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Neuroprotective effect of rotigotine against complex I inhibitors, MPP^+ and rotenone, in primary mesencephalic cell culture

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Abstract

Introduction: *Dopamine agonists are suggested to be more efficacious in treating Parkinson's disease (PD) as they have neuroprotective properties in addition to their receptor-related actions.*

Aim of the study: *The present study was designed to investigate the neuroprotective effects of rotigotine, a $D_3/D_2/D_1$ dopamine receptor agonist, against the two powerful complex I inhibitors, 1-methyl-4-phenylpyridinium (MPP^+) and rotenone, in primary mesencephalic cell culture relevant to PD.*

Material and methods: *Primary mesencephalic cell cultures were prepared from embryonic mouse mesencephala at gestation day 14. Three sets of cultures were treated with rotigotine alone, rotigotine and MPP^+, and rotigotine and rotenone to investigate the effect of rotigotine on the survival of dopaminergic neurons against age-, MPP^+- and rotenone-induced cell death. At the end of each treatment, cultures were fixed and stained immunohistochemically against tyrosine hydroxylase (TH). The effect of rotigotine against rotenone-induced reactive oxygen species (ROS) production was measured using CH-H_2DCFDA fluorescence dye.*

Results: *Rotigotine alone did not influence the survival of tyrosine hydroxylase immunoreactive (THir) neurons except at 10 μM, it significantly decreased the number of THir neurons by 40% compared to untreated controls. Treatment of cultures with 0.01 μM rotigotine rescued 10% of THir neurons against MPP^+-induced cell death. Rotigotine was also found to significantly rescue 20% of THir neurons at 0.01 μM of rotenone-treated cultures. Using of CH-H_2DCFDA fluorescence dye, it was found that rotigotine significantly attenuated ROS production compared to rotenone-treated cultures.*

Conclusions: *Rotigotine provides minor protection against MPP^+ and rescues a significant number of THir neurons against rotenone in primary mesencephalic cell cultures relevant to PD.*

Key words: *dopamine agonists, dopaminergic neurons, MPP^+, neuroprotection, Parkinson's disease, rotenone, rotigotine.*

Introduction

Dopamine replacement therapy using levodopa continues to be the gold standard in the treatment of Parkinson's disease (PD). It has remained the most effective drug for the symptomatic treatment of PD since its introduction by Birkmayer and Horny-

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kiewicz in 1961 [3]. Interestingly, Hurny *et al.* [13] have shown recently that levodopa decreased the CD_{25}/CD_3 ratio, the antigen marker characteristic of apoptotic death within the $T/CD_3(+)$ lymphocyte population in the peripheral blood of PD patients. However, long-term levodopa treatment therapy is accompanied by abnormal involuntary movements known as levodopa-induced dyskinesia [1]. Moreover, there is an enduring belief from pre-clinical studies that levodopa or its metabolite dopamine might be toxic to the remaining substantia nigra (SN) dopaminergic neurons [17]. Accordingly, *de novo* and younger patients might be at high risk for the development of levodopa-induced complications as they require dopaminergic therapy for a longer period of time [8].

Dopamine receptor agonists are the next most effective antiparkinsonian drugs after carbidopa/levodopa medication. They are considered useful alternatives to levodopa in mild or early PD and as an additional drug in moderate to severe late disease [8]. They improve motor outcomes and decrease the risk of levodopa-induced motor complications [32]. In addition to their symptomatic effectiveness, dopamine agonists may have a neuroprotective effect. For instance, recipients of pramipexole and ropinirole showed slower neuronal deterioration compared with levodopa recipients by means of dopamine transporter imaging using single-photon emission computed tomography (SPECT) [21,34].

The non-ergoline dopamine agonist rotigotine is currently approved as monotherapy in early idiopathic PD, in moderate to severe idiopathic restless leg syndrome and as adjunct therapy to levodopa in advanced idiopathic PD. In PD it is supposed to reduce motor fluctuations by constant dopaminergic stimulation using a 24-h transdermal delivery system (patch). Rotigotine exhibits the highest affinity for the D_3 receptor (Ki = 0.71 nM) and acts as a full agonist at dopamine receptors (rank order: $D_3 > D_{2L} > D_1 = D_5 > D_{4.4}$) with potencies 2,600 and 53 times higher than dopamine at dopamine D_3 and D_{2L} receptors [31].

