

Experimental Parkinsonism Alters Anandamide Precursor Synthesis, and Functional Deficits are Improved by AM404: A Modulator of Endocannabinoid Function

Emilio Fernandez-Espejo^{1,*}, Isabel Caraballo¹, Fernando Rodriguez de Fonseca², Belen Ferrer², Fadwa El Banoua¹, Juan A Flores¹ and Beatriz Galan-Rodriguez¹

¹Departamento de Fisiología Médica y Biofísica, Universidad de Sevilla, Sevilla, Spain; ²Fundación Carlos Haya, Hospital Carlos Haya, Málaga, Spain

Modulation of the endocannabinoid system might be useful in treating Parkinson's disease. Here, we show that systemic administration of *N*-(4-hydroxyphenyl)-arachidonamide (AM404), a cannabinoid modulator that enhances anandamide (AEA) availability in the biophase, exerts antiparkinsonian effects in 6-hydroxydopamine-lesioned rats. Local injections of AM404 into denervated striata reduced parkinsonian motor asymmetries, these effects being associated with the reduction of D₂ dopamine receptor function together with a positive modulation of 5-HT_{1B} serotonin receptor function. Stimulation of striatal 5-HT_{1B} receptors alone was observed to ameliorate parkinsonian deficits, supporting the fact that AM404 exerts antiparkinsonian effects likely through stimulation of striatal 5-HT_{1B} serotonin receptor function. Hence, modulation of cannabinoid function leading to enhancement of AEA in the biophase might be of therapeutic value in the control of symptoms of Parkinson's disease. On the other hand, reduced levels of *N*-acyl-transferase (AEA precursor synthesizing enzyme), without changes in fatty acid amidohydrolase (AEA degradative enzyme), were detected in denervated striata in comparison with intact striata. This finding reveals the presence of a homeostatic striatal mechanism emerging after dopaminergic denervation likely tending to enhance low dopamine tone.

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INTRODUCTION

Brain cannabinoid CB₁ receptors are expressed in neural areas that contribute to movement such as the basal ganglia, where their density is high and they colocalize with D₁ and D₂ dopamine receptors, and 5-HT_{1B} serotonin receptors in medium spiny projection neurons of dorsal striatum (Herkenham *et al*, 1990, 1991; Surmeier *et al*, 1996; Tsou *et al*, 1998; Hermann *et al*, 2002). It is known that there is a continual intracerebral release of endogenous cannabinoid receptor agonists such as anandamide (AEA) exhibiting neurotransmitter function (Giuffrida *et al*, 1999; Baker *et al*, 2000), and that the endocannabinoid system is an activity-dependent modulator of intrastriatal dopaminergic neurotransmission (Cadogan *et al*, 1997; Glass *et al*, 1997; Pertwee, 1999; Giuffrida *et al*, 1999; Beltramo *et al*, 2000). Serotonin neurotransmission could also be modulated by the endocannabinoid system, but its interaction with striatal

serotonin receptors such as 5-HT_{1B} is not known. Endogenous cannabinoid CB₁ activation seems to act as a brake of striatal dopaminergic activity, and it has been proposed that it is a homeostatic counter-regulatory mechanism in the basal ganglia (Rodriguez de Fonseca *et al*, 1994, 1998; Giuffrida *et al*, 1999; Beltramo *et al*, 2000).

The interdependence between striatal CB₁ and dopamine receptors has led to propose that CB₁ receptor ligands could be of value for improving motor deficits in neurodegenerative diseases such as Parkinson's disease (Glass *et al*, 1997; Rodriguez de Fonseca *et al*, 1998; Consroe, 1998). The involvement of the endocannabinoid system in Parkinson's disease is also supported by recent findings describing the upregulation of CB₁ receptors in the striatum after dopaminergic denervation, both in animals and humans (Romero *et al*, 2000; Lastres-Becker *et al*, 2001), associated with changes in striatal AEA levels. In this context, enhancement of striatal AEA levels caused by a decrease in AEA degradation (Gubellini *et al*, 2002) or reduction of AEA levels have been reported (Ferrer *et al*, 2003). Hence, the precise physiological motor role of CB₁ upregulation and striatal AEA changes in parkinsonian striata is a matter of controversy.

In the present study, the cannabinoid ligand *N*-(4-hydroxyphenyl)-arachidonamide (AM404) has been tested in a Parkinson's model based on the selective unilateral

*Correspondence: E Fernandez-Espejo, Departamento de Fisiología Médica y Biofísica, Universidad de Sevilla, Av. Sanchez Pizjuan 4, Sevilla E-41009, Spain, Tel: +34-954556584, Fax: +34-954551769, E-mail: efespejo@us.es

