



The effect of antiparkinsonian drugs on oxidative stress induced pathological [³H]dopamine efflux after *in vitro* rotenone exposure in rat striatal slices

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ABSTRACT

An *in vitro* model of mitochondrial dysfunction with subsequent oxidative stress was elaborated and utilized to study the effect of drugs, currently used for the treatment of Parkinson's disease, on pathological H₂O₂-evoked [³H]dopamine efflux and the formation of toxic dopamine metabolites in rat striatal slices. 60 min rotenone (0.1–10 μM) pretreatment decreased dopamine content and [³H]dopamine uptake, as well as ATP level and energy charge of the slices. In addition, a robust potentiation of H₂O₂-evoked [³H]dopamine efflux and the formation of dopamine quinone in the effluent was detected. L-DOPA (200 μM) markedly elevated resting but not 100 μM H₂O₂-evoked and electrically-induced [³H]dopamine efflux. Furthermore, L-DOPA promoted the formation of dopamine quinone. Ropinirole (100 nM) did not affect resting and H₂O₂-evoked [³H]dopamine efflux and inhibited the electrically evoked release only in untreated slices. L-deprenyl, at concentration of 0.01 μM potentiated, whilst between 1 and 50 μM diminished H₂O₂-evoked [³H]dopamine efflux. Rasagiline (0.01–50 μM) slightly inhibited H₂O₂-evoked [³H]dopamine efflux, and it was able to prevent the generation of dopamine quinone. Neither of the drugs was able to suppress both the pathological H₂O₂-evoked [³H]dopamine efflux and the formation of dopamine quinone with simultaneous augmentation of electrically evoked [³H]dopamine release what should be a future concept of antiparkinsonian drug-design.

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, which primarily affects the aging population (Martone and Pangalos, 2005). The core pathological feature of PD is the relatively selective degeneration of the dopaminergic neurons in the *substantia nigra pars compacta*, associated with abnormal protein aggregation. The loss of dopaminergic neurons leads to progressive motor dysfunction, often accompanied by non-motor symptoms such as depression, apathy or dementia. The current treatment of PD relies on the replacement of the precursor of dopamine (DA) by 3,4-dihydroxy-L-phenylalanine (L-DOPA), i.e. a *substitution therapy*. Another mainstream approach is to inhibit the enzymes involved in the metabolic degradation of DA (monoamine oxidase (MAO), catechol-o-methyltransferase, (COMT)) and

thereby elevate DA bioavailability, which is primarily used as a *monotherapy* in the early stage of the disease or as an *add-on therapy* to L-DOPA (Youdim and Riederer, 2004). However, a necessary prerequisite of these approaches are functioning dopaminergic nerve terminals in the striatum. Therefore, in parallel with the progression of the disease L-DOPA and metabolic inhibitors become less effective. Moreover, they have many untoward side effects and long-term complications e.g. the development of dyskinesias or intractable motor fluctuations.

In Parkinson's patients a systemic deficit of mitochondrial complex I is detected (Schapira et al., 1989; Fahn and Sulzer, 2004). Recent studies pointed to the etiopathogenetic role of environmental toxins causing mitochondrial dysfunction in PD. Coincidence of mitochondrial dysfunction and oxidative stress and the consequent dysregulation of neuronal DA pools is believed to play a determinant role in the development of neurodegeneration (Fahn and Sulzer, 2004). Subchronic *in vivo* administration of the complex I inhibitor rotenone in rats was shown to reproduce the major pathological features of PD, including the deposition of Lewy bodies (Betarbet et al., 2000; Sherer et al., 2003; Milusheva et al., 2005). Inhibition of mitochondrial complex I leads to energy depletion and

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serves as a source of reactive species, causing oxidative damage and compromising cellular function at multiple target sites (lipid peroxidation, protein and DNA damage, etc.). As a result, DA is redistributed from vesicles and accumulates in the cytosol (Sai et al., 2008), where it provides an additional source of highly reactive free radicals due to its breakdown by MAO or auto-oxidation. Thus, DA might be oxidized to dopamine quinone (DAQ) (Graham, 1978). DAQ can be adducted by cysteine, and thereby involved in aberrant metabolism and ubiquitination pathways, Lewy body's formation or mediate the synthesis of hydroxyl radical and oxyradical species (Sulzer and Zecca, 2000). In addition, DAQ has been shown to stabilize protofibrils resulting from synuclein aggregation (Conway et al., 2001), and to inhibit the DA transporter (Whitehead et al., 2001).