In contrast to other dopamine agonists, there is only limited knowledge of potential neuroprotective properties of rotigotine. In the acute 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) mouse or progressive MPTP macaque model, the agonist was shown to exert partial protective effects on dopaminergic nerve endings [29,30]. To date there have been no investigations exploring the direct neuroprotective effects of rotigotine towards dopaminergic neurons under conditions of inhibition of the mitochondrial respiratory chain reactions. Accordingly, the present study was carried out in primary dopaminergic cell cultures from embryonic mouse mesencephala stressed with mitochondrial complex I blockers, MPP^+ and rotenone. Such studies are of interest, since the pesticide rotenone was found to be associated with an increased risk of developing PD [5,33] and a subgroup of PD patients were demonstrated to exhibit a defect of complex I activity and structure [15,18,28].

Material and methods

Preparation of primary dopaminergic cell culture

Pregnant mice (OF1/SPF, Himberg, Austria) were cared and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. At gestation day 14, uterine horns were dissected and embryos were collected in Petri dishes containing sterile Dulbecco's phosphate buffered saline (DPBS, Invitrogen, Germany). Under a stereoscope (Nikon SMZ-1B, 10 × magnification), brains were dissected, ventral mesencephala excised and primary cultures were prepared according to Radad *et al.* [23]. Briefly, after careful removal of the meninges, tissues were mechanically cut into small pieces in DPBS and transferred into a sterile test tube containing 2 ml of 0.1% trypsin (Invitrogen, Germany) and 2 ml of 0.02% DNase I (Roche, Germany) in DPBS. The tube was incubated in a water bath at 37°C for 7 min. Then, 2 ml of trypsin inhibitor (0.125 mg/ml in DPBS) (Invitrogen, Germany) were added, the tissue was centrifuged (Hettich, ROTIXA/AP) at 100 g for 4 min. The tissue pellet was triturated with a fire-polished Pasteur pipette in Dulbecco's modified Eagle's medium (DMEM, Sigma, Germany) containing 0.02% DNase I. Dissociated cells were collected in DMEM supplemented with Hepes buffer (25 mM), glucose (30 mM), glutamine (2 mM), penicillin-streptomycin (10 U/ml and 0.1 mg/ml, respectively) and heat inactivated foetal calf serum (FCS, 10%) (all from Sigma, Germany). The cell suspension was plated into four-well multidishes (Nunclon, Germany) pre-coated with poly-D-lysine (50 μg/ml) (Sigma, Germany). Cultures were grown at 37°C in an atmosphere of 5% CO_2/95% air and 100% relative

humidity. The medium was exchanged on the 1st day *in vitro* (DIV) and on the 3rd DIV. On the 5th DIV half of the medium was replaced with serum-free DMEM containing 0.02 ml of B-27/ml (Invitrogen, Germany). Serum-free supplemented DMEM was used for feeding from the 6th DIV and subsequently replaced every 2nd day.

Treatment of cultures with rotigotine, MPP^+ and rotenone

To investigate the effect of rotigotine (UCB, Belgium) on the survival of THir neurons, primary mesencephalic cell cultures were treated with different concentrations of rotigotine (0.01, 0.1, 1 and 10 μM) from the 6th DIV for 8 consecutive days. Rotigotine was added with each change of the culture medium every second day from a fresh stock solution (10 mM) prepared in DMSO (0.1% final concentration). To investigate the effect of rotigotine against MPP^+ or rotenone-induced cell death, rotigotine was added from the 6th DIV until the 14th DIV as described above and MPP^+ (10 μM) or rotenone (20 nM) were co-administered on the 12th DIV for 48 h.