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destruction of nigral neurons in rats. AM404 is a modulator of AEA function that enhances AEA availability in the biophase by blocking AEA reuptake and inhibiting fatty acid amidohydrolase (FAAH), AEA degradative enzyme (Beltramo *et al*, 1997, 2000; Compton and Martin, 1997; Glaser *et al*, 2003). AM404 also activates vanilloid receptors, and seems to enhance the availability of 2-arachidonoyl-glycerol as well, another endocannabinoid substance (Zygmunt *et al*, 2000; Bisogno *et al*, 2001). The animal model used in this study produces a suitable hemiparkinsonian syndrome (Marshall, 1979; Schwarting and Huston, 1996; Fornaguera *et al*, 1994; Cenci *et al*, 2002), and it has been extensively used to monitor the effects of grafts, neuroprotective agents, and the antiakinetik potential of candidate antiparkinsonian drugs (Schwarting and Huston, 1996; Cenci *et al*, 2002). Considering controversial results on striatal AEA levels after dopaminergic denervation, changes in enzymatic activity of FAAH and *N*-acyl-transferase (NAT, AEA precursor synthesizing enzyme) have also been studied, including an intact group of rats for comparisons, taking into consideration the well-known described effects of brain lesioning on the dynamics of the endogenous cannabinoid system (Hansen *et al*, 2002).

Here, we show that AM404 ameliorates most antiparkinsonian deficits in parkinsonian rats. Reduced levels of NAT were also detected in the denervated striatum *vs* intact striata, indicating that AEA neurotransmission appears to be reduced after dopaminergic denervation likely as a homeostatic mechanism tending to enhance low dopamine tone. In this scenario, AM404 enhanced striatal 5-HT_{1B} serotonin receptor function. Direct stimulation of 5-HT_{1B} receptors was also observed to improve striatal motor function, supporting the fact that AM404 might ameliorate functional deficits in parkinsonian rats through enhancement of striatal 5-HT_{1B} serotonin receptor function.

MATERIALS AND METHODS

Subjects

Male Wistar rats (275–325 g) from the breeding colony of the Faculty of Medicine of the University of Seville, Spain were used. Laboratory temperature was kept at $22 \pm 1^\circ\text{C}$, and a 12 h light–dark cycle (lights on at 0800) was maintained throughout the experiment. Food (lab chow) and water were available *ad lib*.

Unilateral 6-Hydroxydopamine-Induced Nigra Lesion

At 30 m before 6-hydroxydopamine (6-OHDA, RBI) infusion, rats were injected with the antibiotic ceftriazone (10 mg/0.3 ml *i.m.*) and desipramine (15 mg/kg *IP*, Bioline) in order to protect noradrenergic terminals from 6-OHDA toxicity. Rats were anesthetized with chloral hydrate (425 mg/kg *i.p.*) and placed in a Kopf stereotaxic apparatus. Saline solution (1.2 μl per site) containing 6-OHDA (5 $\mu\text{g}/\mu\text{l}$, free base) and 0.2% ascorbic acid (Sigma) was injected over 5 min with a blunted 30-gauge cannula at the following coordinates: AP = -5.2 , -5.4 , L = -2.2 , and V = -8.2 mm (substantia nigra (SN), Paxinos and Watson, 1997). Control rats followed the same protocol except that the injected solution lacked 6-OHDA.

Guide Cannula Surgery and Intracerebral Injection

Anesthetized rats were given prophylactic ceftriazone and placed in a Kopf stereotaxic apparatus. A hole was drilled over the injection site, and a 22-gauge stainless-steel guide cannula (small parts) was aimed 2 mm above the dorsal striatum (AP = $+0.5$, L = ± 3 , and V = -5.5) (Paxinos and Watson, 1997). The guide cannula was fastened to the skull with stainless-steel screws (small parts) and dental cement, and was fitted with a 30-gauge stainless-steel obturator, protruding 1.5 mm out of the tip of the guide cannula (small parts). Rats were allowed to recover 48 h after surgery. Injections were performed in the home cage after removing the obturator cannula, which was replaced by a 30-gauge stainless-steel internal cannula (Small Parts) connected to a Hamilton syringe and a delivery pump (Stoelting). Solutions were slowly injected over 5 min, and afterwards the internal cannula was carefully removed and the obturator cannula was replaced. After completion of all the experiments, brains were removed and sectioned. Cannula placements were verified under microscope and were mapped onto a stereotaxic atlas (Paxinos and Watson, 1997), and confirmed to be in the dorsal striatum.

Chemicals and Doses

AM404 and AM251 were provided by Tocris (Biogen). D-amphetamine was provided by RBI. AM404 was dissolved in 30% ethanol/70% distilled water. AM251 was dissolved in 50% DMSO/50% distilled water. D-amphetamine (5 mg/kg) was dissolved in saline solution (0.9% NaCl). With regard to dopamine receptor ligands, SKF 38393 (D₁ dopamine receptor agonist), SCH 23390 (D₁ dopamine receptor antagonist), and quinpirole (D₂ dopamine receptor agonist) were provided by Tocris. Eticlopride (D₂ dopamine receptor antagonist) was provided by Sigma. All these dopaminergic ligands are highly selective (Seeman and Van Tol, 1994; Sullivan *et al*, 1998). SKF 38393 and SCH 23390 were dissolved in double distilled water. Eticlopride and quinpirole were dissolved in 30% ethanol/70% distilled water. For serotonergic receptor ligands, 8-OH-DPAT (5-HT_{1A} receptor agonist), CGS12066B (5-HT_{1B} agonist), NAN190 (5-HT_{1A} antagonist), and GR55562 (5-HT_{1B} antagonist) were provided by Tocris. All these serotonergic compounds are highly selective (Middlemiss and Fozard, 1983; Neale *et al*, 1987; Glennon *et al*, 1988; MacLean *et al*, 1996). 8-OH-DPAT, CGS12066B, and NAN190 were dissolved in double distilled water, and GR55562 was dissolved in 30% ethanol/70% distilled water. The corresponding vehicle was used for the control group in every treatment.

