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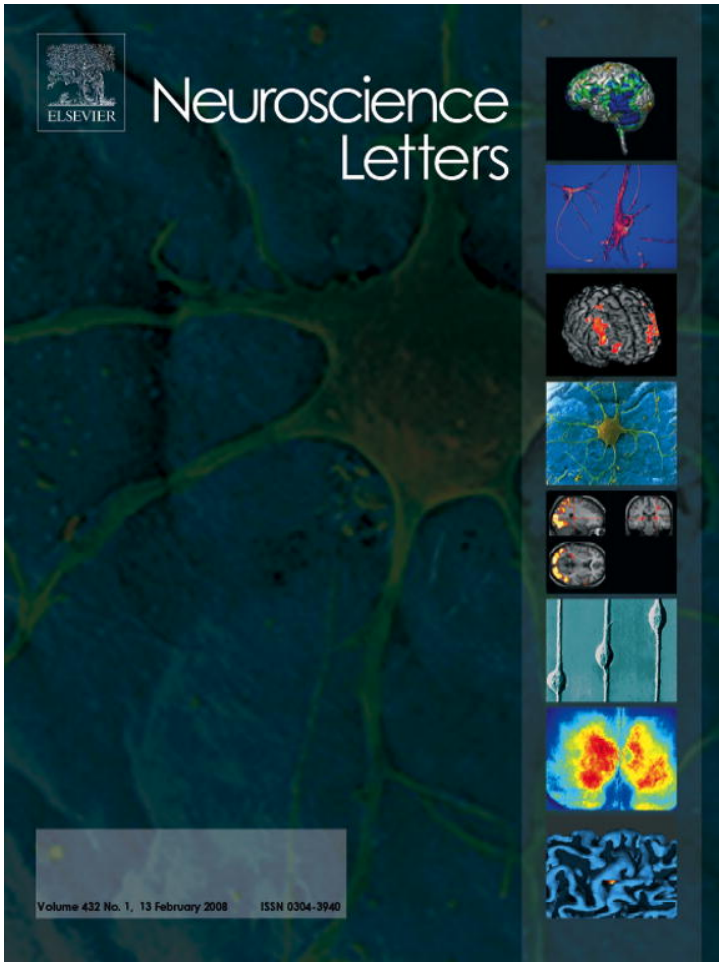

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Neuroprotective effects of rotigotine in the acute MPTP-lesioned mouse model of Parkinson's disease

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Abstract

Dopamine agonists used to manage Parkinsonian motor symptoms have been suggested to be neuroprotective. The study was designed to assess the neuroprotective potential of the $D_3/D_2/D_1$ dopamine receptor agonist rotigotine in the acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned (MPTP) mouse model of Parkinson's disease by measuring mesencephalic degenerating neurons using FluoroJade staining and the remaining dopaminergic nerve endings in the striatum using dopamine transporter binding. Continuous administration of rotigotine at a dose of 3 mg/kg significantly attenuated MPTP-induced acute cell degeneration in the FluoroJade-staining paradigm. Rotigotine (0.3–3 mg/kg) partially protected dopamine nerve endings from MPTP-induced degeneration in a dose-dependent manner. These data suggest that rotigotine, at the doses employed, significantly protected dopamine neurons from degeneration in an acute mouse model of MPTP intoxication.
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Keywords: Rotigotine; Dopamine agonist; Continuous administration; Striatum; Substantia nigra pars compacta; Transdermal patch; Dopamine transporter

In contrast to other neurodegenerative conditions, symptomatic therapy exists for Parkinson's disease (PD). Unfortunately, there is no proven therapy to prevent or slow the progressive neuronal cell death, or restore abnormally behaving neurons to a normal state [7]. There has been increasing interest in the development of drugs to modify the biochemical abnormalities that cause the neurodegeneration and thus alter the course of PD, either by retarding the rate of cell death or by restoring function to neurons that are likely to undergo degeneration [6]. In this context, dopamine (DA) receptor agonists have shown promise. Not only do these drugs provide symptomatic relief of PD but they also appear to be associated with a significant decrease in the rate of motor complications and to be capable of protecting against some of the adverse consequences of levodopa use (for review, see Ref. [23]). Evidence based largely on experimental *in vitro* and *in vivo* rodent studies is emerging suggesting that some DA agonists may have neuroprotective properties in addition to their symptomatic effects (for review, see Refs. [19,24]). In this context, the present study was designed to investigate the potential of the $D_3/D_2/D_1$ DA receptor agonist rotigotine to slow down PD progression in an acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse model of Parkinson's disease. As neurodegeneration is occurring stochastically over some period of time, rotigotine was administered continuously using a slow release formulation mimicking the release from a transdermal patch formulation as used clinically.