Identification of THir neurons

Dopaminergic neurons were identified immunocytochemically by staining tyrosine hydroxylase. Cultures were rinsed carefully with phosphate buffered saline (PBS, pH 7.2) at the end of each treatment and fixed in 4% paraformaldehyde for 45 min at 4°C. After washing with PBS, cells were permeabilized with 0.4% Triton X-100 for 30 min at room temperature. Cultures were washed 3 times with PBS and incubated with 5% horse serum (Vectastain ABC Elite kit) for 90 min to block nonspecific binding sites. To determine the number of THir in cultures, cells were sequentially incubated with anti-TH primary antibody overnight at 4°C, biotinylated secondary antibody (Vectastain) and avidin-biotin-horseradish peroxidase complex (Vectastain) for 90 min at room temperature and washed with PBS between stages. The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS containing 3.3 mM of hydrogen peroxide (H_2O_2) and stained cells were counted with a Nikon inverted microscope in 10 randomly selected fields (1.13 mm^2 = field) in each well at 10× magnification. On the 14th DIV, the average number of THir cells in the various experiments was between 1000 and 1500 cells/well.

Measurement of ROS with CH-H_2DCFDA

When the production of ROS was measured, cultures were treated with rotigotine (0.01 μM, the protective concentration against rotenone) from the 6th DIV until the 12th DIV. Rotenone (20 nM) was co-administered on the 12th DIV for 48 h. On the 14th DIV cultures were washed and incubated with 5 μM of the fluorescence indicator, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CH-H_2DCFDA), for 30 min and ROS was measured according to Radad *et al*. [24]. In short, cultures were rinsed twice with colourless medium and photographed on a Nikon inverted microscope equipped with epifluorescence attachment using a rhodamine filter set (510 DM/520 BA, B-2A) and a Coolpix 990 digital camera (Nikon, Japan). In each experiment, four wells were cultivated for each treatment condition. Five micrographs were taken from each well. Ten cell bodies were randomly selected by a blinded researcher from each micrograph (meaning that 200 cells were evaluated for each treatment condition in each of three independent experiments) and the average density for 100 pixels in individual cell bodies was determined using Adobe Photoshop software®.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). Statistical significance was calculated using the Kruskal-Wallis (H)-test followed by χ^2. Differences with $p < 0.05$ were regarded as statistically significant.

Results

Effect of rotigotine on the survival of dopaminergic neurons

Addition of rotigotine (0.01, 0.1, 1 μM) for 8 consecutive days produced no effect on the number of THir neurons in primary mesencephalic cell culture compared to untreated controls. However, rotigotine decreased the number of THir neurons by 40% when added at an unphysiological high concentration of 10 μM (Fig. 1).

Rotigotine slightly protected dopaminergic neurons against MPP^+ toxicity

MPP^+ (10 μM) decreased the number of THir neurons by 43% compared to untreated control cultures

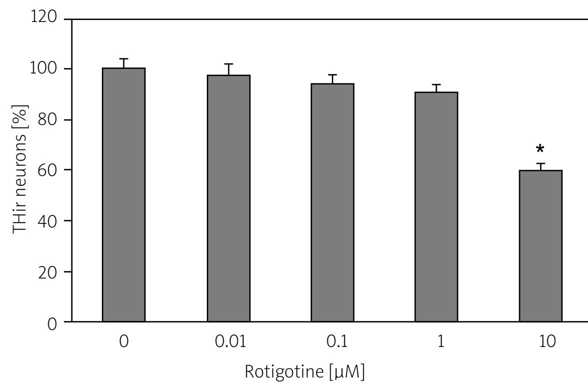
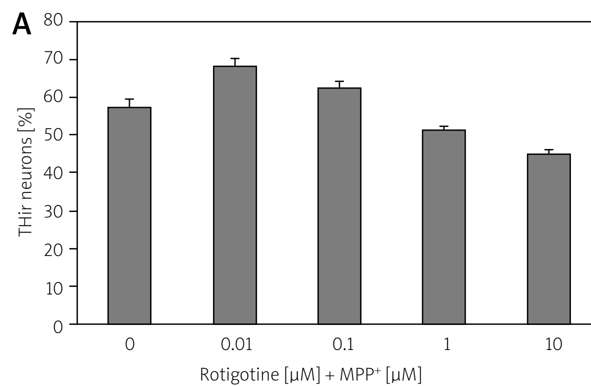
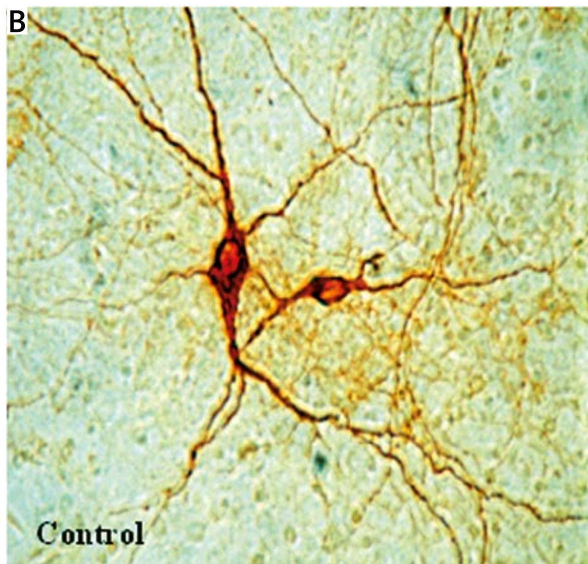