AM404 was injected at systemic doses of 0, 1, and 5 mg/kg *i.p.* (v/w, 1 ml/kg); and at intracerebral doses of 0, 1, and 5 $\mu\text{g}/\mu\text{l}$ (volume of infusion, 1.5 μl). AM251 was coinjected with AM404 at systemic doses of 0, 1, and 5 mg/kg or at intracerebral doses of 0, 1, and 5 $\mu\text{g}/\mu\text{l}$ (1:1 cotreatment). For intrastriatal infusions of dopaminergic ligands (volume, 1.5 μl), SCH-23390 and eticlopride were injected at 0, 1, and 2 $\mu\text{g}/\mu\text{l}$ doses (free base, SCH-23390, 4.6 and 9.1 nmol; eticlopride, 2.6 and 5.3 nmol), and SKF-38393 and quinpirole were each administered at 0, 0.5, and 1 $\mu\text{g}/\mu\text{l}$ doses (free base, SKF-38393, 2.2 and 4.4 nmol; quinpirole, 2.9 and 5.8 nmol). With regard to intrastriatal infusions of

serotonergic ligands, 8-OH-DPAT and CGS12066B were administered at 0, 5, and 10 nmol (free base), and NAN190 and GR55562 were injected at 0, 4, and 8 nmol. For cotreatments, AM404 was injected at 1 µg/µl (1.5 µl).

Groups and General Protocol

For systemic administration studies, animals belonged to two groups: (i) hemiparkinsonian rats ($n=18$) and (ii) sham-lesioned control rats ($n=10$). All parkinsonian rats presented a strongly positive response to amphetamine (>420 turns/h) 15 days after lesion. At 1 month after lesion, the CB₁ ligand was administered every day following a 'Latin-square-' type design. With regard to intracerebral injections, two groups of parkinsonian rats were studied, each group with a cannula aimed at the left ($n=10$) or right ($n=9$) dorsal striatum. At 1 month after lesion, AM404 was administered daily through the cannula at several doses following a 'Latin-square-' type design, and changing the initial dose for every rat, immediately before amphetamine (one test per day). Different groups of parkinsonian rats were also subjected to intrastriatal injections of dopaminergic ligands alone or in combination with AM404 (D₁ receptor ligands, $n=9$; D₂ receptor ligands, $n=10$), or serotonergic ligands alone or in combination with AM404 (5-HT_{1A} receptor ligands, $n=9$; 5-HT_{1B} receptor ligands, $n=10$), following a 'Latin-square-' type design, and changing the initial dose for every rat. If dopaminergic or serotonergic ligands were coinjected with AM404, they were administered 5 min before AM404 infusion.

Behavioral Tests and Statistics

For behavioral study, we followed a methodology described previously (Ungerstedt and Arbuthnott, 1970; Marshall, 1979; Schwarting and Huston, 1996; Fornaguera *et al*, 1994; Espejo *et al*, 1998). Thus, locomotor directional bias was evaluated by quantifying either ipsiversive rotations induced by amphetamine (5 mg/kg i.p.), from 30 to 90 min after amphetamine. Akinesia was quantified in a 10-min open-field test ($1 \times 1 \text{ m}^2$) through distance travelled (cm). Sensorimotor orientation was evaluated by means of the odor test (Stricker and Zigmond, 1986). A probe with the tip impregnated in amoniac was approached from the right side near the nose, and the latency for shaking the head off the probe was quantified. Forelimb asymmetry was evaluated by the cylinder test (Kirik *et al*, 2000), where the animal is allowed to move freely in a transparent cylinder ($50 \times 30 \text{ cm}^2$) until it has displayed 20 rearing postures. The numbers of left and right forepaw contacts are counted, and the data are presented as the percentage of right forepaw contacts (right paw use ratio). Hemiparkinsonian rats with lesion in the left SN present a significant impairment in the contralateral (right) paw use.

With regard to intracerebral injections, the number of amphetamine-induced turning was considered as the dependent variable in order to detect changes in the functional activity of basal ganglia. Percent changes in the number of turnings with respect to vehicle-treated rats were measured, considering vehicle-treated rats as percentage 0 (positive values, reduction; negative values, enhancement of turning).

Behavioral data after systemic injections were studied by two-way ANOVA (group, between variable; drug dose, within variable), followed by one-way ANOVA (drug dose as within variable) and *post hoc* Newman-Keuls tests. Behavioral data after intracerebral injections were analysed by using one-way ANOVA (treatment as between variable) followed by *post hoc* Newman-Keuls test for comparison between groups. When variance was not homogeneous, data were logarithmically ($\log(x)$) transformed prior to analysis. Student's *t*-test (independent measures) were used for comparison between groups at the same dose point. Experiments were performed according to the animal care guidelines of the European Communities Council (86/609/EEC).