Experiments were carried out in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC) for care of laboratory animals. All efforts were made to minimise animal suffering and to use the minimum number of animals necessary to perform statistically valid analysis. C57BL/6J@RJ male mice (Breeder: Elevage Janvier, 53940 Le Genest, France) weighing 26–29 g were housed in a temperature-controlled room under a 12 h light/dark cycle with free access

Abbreviations: DA, dopamine; DAT, dopamine transporter; FJ, FluoroJade; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SD, standard deviation; SN, substantia nigra.

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Table 1
Experiment no. 1 design

Groups	Day 1		Days 2–8	Day 8	
	Rot/Veh	MPTP/sal	Rot/Veh	*n*	Brain
A1	–	–	–	10	FF
A2	Veh	Sal	Veh	11	FF
A3	Veh	MPTP	Veh	10	FF
A4	0.3	MPTP	0.3	6	FF
A5	1.0	MPTP	1.0	6	FF
A6	3.0	MPTP	3.0	7	FF
A7	3.0	Sal	3.0	12	FF

Rot: rotigotine (mg/kg); Veh: vehicle; Sal: Saline; FF: fresh frozen. On day 1, animals received rotigotine or its vehicle followed by four injections of MPTP or saline (i.p.) at 2 h intervals. They then received rotigotine or its vehicle once daily on days 2–8. Brains were collected fresh frozen on day 8.

to food and water. To induce neurodegeneration of half of the dopaminergic neurons in the substantia nigra, mice were treated with MPTP hydrochloride on day 1 (four injections of MPTP; 20 mg/kg i.p. at 2 h intervals; saline as vehicle) as previously described [2,4,18,20]. Experimental groups received an oily suspension of rotigotine (s.c., SPM 962, batch no. 20004007, Schwarz BioSciences GmbH) or rotigotine vehicle (s.c., batch no. 20104016, Schwarz BioSciences GmbH). Two experimental designs were used. In experiment 1 (Table 1), mice received rotigotine/vehicle slow release formulation shortly before 1st MPTP/saline administration (day 1) and subsequently once daily from days 2 to 8. They were euthianzed 4 h after the last rotigotine or vehicle administration on day 8, i.e. 7 days after the MPTP injections [12], by cervical dislocation followed by decapitation. In experiment 2 (Table 2), mice received rotigotine/vehicle slow release formulation on day 0, i.e. 18 h prior to MPTP/saline (day 1). They were then anesthetized and euthanized by paraformaldehyde perfusion 24 h later.

DAT binding using [^{125}I]-(E)-*N*-(3-iodoprop-2-enyl)-2ß-carboxymethyl-3ß-(4′-methylphenyl)-nortropane (PE2I; Chelatec, France) was measured on fresh frozen cryostat-cut 20 μm thick slide-mounted coronal sections as previously described [2,3,9]. Briefly, sections were incubated for 90 min at 25 °C with 100 pM [^{125}I]PE2I in pH 7.4 phosphate buffer (NaH_2PO_4 10.14 mM, NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.76 mM). After thorough rinsing and drying, sections were exposed to β radiation-sensitive film (Hyperfilm ßmax, Amersham, UK) in X-ray cassettes, for 2 days. Densitomeric analysis of autoradiographs was performed using an image analysis system (Densirag V. D2.99, Biocom) as previously described [2,3,9]. The optical density was assessed in both the dorsal and ventral part of the rostral caudate-putamen defined according to a mouse brain atlas [8]. Four sections per animal were analysed by an examiner blinded to the experimental conditions. Optical densities were averaged in each animal and converted to amount of radioactivity bound by comparison to the previously measured sections from control animals [9]. Mean radioactivity bound and S.D. were then calculated for each group. Data are expressed in fmol/mg of tissue equivalent.