Fig. 1. Effect of incubation with various concentrations of rotigotine on the survival of THir neurons. Treatment was begun on the 6th DIV for 8 consecutive days with a change of medium together with rotigotine every 2nd day. 100% corresponds to the total number of dopaminergic neurons after a cultivation period of 14 days without rotigotine treatment. Values represent the mean ± SEM of three independent experiments in quadruplicate. $*p < 0.0001$ compared to untreated control.





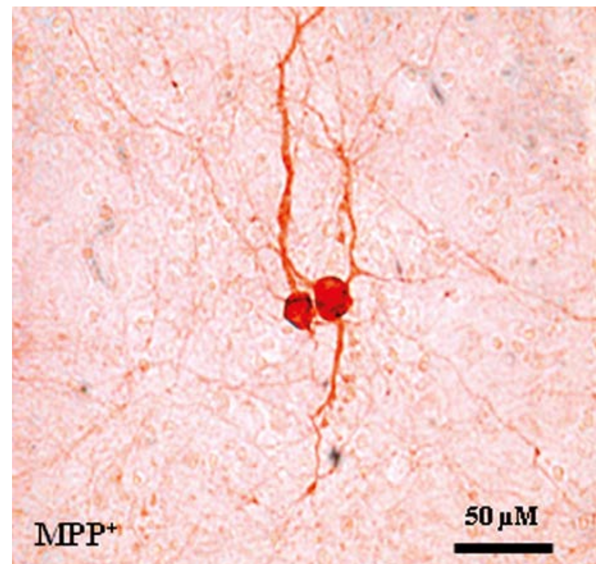


Fig. 2. A) Effect of rotigotine on the survival of MPP^+-treated dopaminergic neurons. Treatment with rotigotine was begun on the 6th DIV for 8 consecutive days with a change of medium together with rotigotine every 2nd day. MPP^+ (10 µM) was additionally added for 48 h from 12-14th DIV. 100% corresponds to the total number of dopaminergic neurons after a cultivation period of 14 days without any treatment. Values represent the mean ± SEM of three independent experiments in quadruplicate. **B)** Representative micrographs of THir neurons after 14 DIV. Control cultures were cultivated without any treatment while for MPP^+ treatment, cultures were incubated with MPP^+ for 48 h from 12-14th DIV.

(Fig. 2A). After MPP^+ treatment, surviving neurons had shortened and thickened neurites as well as atrophied cell bodies (Fig. 2B). The treatment of cultures with 0.01 μM rotigotine attenuated MPP^+-induced toxicity by increasing the number of dopaminergic neurons by 10%, although this effect did not reach significance (Fig. 2A).

Rotigotine significantly protected dopaminergic neurons against rotenone-induced cell death

The treatment of cultures with 20 nM rotenone decreased the number of THir neurons by 55% compared to untreated control cultures (Fig. 3A). After rotenone treatment, surviving neurons exhibited less and shortened neurites (Fig. 3B). On the other hand, 0.01 μM rotigotine was able to counteract rotenone-induced toxicity by significantly increasing the number of dopaminergic neurons by 20% (Fig. 3A).

