us in the S of lesioned rats is consistent with the higher spontaneous glumatergic transmission reported in these animals (Gubellini *et al*., 2002), which can be linked to the ability of endocannabinoids to negatively modulate corticostriatal glutamatergic transmission (Gerdeman & Lovinger, 2001).

In the course of our investigations on 6-OHDA-lesioned rats, we never found changes in the concentrations of the second endogenous cannabinoid 2-AG. These observations differ from previous studies carried out in the reserpine-treated rats, another model of PD. In these animals, elevated 2-AG levels were found in GP, and administration of dopaminergic agonists decreased both anandamide and 2-AG concentrations in the same area (Di Marzo *et al*., 2000). Neurochemical and electrophysiological differences in the basal ganglia circuitry between 6-OHDA-lesioned and reserpinized rats may account for

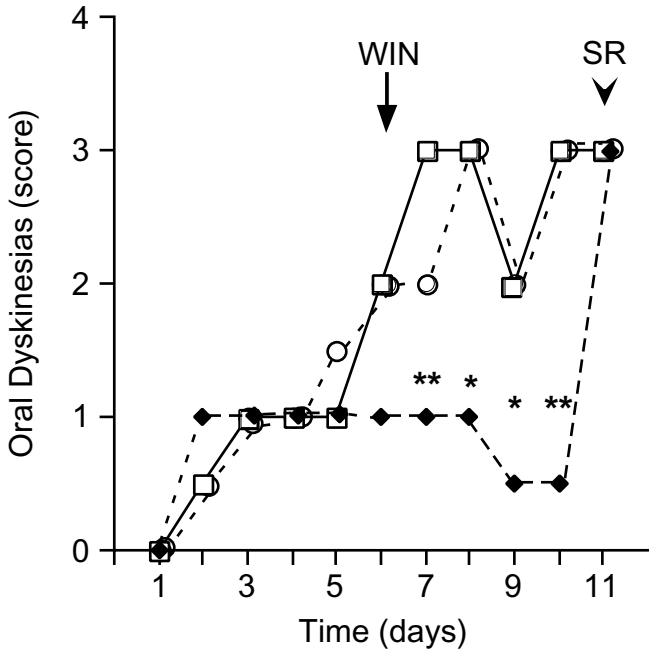

FIG. 4. Time course of the effects of i.p. administration of the CB_1 agonist WIN (◆, 1.0 mg/kg, 1 injection/day, $n = 11$) and its inactive enantiomer S(−)-WIN55,212-3 (○, 1.0 mg/kg, one injection/day, $n = 11$) on involuntary oral movements induced by chronic levodopa (50 mg/kg, one injection/day for 11 days) in 6-OHDA-lesioned rats. □, lesioned rats treated with levodopa only ($n = 11$). Each enantiomer was given to distinct groups of rats from day 6 to day 10, 20 min before levodopa (arrow), whereas rats treated with levodopa only (□) received an injection of vehicle (5% PEG + 5% Tween-80 in saline) starting from day 6. On day 11, the CB_1 antagonist SR141617A (SR, 0.3 mg/kg, i.p.; 15 min before WIN) was given to rats treated with WIN (arrowhead), whereas all other groups received an injection of vehicle. Values represent median score. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to lesioned rats treated with levodopa only (Kruskal–Wallis test followed by Dunn's multiple comparison test).

these divergences. Indeed, electrophysiological responses recorded in the basal ganglia of 6-OHDA-lesioned animals challenged with D_1 dopamine agonists are opposite to those observed in intact or reserpinized animals (Huang & Walters, 1994).

No endocannabinoid changes were found either in the intact or in the lesioned side of 6-OHDA-lesioned rats after levodopa administration. However, systemic administration of the mixed D_1/D_2 agonist apomorphine in these rats caused a significant increase in anandamide in the S and GP of both hemispheres, indicating a normal function of postsynaptic dopamine receptors. This observation excludes the possibility that an uncoupling of D_1/D_2 synergism, as reported in 6-OHDA-lesioned rats (Hu *et al*., 1990; La Hoste *et al.*, 1993), may prevent levodopa from exerting its stimulatory action on anandamide production. Although we cannot exclude a modest presynaptic release of striatal anandamide from the nigro-striatal afferents spared by the lesion, the significant anandamide elevation following apomorphine administration indicates that dopamine-responsive elements located postsynaptically in S most probably represent the major source of this signalling lipid. In agreement with our observations, biochemical (Di Marzo *et al*., 1994) and electrophysiological (Gerdeman *et al*., 2002) data support the existence of a postsynaptic release of endocannabinoids in the striatum. However, like levodopa, apomorphine failed to elevate anadamide in the SN of both hemispheres of lesioned rats, suggesting that unknown compensatory mechanisms may contribute to the unresponsiveness of the intact SN to dopaminergic agonists.