Immunohistochemistry and Cell Counting

Half of the rats subjected to systemic injections were killed by decapitation and brains carefully removed. Brains were postfixed and stored in 4% paraformaldehyde in phosphate buffer (PB) 0.1 M (pH 7.2–7.4) at 4°C. Coronal brain sections (50 µm thick) were cut on a vibratome and collected in PBS. Thereafter, endogenous peroxidase activity was quenched by placing sections into 0.3% H₂O₂ in 0.05 Tris buffer (pH=7.6) for 2 h. Then sections were incubated in PBS/0.1% Triton X-100 (PBS-T) with 10% FCS (Vector) and BSA (1 mg/ml, Sigma) for 4 h to block nonspecific sites. Sections were incubated overnight with rabbit anti-tyrosine-hydroxylase polyclonal antibody (1:1000, Chemicon) in PBS-T. After washing they were incubated for 1 h with anti-rabbit biotin-conjugated antibody (1:200, Chemicon). Then, sections were incubated with the ABC kit, and specifically bound antibody were revealed by using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as chromogen and 0.05% hydrogen peroxide (Merck). Negative control sections were incubated following the same protocol, with the exception that the primary antibody solution was replaced by a PBS-T solution containing 10% FCS and BSA (1 mg/ml). Sections were washed in PBS and mounted on glass slides and coverslipped with DPX. A quantitative assessment of dopaminergic neurons in SN was carried out to confirm lesion degree. Thus, the number of tyrosine hydroxylase (TH) immunoreactive neurons in the SN pars compacta was assessed by a blinded observer. In all rats 15 consecutive sections were used, and stained neurons in the SN were counted at $\times 4$ magnification ($n=5$ per group), on the basis of Paxinos and Watson (1997). TH positive neurons were counted when displaying a nucleus surrounded by TH-positive cytoplasm.

Preparation of Rat Tissue Subcellular Fractions and Enzymatic Activities of FAAH and N-Acyltransferase

For these measures, apart from sham and lesioned rats, a group of intact naive rats was also used for better discernment of enzymatic changes. The brain endogenous cannabinoid system has been involved in inflammatory processes associated with brain lesioning like that associated with craniotomy and cannula implantation (Hansen *et al*, 2002). Half of the lesioned and sham rats of systemic studies were used for this study. After rapid inspection of cannula location, brain tissues were homogenized in 50 mM

Tris buffer, pH 8, containing 0.32 M sucrose. Homogenates were centrifuged first at 1000g (5 min), the pellet discarded and the supernatant centrifuged at 45 000g (30 min). The pellets obtained were solubilized at 0–4°C in Tris buffer. Protein content in the membrane fraction was measured with the Bradford method. All tissue samples and membrane fractions were stored at –70°C until used. Both enzymatic assays were run under conditions that were linear with time and protein concentration. We assayed membrane-bound FAAH activity using arachidonoyl-[1-³H]ethanolamide as a substrate, and measuring metabolized [³H]AEA (as [³H]ethanolamine) in the aqueous phase after chloroform extraction, as described (Desarnaud *et al*, 1995; Rodríguez de Fonseca *et al*, 2001). Microsomal NAT activity assays were performed using 1,2-Di[¹⁴C]palmitoyl-*sn*-glycerophosphocholine as substrate (Cadas *et al*, 1996, 1997). Assays were carried out for 1 h at 37°C in 0.5 ml of Tris buffer (50 mM, pH 8), to which we added 0.3 mg protein, 3 mM CaCl₂, and 1 × 10⁶ dpm radioactive substrates. Parallel incubations carried out in the absence of tissue contained on average 100 dpm/sample; these blank values were subtracted in the calculations of enzyme activity. Incubations were stopped by adding chloroform/methanol (2:1), and the *N*-acyl PEs were fractionated by column chromatography.

RESULTS

Induction of Hemiparkinsonism

Rats were rendered hemiparkinsonian by injecting the toxin 6-OHDA into the left SN. Those animals which showed a strong ipsiversive rotational behavior after the administration of amphetamine (>420 turns/hour), indicative of depletion of more than 85% of striatal dopamine (Fornaguera *et al*, 1994), were selected for the study. These animals presented an overt hemiparkinsonian syndrome characterized by drug-induced turning, spontaneous ipsiversive turning, akinesia, contralateral sensorimotor neglect, and forepaw use asymmetry. TH immunohistochemistry indicated that intranigral 6-OHDA injection destroyed 88 ± 3% of the TH-containing cell bodies in the affected unilateral site (as measured through cell counting), leaving intact the cells of the contralateral nucleus, and dopaminergic innervation in the corresponding ipsilateral striatum was nearly absent.