Table 2
Experiment no. 2 design

Groups	Day 0	Day 1	Day 2	
	Rot/Veh	MPTP/sal	*n*	Brain
B1	Veh	Sal	5	PFA
B2	Veh	MPTP	5	PFA
B3	0.3	MPTP	5	PFA
B4	1.0	MPTP	5	PFA
B5	3.0	MPTP	5	PFA
B6	3.0	Sal	3	PFA

Rot: rotigotine (mg/kg); Veh: vehicle; Sal: Saline; PFA: perfused with paraformaldehyde. On day 0, animals received rotigotine or its vehicle 18 h prior four injections of MPTP or saline (i.p.) at 2 h intervals. They were then anesthetized and killed by perfusion 24 h later.

FluoroJade-staining was performed as in Schmued and Hopkins [27]. Deparaffinized and rehydrated 18 μm-thick sections were incubated in a solution of 0.01% FluoroJade (Histo-Chem, Jefferson; AR, USA) in 0.1% acetic acid for 30 min and were visualized under epifluorescence (with a fluorescein/FITC filter). Images were captured with an imaging system (Sony MC3255 camera, KS100 Rel.3.0). For final output, images were processed using Photoimpact 4 software. For each animal, FluoroJade-positive (FJ+) cells were counted by two independent examiners, one being blind to the treatment schedule. FJ+-cells were counted in two representative mesencephalic planes. All fluorescent profiles irrespective of the presence of a nucleus were counted. Mean number of FJ+-cells ±S.D. were calculated for each group.

Rotigotine vehicle/MPTP treatment (A3) elicited a significant reduction in DAT binding in both the dorsal (84% decrease) and ventral (84% decrease) parts of the striatum, compared to animals receiving rotigotine vehicle/MPTP vehicle (A2) (both $P < 0.001$; Figs. 1 and 2). In the dorsal part of the striatum, rotigotine (0.3, 1 and 3 mg/kg)/MPTP-treated groups (A4, A5 and A6, respectively) had significantly higher DAT binding than animals treated with rotigotine vehicle/MPTP (A3) (100%, 340% and 365% higher, respectively, $P < 0.05$, $P < 0.001$ and $P < 0.001$,

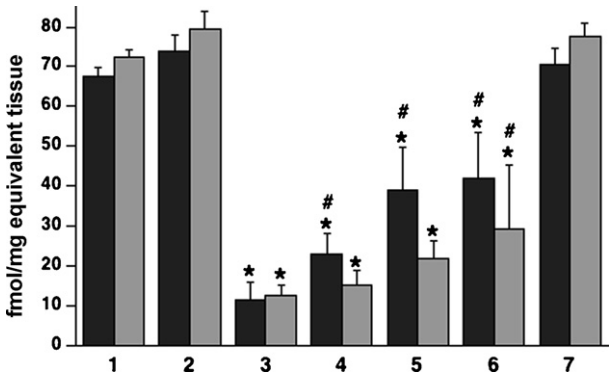

Fig. 1. Effect of rotigotine on DAT binding. DAT binding (fmol/mg equivalent tissue) in dorsal (dark columns, $F_{(6,61)} = 155.23$, $P < 0.0001$) and ventral part (grey columns, $F_{(6,61)} = 222.27$, $P < 0.0001$) of the striatum in the seven groups of experiment 1 animals. (*) Significant decrease in dopamine transporter binding compared to Group 2; (#) significant preservation of binding in comparison with Group 3. Data were analysed with a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison's test (Intercooled Stata 6.0, Stata Corporation, College Station, TX).