Taken together, our data indicate that a dysregulation of anandamide signalling occurs in 6-OHDA-lesioned rats as a consequence of striatal deafferentation. Given the ability of CB_1 receptor stimulation to counterbalance dopamine-induced motor activity (Giuffrida *et al*., 1999; Masserano *et al*., 1999; Meschler *et al*., 2000), this dysregulation may cause a deficient modulation of dopamine transmission which, in turn, may result in motor disturbances. Indeed, the reduced endocannabinoid tone in S may contribute to increased D_1 receptor-mediated signalling (Bezard *et al*., 2001; Meschler & Howlett, 2001) and enhanced glutamatergic transmission (Gerdeman *et al*., 2002; Gubellini *et al*., 2002). These two events may, following levodopa treatment, produce overactivation of striatal efferents to medial GP and SN pars reticulata and contribute to the generation of dyskinesias (Brotchie, 2003). In keeping with this possibility, we found that the oro-lingual involuntary movements elicited in lesioned rats by repeated administration of levodopa were reduced by systemic application of the cannabinoid agonist WIN. This effect was obtained with a dose of WIN (1 mg/kg) which did not affect apomorphine-induced circling behaviour or induce catalepsy (Anderson *et al*., 1995), thus indicating that the reduction in oral dyskinesias was not due to a generalized motor depression. The implication of CB_1 receptor activation in this response was confirmed by the lack of effect of the WIN enantiomer S(−)-WIN55,212-2, which is less active on CB_1 receptors (D'Ambra *et al*., 1992), as well as by the ability of the CB_1 antagonist rimonabant (SR141716A) to reverse the action of WIN. The effects of WIN may be predictive of an antidyskinetic action in humans, because levodopa-induced abnormal movements in rats are suppressed by compounds which have antidyskinetic efficacy in parkinsonian patients and nonhuman primates (Lundblad *et al*., 2002). In addition, CB_1 receptor stimulation has been shown to reduce levodopa-dependent motor complications in MPTP-lesioned monkeys (Fox *et al*., 2002) and PD patients (Sieradzan *et al*., 2001).

Based on our results, long-term treatment with levodopa is not sufficient to cause abnormal oro-lingual movements by itself. Indeed, chronic levodopa failed to sensitize oro-lingual movements in intact rats. Thus, the presence of a lesion in the nigro-striatal pathway represents a prerequisite for the generation of levodopa-dependent behavioural sensitization (Bordet *et al*., 1997; Bordet *et al*., 2000) and dyskinesias (Cenci *et al*., 1998), most probably as consequence of the cascade of neurochemical and functional changes following dopamine depletion (Gerfen *et al*., 2002). In this context, disturbances in endocannabinoid signalling can be seen as a new component of the neurotransmitter unbalances associated with striatal denervation, which collectively contribute to the pathogenesis of levodopa-associated dyskinesias (for review see Bezard *et al*., 2001).

In conclusion, our findings indicate that endocannabinoid transmission is dysfunctional in 6-OHDA-lesioned rats, and suggest that enhancement of the endocannabinoid system may represent a promising adjunctive therapy to suppress or prevent levodopa-induced motor complications.

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Abbreviations

2-AG, 2-arachidonylglycerol; 6-OHDA, 6-hydroxydopamine; C, motor cortex; FAAH, fatty acid amidohydrolase; GP, lateral globus pallidus; HPLC/MS, high

performance liquid chromatography/mass spectrometry; levodopa, L-3,4-dihydroxyphenylalanine; PD, Parkinson's disease; S, caudate–putamen; SN, substantia nigra; TH, tyrosine hydroxylase; WIN, R(+)-WIN55,212-2.

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