Rotigotine significantly inhibited ROS production by rotenone

The formation of ROS in cultures was measured using CM-H_2DCFDA fluorescence staining. The treatment of cultures with 20 nM rotenone on the 10th DIV for 48 h significantly elevated overall

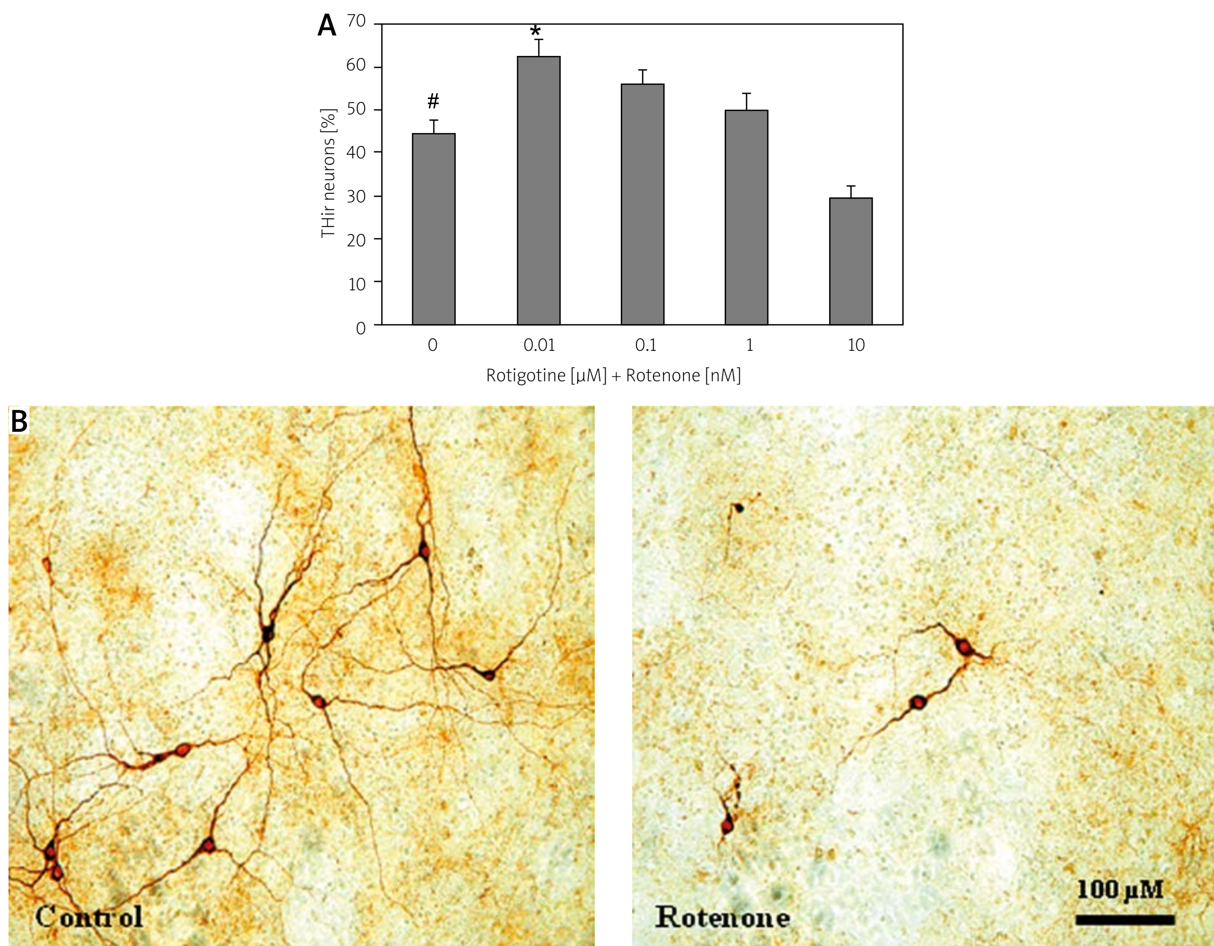


Fig. 3. A) Effect of rotigotine on the number of dopaminergic neurons after treatment with rotenone. Treatment with rotigotine was begun on the 6th DIV for 8 consecutive days with a change of medium together with rotigotine every 2nd day. Rotenone (20 nM) was additionally added for 48 h from 12-14th DIV. 100% corresponds to the total number of dopaminergic neurons after a cultivation period of 14 days without any treatment. Values represent the mean ± SEM of three independent experiments in quadruplicate. ($^{\#}p < 0.0001$ compared to untreated controls, $^{*}p < 0.0001$ compared to rotenone-treated cultures). **B)** Representative micrographs of THir neurons after 14 DIV. Control cultures were cultivated without any treatment while for rotenone treatment, cultures were incubated with rotenone for 48 h from 12-14th DIV.