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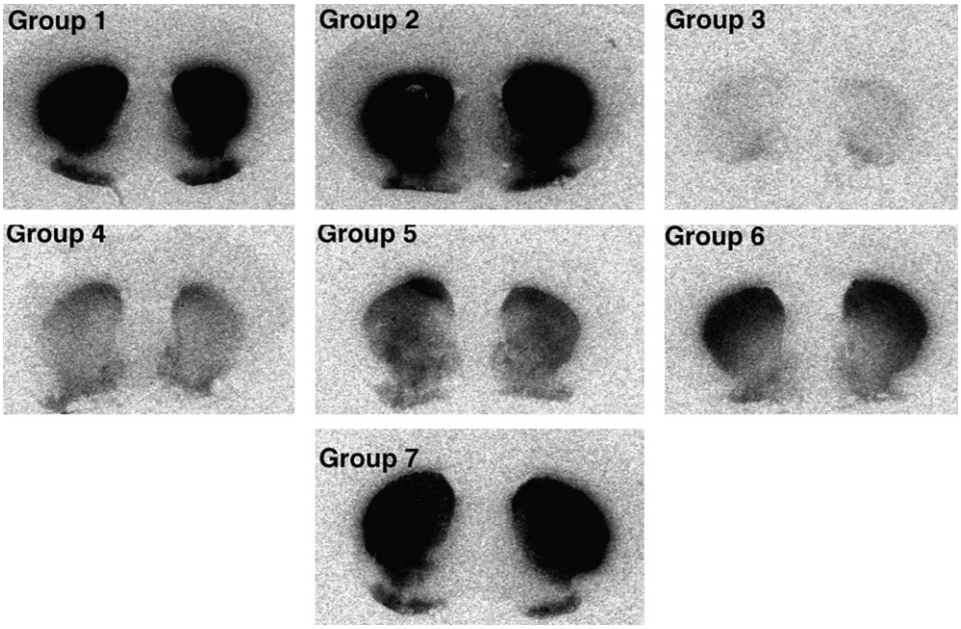

Fig. 2. Representative examples of DAT binding in the striatum of the seven groups of animals. Note that the dorsal part of the striatum is more protected than the ventral part.

respectively; Figs. 1 and 2). In addition, the two highest doses of rotigotine (1 and 3 mg/kg; Groups 5 and 6, respectively) had significantly higher DAT binding than the lowest dose (0.3 mg/kg; Group 4) ($P < 0.01$ and $P < 0.001$, respectively; Figs. 1 and 2), suggesting a dose-dependent effect. In the ventral part of the striatum, there was a trend towards a comparable dose–response curve. However, only the group treated with rotigotine (3 mg/kg)/MPTP (A6) had significantly higher DAT binding than the group treated with rotigotine vehicle/MPTP (A3) (231% higher, $P < 0.001$; Figs. 1 and 2).

To ensure that protection of DA terminals was observable at the cell body level, we counted the number of FJ+-cells in the mesencephalon 24 h after MPTP administration, i.e. at a time point where tyrosine hydroxylase is anyway down-regulated even in the neurons that would eventually survive the toxic insult [12]. As expected, a large number of FJ+-cells was observed in the MPTP-treated groups B2, B3, B4 and B5, while in the control groups B1 and B6 FJ+-cells were seen only occasionally (for group definition see Table 2) (Fig. 3A). Only the group treated with rotigotine (3 mg/kg)/MPTP (B5) had significantly lower number of FJ+-cells than the group treated with rotigotine vehicle/MPTP (B2) ($P < 0.05$; Fig. 3A–B).

These results show that the $D_3/D_2/D_1$ receptor agonist rotigotine dose-dependently protected DA nerve endings in the mouse striatum from MPTP insult (Figs. 1 and 2) and decreased the number of degenerating cells at the highest dose (Fig. 3). The autoradiographic technique used allowed assessing the preservation of DAergic terminals. Acute MPTP treatment regimens are known to induce a uniform denervation of the striatum (e.g. see Group 3) in mouse, monkey [3] and human [28] occurring subsequently to the degeneration of the dopaminergic neurons of the SN. At variance with this feature, PD affects predominantly the dorsal striatum and, to a lesser extent, the ventral striatum [5,17]. The present results clearly show a preferential neuroprotection of the dorsal striatum. Relative DAT up-regulation is unlikely to occur since it would decrease the extracellular DA content [11] and precipitate the Parkinsonian manifestations [15]. Furthermore, D_3 agonists are known to putatively down-regulate DAT expression [16], therefore suggesting that we might underestimate the rotigotine-mediated protection and definitvely not overestimate. Such DAT down-regulation, however, could account. The possibility that