Enzymatic Activities of Striatal FAAH and *N*-Acyltransferase

As shown in Table 1, in rats with 6-OHDA-induced lesion, striatal NAT enzymatic activity was significantly reduced *vs* intact (not sham) rats ($p < 0.05$), but no significant changes were observed in FAAH activity. However, sham rats showed enhanced levels of FAAH in both ipsilateral and contralateral striata ($p < 0.05$) *vs* the remainder groups.

Systemic Administration of AM404

Following AM404 treatment, two-way ANOVA indicated a significant interaction ($F(2, 40) = 12.1$, $p < 0.01$) effects on amphetamine-induced turning. Thus, turning behavior was significantly reduced in parkinsonian rats after 1 (48.1% reduction, $p < 0.05$) and 5 mg/kg AM404 (48.7%, $p < 0.05$). Pretreatment of these rats with AM251 (selective CB₁ receptor antagonist, 0, 1, and 5 mg/kg doses, respectively) eliminated the capacity of AM404 to induce functional changes on drug-induced turning, confirming the existence of a CB₁-mediated modulation of the intrastriatal dopaminergic tone (Figure 1).

For spontaneous parkinsonian deficits, one-way ANOVA indicated significant dose effects for akinesia ($F(2,$

Table 1 Enzymatic Activities of FAAH and NAT in the Brain Membrane Fraction from Intact Animals, Animals with Sham Lesion in the Substantia Nigra (Sham), and Animals Lesioned with 6-OHDA (Lesion)

	Intact	Sham	Lesion
FAAH (pmol/mg/min)			
Left striatum	489 ± 13	535 ± 14*	494.1 ± 34.5
Right striatum	466 ± 9	529 ± 10*	431 ± 46.2
NAT (pmol/mg/min)			
Left striatum	502 ± 29	466 ± 133	310.2 ± 61 ^{#,o}
Right striatum	472 ± 64	529 ± 112	536 ± 194

Left striatum was the denervated one in lesioned rats. Mean ± SEM of at least five samples per group.

* $p < 0.05$ *vs* intact and lesioned rats.

[#] $p < 0.05$ *vs* intact rats.

^o $p < 0.05$ *vs* corresponding contralateral right striatum.

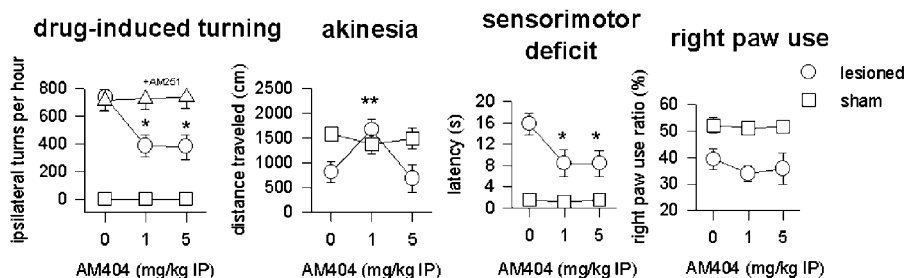


Figure 1 Effects of AM404 treatment on amphetamine-induced turning, and spontaneous motor and sensorimotor deficits in hemiparkinsonian rats and sham control animals (□). AM404 ameliorated functional deficits in parkinsonian rats (O), as revealed by significant dose effects on amphetamine-induced turning ($p < 0.01$), akinesia (distance travelled, $p < 0.05$), and sensorimotor orientation (odor test, $p < 0.01$). Cotreatment with AM251 (CB₁ agonist) eliminated functional effects on turning of AM404 (Δ). The data show that AM404 is able to improve significantly drug-induced asymmetries and spontaneous functional deficits in hemiparkinsonian rats, except forelimb asymmetry. Mean ± SEM, * $p < 0.05$, ** $p < 0.01$ *vs* dose 0 (Newman-Keuls test).

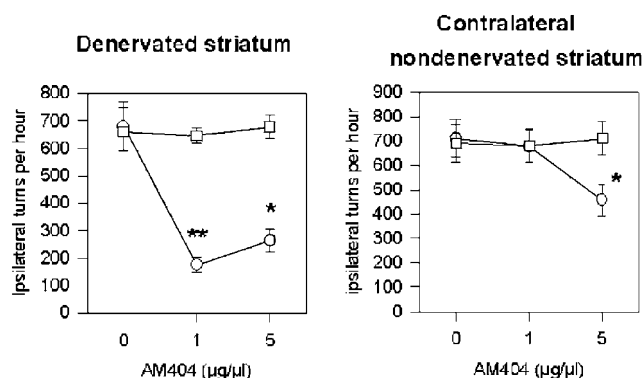


Figure 2 Effects on drug-induced ipsilateral turning (towards the site of nigral lesion) of left (denervated) and right (nondenervated) intrastriatal injections of AM404 in hemiparkinsonian rats (○). Intrastriatal injections into denervated striata significantly attenuated drug-induced turning after 1 and 5 µg/µl AM404. Intrastriatal injections into nondenervated contralateral striata reduced ipsilateral turning after 5 µg/µl AM404. Cotreatment with AM251 (CB₁ agonist) eliminated functional effects on turning of AM404 (□). Mean ± SEM, * $p < 0.05$, ** $p < 0.01$ vs dose 0 of corresponding group (Newman-Keuls test).