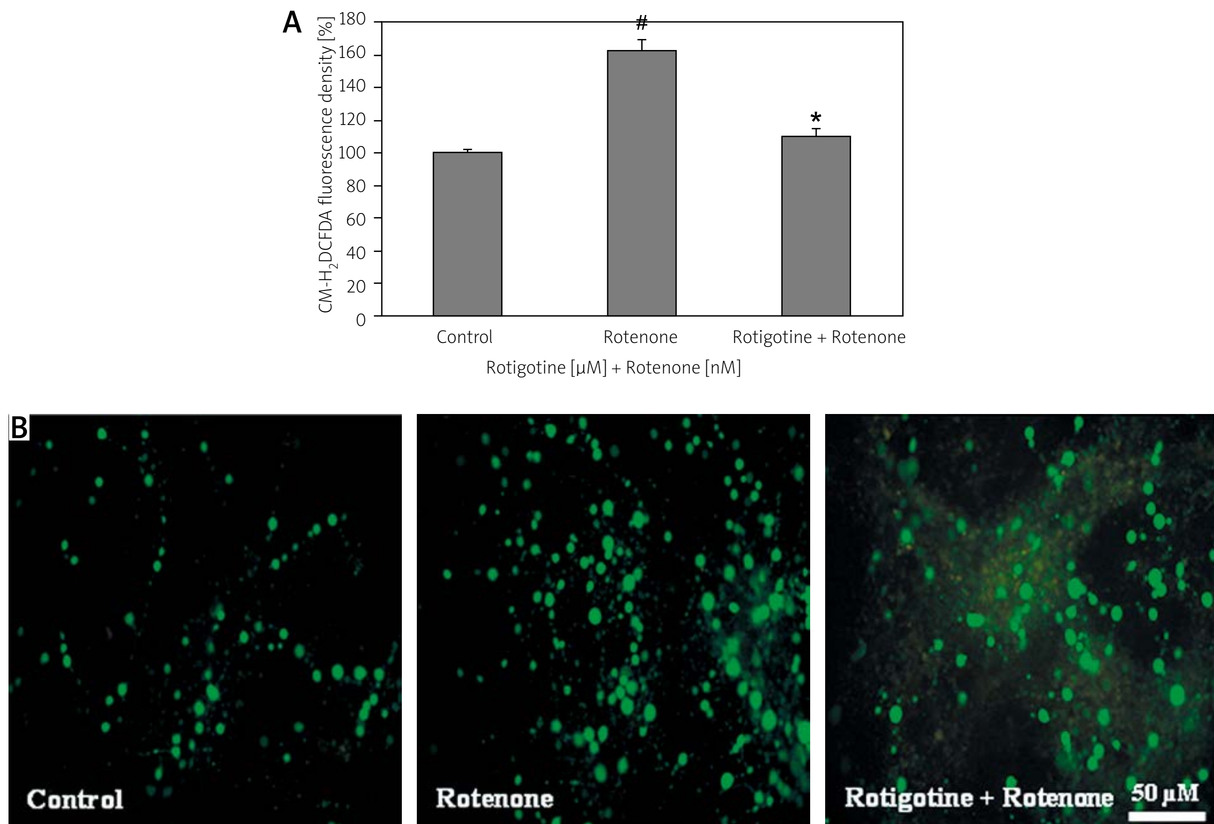


Fig. 4. A) Effect of rotigotine on ROS production by rotenone. Treatment with rotigotine (0.01 µM) was begun on the 6th DIV for 8 consecutive days with a change of medium together with rotigotine every 2nd day. Rotenone (20 nM) was added for 48 h from 12-14th DIV. 100% corresponds to the fluorescence density of the dye in untreated control cultures. Values represent the mean ± SEM of three independent experiments in quadruplicate. ($^{\#}p < 0.0001$ compared to untreated control cultures, $^{*}p < 0.0001$ compared to rotenone-treated cultures). **B)** Representative micrographs of fluorescence density of CM-H_2DCFDA in control, rotenone-treated and rotigotine plus rotenone-treated cultures. Higher fluorescence density indicates higher ROS production.