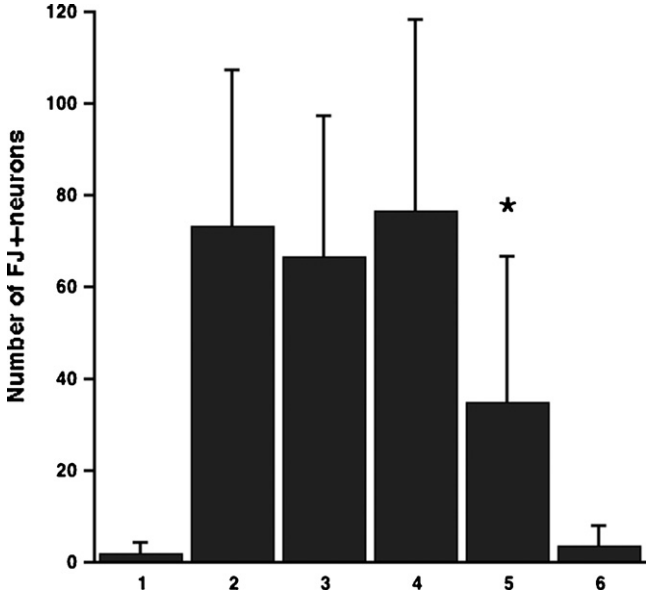

Fig. 3. Effect of rotigotine on mesencephalic FluoroJade-stained neurons. Number of FJ+-neurons in the mesencephalon of the six groups of experiment 2 animals. (*) Significant decrease in the number of FJ+-stained cells compared to Group 2. Data were analysed by *U*-test (Mann–Whitney).

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rotigotine could down-regulate DAT expression and that part of the decreased MPTP toxicity may result from this down-regulation [4,13], however, exists. In the same way, spontaneous neuroregeneration (due to regenerative capacity of the brain) is unlikely to occur given the time frame of the experiments while first signs of sprouting have been reported as starting from day 14 at the earliest [10]. These DAT binding results, however, are further supported by the observation that rotigotine at 3 mg/kg decreased the number of degenerating neurons in the mesencephalon as evidenced by the FluoroJade staining (Fig. 3) 24 h after the toxic insult. Those data extends findings in a macaque model of PD mimicking progressiveness of the degeneration where DA terminals were partially preserved but protection of cell bodies could not be observed [25].

Several studies have suggested that DA agonists may have neuroprotective effects via direct scavenging of free radicals or increasing the activities of radical-scavenging enzymes, and enhancing neurotrophic activity [19,24]. However, DA agonists also can induce neuroprotective activities *in vitro* via activation of specific intracellular signalling cascade [22]. Regarding its binding profile, rotigotine is a DA agonist with a preference for D_3 receptors over D_2 and D_1. However, at a functional level, the intrinsic activity via these three receptors is similar; *in vitro*, rotigotine even shows a supra-activation of the D_2 receptor in comparison to dopamine [26]; thus, under certain circumstances, rotigotine may act preferentially via D_2 or D_3 receptors [14,22,26]. Both receptors have been suggested to mediate neuroprotection although the mechanisms of protection have not been investigated in more detail *in vivo*.

We are fully aware that the acute MPTP intoxication does not replicate the aetiology of PD and that drugs should also be tested in clinically driven experimental designs [21]. However, important clues can arise from studies using this toxin and to acutely and selectively eliminate DAergic neurons in order to demonstrate neuroprotective properties of compounds in an early stage of development [1,29,30]. In addition, we consider the use of acute models also as of value to investigate the effect of administration regimens: the time of exposure of the tissue with the potential protective drug seems to be of importance as the degeneration of the individual neurons is a stochastic process and likely to occur continuously over a prolonged period of time. A neuroprotective drug, therefore, should have either a long half-life or should be administered continuously in order to influence as many degenerating cells as possible at any time. The oily crystal suspension of rotigotine as used here provides a continuous delivery of the compound thus mimicking the release from a transdermal (patch) formulation; future experimental and clinical work is necessary to substantiate that hypothesis.

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