21) = 4.56, $p < 0.05$) and sensorimotor orientation ($F(2, 21) = 3.2$, $p < 0.05$) after AM404 (Figure 1). Thus, significant amelioration of akinesia occurred following 1 mg/kg dose ($p < 0.01$), without differences in control rats. Akinesia reappeared after the highest dose tested (5 mg/kg i.p.). Sensorimotor orientation was improved after 1 and 5 mg/kg AM404 ($p < 0.05$). Right forepaw use was not improved following AM404.

Intrastriatal AM404 Acts Reducing D₂ Dopamine Receptor Function, and Enhancing 5-HT_{1B} Serotonin Receptor Function

With regard to infusions into denervated striata, one-way ANOVA indicated significant dose effects ($F(2, 18) = 3.2$, $p < 0.05$) on rotation after AM404 (Figure 2). Thus, turning behavior after amphetamine was significantly reduced following 1 (74.2% reduction, $p < 0.01$) and 5 µg/µl AM404 (61.4% reduction, $p < 0.05$). This effect was blocked after pretreatment with AM251 (1 and 5 µg/µl doses, respectively). After injections into nondenervated contralateral striatum, only the highest dose of AM404 (5 µg/µl) significantly reduced ipsilateral turning ($p < 0.05$), as shown in Figure 2.

As observed in Table 2, one-way ANOVA did not indicate significant treatment effects after local infusions of the D₁ dopamine receptor ligands in combination with AM404 (1 µg/µl; dose exerting maximum effect) in denervated striata. Neither SKF-38393 (D₁ receptor agonist) nor SCH23390 (D₁ receptor antagonist) plus AM404 altered functional effects of AM404 or D₁ dopaminergic ligands given alone. However, one-way ANOVA indicated significant treatment effects after local infusions of the D₂ dopamine receptor agonist in combination with AM404. Thus, quinpirole (2.9 and 5.8 nmol) plus AM404 significantly attenuated functional effects of AM404 or quinpirole alone ($F(4, 36) = 5.8$, $p < 0.01$). These findings indicate that

AM404 negatively modulate D₂ dopamine receptor function in denervated striata.

As observed in Table 2, one-way ANOVA indicated significant treatment effects after local infusions of 5-HT_{1B}, but not 5-HT_{1A}, serotonin receptor ligands in combination with AM404. Thus, GR55562 (5-HT_{1B} receptor antagonist, 4 and 8 nmol) plus AM404 significantly antagonized functional effects of both compounds given alone ($F(4, 36) = 6.7$, $p < 0.01$). Interestingly, the number of turnings was further enhanced after cotreatment with GR55562 with respect to vehicle-treated rats. CGS12066B (5-HT_{1B} receptor agonist, 4 and 8 nmol) plus AM404 significantly enhanced the functional effects of AM404 or CGS12066B alone ($F(4, 36) = 6.2$, $p < 0.01$). These findings clearly indicate that AM404 acted stimulating striatal 5-HT_{1B} serotonin receptor function. Stimulation of 5-HT_{1B} serotonin receptors also reduced *per se* the number of turnings after amphetamine (see Table 2).

Local Injections: Histology

Only those animals where injection site was found to be correct (within the dorsal striatum) were included for analyses. Inspection of the brain tissue revealed slight evidence of gliosis at the site of injection, although surrounding tissue was generally intact.

DISCUSSION

NAT Activity is Reduced in Denervated Striata

The lesion of dopamine neurons of the SN with the toxin 6-OHDA resulted in a decrease in the activity of striatal NAT, the enzyme responsible for AEA precursor synthesis, if compared with striata of intact rats. However, no significant changes were observed with respect to sham rats, a result that is otherwise in accordance with Gubellini *et al* (2002), who reported no changes of NAT activity in 6-OHDA-lesioned rats after comparing with sham rats. However, the findings of the present study reveal that NAT activity is actually reduced after denervation if compared with intact striata, representing a novel result. It is possible that sham nigra lesion somehow affected striatal NAT activity explaining the lack of differences with denervated striata. On the other hand, striatal FAAH activity was not affected if compared with intact animals but, again, FAAH activity was reduced if compared with sham rats. This latter result is also in accordance with Gubellini *et al* (2002), who reported a decrease of FAAH activity after 6-OHDA-induced nigral lesion with respect to sham rats. However, if intact rats are employed for comparisons, no changes in FAAH activity can be observed. It is worth noting that FAAH activity was found to be enhanced in denervated striata of sham rats long after sham lesion in the SN, as well as in contralateral striata. Observed effects on NAT and FAAH activity in sham rats could be accounted for by a long-term overall inflammatory process caused by microcannula lesion because the brain endogenous cannabinoid system has been involved in inflammatory processes associated with brain lesioning and craniotomy (Hansen *et al*, 2002), suggesting that sham rats do not represent an adequate control group for comparisons. Besides, since striatal FAAH

Table 2 Effects of Intrastriatal Dopaminergic and Serotonergic Ligands and AM404 on Amphetamine-Induced Turning (Percent Change vs Control) in Parkinsonian Rats*AM404 and dopamine receptor ligands**D₁ antagonism*