ROS production by 63% (Fig. 4A) as reflected in higher fluorescence density compared to untreated control culture (Fig. 4B). Rotigotine (0.01 µM, on the 6th DIV for six days) significantly decreased the intracellular production of ROS by 17% compared to rotenone-treated cultures (Fig. 4A). Figure 4B showed that treatment of cultures with rotigotine (0.01 µM) decreased the fluorescence density of CM-H_2DCFDA compared to rotenone-treated cultures.

Discussion

Protection of dopaminergic neurons by dopamine agonists has been a topic of interest for the past 20 years. While the lack of placebo groups in clinical studies did not allow a definite conclusion with respect to the neuroprotective effect of the dopamine agonists ropinirole and pramipexole [21,34], numerous preclinical studies were able to demonstrate distinct neuroprotective effects in animal or cell culture models [22,26,27]. In the present study, primary mesencephalic cell cultures were used to investigate the neuroprotective potential of the non-ergot dopamine agonist rotigotine against MPP^+ and rotenone toxicity. Both components are known inhibitors of complex I of the mitochondrial respiratory chain [20,25] and the detection of a defect in complex I in the SN of idiopathic PD cases provided a direct link to the MPTP (respectively MPP^+) and rotenone toxicity models.

In preliminary experiments, the effect of rotigotine on the survival rate of THir neurons was investigated. In a concentration range up to 1 µM, rotigo-

tine, when given for eight consecutive days, did not affect the survival of THir neurons. These results are similar to those achieved with the D_2 receptor agonist α-dihydroergocryptine that showed no stimulation of THir neurons, but differ from the D_2 receptor agonist lisuride which significantly enhanced the number of THir neurons under the same treatment conditions [9,11]. Treatment with 10 μM rotigotine decreased the number of THir neurons, which is similar to the behaviour of the D_2 agonist pergolide and α-dihydroergocryptine [9,10]. However, the high unphysiological concentration of 10 μM exceeds by far the therapeutic doses of 1-16 mg/day and the achieved plasma concentrations of unconjugated rotigotine which amount up to 1 ng/ml [7].

Some dopamine agonists have exhibited neuroprotection for dopaminergic neurons against MPTP in mice, for example bromocriptine [19] and pramipexole [16]. Neuroprotection against MPP^+ in primary dopaminergic culture from embryonic mouse or rat mesencephalon was demonstrated for lisuride [11], and pramipexole and ropinirole, respectively [6]. In contrast to these findings, rotigotine was not able to significantly counteract MPP^+-induced toxicity for dopaminergic neurons.

Since Betarbet *et al*. used the highly selective complex I inhibitor rotenone to reproduce PD's features in rats [2], rotenone has been considered a model neurotoxin for PD both *in vitro* and *in vivo*. Similar to our previous work [24], treatment of primary mesencephalic cell culture with 20 nM rotenone killed about 50% of dopaminergic neurons. Rotenone-induced cell death was shown to be attributed to overproduction of ROS and a decreased mitochondrial membrane potential [24]. Here we show that rotigotine was able to protect dopaminergic neurons from rotenone toxicity which might at least be partially attributed to a significant suppression of ROS production induced by rotenone treatment. Other dopamine agonists like pramipexole and ropinirole protected against rotenone-induced cell death in the dopaminergic neuroblastoma cell line SHSY-5Y [4,12] and pramipexole protected also against nigrostriatal dopaminergic degeneration and motor deficits in rotenone-treated mice [14].

In conclusion, rotigotine significantly protects dopaminergic neurons against rotenone toxicity in primary mesencephalic cell culture. The mechanism of protection involves counteracting of mitochondrial dysfunction as rotenone-induced radical production can be prevented by rotigotine.

Disclosure

Authors report no conflict of interest.

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