AM404	SCH (4.6 nmol)	SCH (4.6 nmol)+AM404	SCH (9.1 nmol)	SCH (9.1 nmol)+AM404
62.3 ± 9	84.4 ± 4	44 ± 16	90 ± 4	57 ± 18
AM404	SKF (2.2 nmol)	SKF (2.2 nmol)+AM404	SKF (4.4 nmol)	SKF (4.4 nmol)+AM404
62.2 ± 5	39.4 ± 8	57 ± 16	47.7 ± 7	50.5 ± 15

D₂ antagonism

AM404	Eti (2.6 nmol)	Eti (2.6 nmol)+AM404	Eti (5.3 nmol)	Eti (5.3 nmol)+AM404
61.9 ± 6	21 ± 30	10 ± 36	34 ± 15	47.2 ± 13
AM404	Qui (2.9 nmol)	Qui (2.9 nmol)+AM404	Qui (5.8 nmol)	Qui (5.8 nmol)+AM404
62 ± 6* #	72.8 ± 4.8*	47.8 ± 9	68 ± 8#	32 ± 12

*AM404 and serotonin receptor ligands**5-HT_{1A} antagonism*

AM404	NAN (10 nmol)	NAN (10 nmol)+AM404	NAN (10 nmol)	NAN (10 nmol)+AM404
62.3 ± 6	87 ± 10	78 ± 17	89 ± 4	77 ± 16
AM404	DPAT (5 nmol)	DPAT (5 nmol)+AM404	DPAT (10 nmol)	DPAT (10 nmol)+AM404
62 ± 6	65.4 ± 8	59.4 ± 10	70.4 ± 8	49 ± 16

5-HT_{1B} antagonism

AM404	GR (4 nmol)	GR (4 nmol)+AM404	GR (8 nmol)	GR (8 nmol)+AM404
61.2 ± 5** #	44.8 ± 10**	-57.1 ± 7	56.4 ± 5.5##	-26 ± 20
AM404	CGS (4 nmol)	CGS (4 nmol)+AM404	CGS (8 nmol)	CGS (8 nmol)+AM404
62.1 ± 4* #	23.3 ± 5**	79 ± 8	39.5 ± 9##	87.5 ± 14

Percent changes in amphetamine-induced turning (positive values, reduction of turning; negative values, increase) with respect to vehicle-treated rats (percent value = 0) are shown. Mean ± SEM.

SKF, SKF38393; SCH, SCH23390; Eti, eticlopride; Qui, quinpirole; NAN, NAN190; DPAT, 8-OHDPAT; GR, GR55562; CGS, CGS12066B.

**p* < 0.05.

***p* < 0.01 vs the corresponding combination of AM404 and dopaminergic or serotonergic ligand (lower dose).

#*p* < 0.05.

##*p* < 0.01 vs the corresponding combination of AM404 and dopaminergic or serotonergic ligand (higher dose). AM404 was injected at 3.79 nmol (1 µg/µl; 1.5 µl).

levels were found to be normal in parkinsonian rats vs intact animals, it seems that 6-OHDA-induced dopaminergic degeneration would oppose FAAH activity enhancement caused by inflammation.

To sum up, the findings of the present study allow proposing that striatal NAT activity is actually reduced after 6-OHDA-induced nigra lesion, and FAAH activity is not affected, by using intact rats as controls. As a consequence, the net effect of denervation on AEA availability in the biophase would be a reduction since AEA precursor synthesis is impaired. This conclusion is in line with recent findings by Ferrer *et al* (2003), but in clear contrast with previous reports showing enhancement of AEA levels in denervated striata in two different models of parkinsonism (Gubellini *et al*, 2002; Di Marzo *et al*, 2000). These differences can stem from either the employment of sham rats for comparisons or different procedures to detect levels of AEA. The reduction of NAT enzymatic activity in denervated striata could represent a homeostatic mechanism tending to diminish AEA neurotransmission and to enhance dopamine release, because endogenous AEA activation acts as a brake of striatal dopaminergic activity (Rodriguez de Fonseca *et al*, 1998). The fact that AEA

precursor synthesis is reduced in denervated striatum could be of importance for the development of potential treatments for Parkinson's disease.

Systemic AM404 Exerts Antiparkinsonian Effects

The data indicated that, within a dose range (1–5 mg/kg), the systemic administration of AM404 induced significant antiparkinsonian effects in parkinsonian rats, as revealed by improvement of akinesia and sensorimotor orientation, as well as a reduction of drug-induced turning. It is worth noting that akinesia did not show significant differences with control rats after 1 mg/kg dose, indicating that at this dose the cannabinoid ligand was particularly effective in ameliorating this symptom. Since the unilateral 6-OHDA-induced lesion model of Parkinson's diseases in rats parallels the human disorder well (Cenci *et al*, 2002), these findings are remarkable because sensorimotor orientation and mostly akinesia are difficult to alleviate in human Parkinson's disease, and the antiakinetik effect is highly consistent with an antiparkinsonian profile of AM404.

It is known that cannabinoid agonists have a biphasic effect on movement, with an activational role at low doses

until immobility and catalepsy appears at higher doses (Fride *et al*, 1994; Rodriguez de Fonseca *et al*, 1998; Zimmer *et al*, 1999; Sañudo-Peña *et al*, 2000). The results allow proposing that, within a dose range, systemic administration of cannabinoid ligands (such as AM404) enhancing AEA availability through endocannabinoid deactivation (and particularly transport) might be useful for the treatment of Parkinson's disease. It is important to keep in mind that AM404 induced hypokinesia at high doses, which suggests the existence of a 'therapeutic window' and the need of a 'rational' use of this compound as potentially antiparkinsonian tool. However, further clinical research is needed on this topic since the findings of the present study refer to an animal model of parkinsonism and not to an actual human syndrome.

Intrastriatal AM404 Improves Motor Deficits Through Enhancement of 5-HT_{1B} Serotonin Receptor Function

In order to further analyze AM404-mediated action within the dorsal striatum, regional injections were performed into this structure, where CB₁ receptors are known to be highly expressed (Rodriguez de Fonseca *et al*, 1998). Regional injections induced different and stronger effects on denervated basal ganglia circuit than in nondenervated one, in accordance with previous data where intranigral cannabinoid agonists were found to produce more vigorous turning behavior in 6-OHDA-lesioned rats than in non-lesioned animals (Sañudo-Peña *et al*, 1996, 1998). With regard to nondenervated circuit, basal ganglia motor function was reduced after the highest AM404 dose as revealed by reduced ipsilateral rotation. This fact could be explained by a negative modulation of dopamine neurotransmission after AM404, thereby reducing the dopamine disbalance between both striata (Cadogan *et al*, 1997; Glass *et al*, 1997; Rodriguez de Fonseca *et al*, 1994, 1998; Pertwee, 1999; Giuffrida *et al*, 1999; Beltramo *et al*, 2000). However, parkinsonian asymmetries were also reduced after AM404 injections into denervated striata, indicating the participation of different mechanisms. Besides, rotation was reduced in a dose-dependent manner, pointing to a critical role for dorsal striatum in motor effects following systemic AM404 in parkinsonian rats. Functional effects of AM404 in denervated striata could also be related to the upregulation of CB₁ receptors, a phenomenon that has been described both in animals and humans (Romero *et al*, 2000; Lastres-Becker *et al*, 2001).

Following injections into denervated striata, the findings revealed that AM404 acted reducing D₂ dopamine receptor function, because motor actions of the D₂ receptor agonist quinpirole were diminished after coinjection with AM404. In this context, AEA-induced effects within the striatum have been explained by an inhibitory influence of endogenous CB₁ receptor agonists on striatal dopamine receptors (Rodriguez de Fonseca *et al*, 1998; Giuffrida *et al*, 1999), concretely D₂ dopamine receptors, since D₂ receptor activation enhances AEA release and motor effects of AEA are blocked by quinpirole, D₂ receptor antagonist. The data hence supports previous findings indicating that D₂ dopamine receptor function is negatively affected by AM404. Besides, and of greater importance, AM404 also seems to act through an enhancement of 5-HT_{1B} (but not 5-

HT_{1A}) serotonin receptor function in denervated striata, because motor actions of the highly selective 5-HT_{1B} receptor agonist CGS12066B or antagonist GR55562 were either enhanced or reduced, respectively, after coinjection with AM404. Our study also demonstrated that stimulation of striatal 5-HT_{1B} receptors alone reduced parkinsonian asymmetries, supporting the fact that stimulation of these serotonin receptors improves motor function. In this context, it has been reported that 5-HT_{1B} receptors colocalize with CB₁ receptors in striatal GABAergic medium spiny neurons (Hermann *et al*, 2002), pointing to an interrelation between cannabinoid and serotonin activity within striatum, as confirmed for the first time in the present study, and at the level of dorsal striatum, 5-HT_{1B} receptors play a major role regulating serotonin release (Wong *et al*, 1995). How AM404 interacts with 5-HT_{1B} receptors deserves further investigation, but modulation of 5-HT_{1B} receptor could be indirect (via an endocannabinoid substance or communication between transduction pathways) or through receptor-receptor interaction. Whatever the mechanism, a positive modulation of 5-HT_{1B} receptor function could account for antiparkinsonian motor effects of AM404. These effects would lead to less disbalance between left and right basal ganglia circuits as indicated by the reduction of amphetamine-induced turning, and amelioration of spontaneous parkinsonian symptoms. It is interesting that NAT enzymatic activity was reduced in denervated striata, likely as a homeostatic mechanism tending to enhance dopamine release. Although AM404 would enhance striatal AEA availability in the biophase thereby reducing dopamine neurotransmission (Giuffrida *et al*, 1999; Beltramo *et al*, 2000; Gubellini *et al*, 2002), this cannabinoid ligand seems to exert antiparkinsonian effects likely through a different mechanism: positive modulation of 5-HT_{1B} receptor function. Functional effects of AM404 validates endocannabinoid deactivation (particularly transport) as a target for anti-parkinsonian therapy.

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