In our previous study we showed that DAQ is generated from released DA in striatal slices of rats, *in vivo* subchronically pretreated with the irreversible complex I inhibitor rotenone, but only under conditions of coincident oxidative stress (Milusheva et al., 2005; Baranyi et al., 2006). Moreover, rotenone-induced mitochondrial dysfunction and oxidative stress had supra-additive impact on the pathological, presumably cytoplasmic release of DA (Milusheva et al., 2005; Baranyi et al., 2006). Thus, even a minimal mitochondrial deficit can predispose dopaminergic neurons to the harmful effect of subsequent oxidative stress and thereby aggravate the generation of toxic DA metabolites and the processes leading to neuronal death.

In fact, it is not known how antiparkinsonian drugs affect this vicious cycle. *In vitro* such process can be mimicked in rat striatal slice pretreated with rotenone and then subjected to mild oxidative challenge with low dose of H₂O₂ (Milusheva et al., 2005, 2008). Therefore, we examined *in vitro* the effects of L-DOPA, the golden standard of current PD therapy, ropinirole, a widely used D2 dopaminergic agonist, and two MAO-B inhibitors, L-deprenyl and rasagiline, on pathological [³H]dopamine ([³H]DA) release and the formation of oxidized metabolites of DA. We also compared the effect of these drugs on resting and on electrical field stimulation-induced [³H]DA release in untreated and rotenone-pretreated striatal slices.

2. Methods

All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Hungarian Academy of Sciences.

2.1. [³H]Dopamine release experiments

Male Wistar rats (180–220 g, bred in the local animal house) were decapitated and the brain was quickly removed into ice-cold Krebs' solution. Then the striatum was dissected out and sliced into 400 μm-thick sections with a McIlwain tissue chopper. The release experiments were performed as described earlier (Milusheva et al., 2005, 2008). Briefly, slices were incubated in 1 ml of Krebs' solution containing 5 μCi [³H]DA for 45 min and were continuously gassed with a mixture of 95% O₂ and 5% CO₂ at 37 °C. After incubation the slices were rinsed and transferred to tissue chambers and perfused continuously with modified Krebs solution at a rate of 0.5 ml/min. After a 60-min preperfusion 3-min samples were collected and assayed for [³H]DA. The samples for high performance liquid chromatography (HPLC) analysis of [³H]metabolites were acidified by 15 μl of 1.9 M perchloric acid (PCA) during the collection.

Oxidative stress was mimicked by addition of H₂O₂ (100–250 μM) to the Krebs solution starting at the 9th min of collection. Drugs (ropinirole, rasagiline, L-deprenyl, GBR12909) were added to the perfusion fluid 18 min before H₂O₂ application, with the exception of L-DOPA, which was preperfused for 60 min. In some experiments, the tissue chambers were fast cooled to 12 °C as described earlier (Vizi, 1998) by the Frigomix R thermoelectric device (Braun Instruments, Darmstadt, Germany). Low temperature was reached 30 min before the beginning of the sample collection period and lasted till the end of experiment.

In other experiments electrical field stimulation (EFS, S1, S2) was delivered by a Grass S88 stimulator twice, during the 3rd and 13th sample of the collection

period, using platinum ring electrodes attached to the top and bottom of tissue chambers, with the following parameters: 25 V, 1 ms, 2 Hz, 240 shocks. Previous studies showed that the electrically evoked efflux of [³H]DA under similar conditions is [Ca²⁺]_o-dependent and sensitive to the inhibition of voltage activated sodium channels by tetrodotoxin, reflecting axonal activity (e.g. Jin et al., 1993). In this set of experiments drugs were administered 18 min before the second stimulation period (S2) and onwards.

In all experiments with H₂O₂ application and in part of experiments with EFS, the slices were pretreated with rotenone (0.1–10 μM) for 60 min, and then rinsed with normal Krebs solution before loading with the isotope.

At the end of the experiment slices were homogenized in 0.5 ml of 10% trichloroacetic acid. A 0.5-ml aliquot of the superfusate and 0.1 ml of the tissue supernatant were added to 2 ml of scintillation cocktail (Ultima Gold, Packard). Tritium was measured with a Packard 1900 TR liquid scintillation counter using an internal standard. The release of tritium was calculated in Bq/g and expressed as percentage of the amount of radioactivity in the tissue at the time of sample collection (fractional release, FR%). The net release evoked by H₂O₂ or EFS (FRS1, FRS2) was calculated by the area-under-the-curve (AUC) method, i.e. subtracting the resting release calculated from the pre-stimulation period, from the release measured during H₂O₂/EFS. The effect of drugs on EFS-evoked release of [³H]DA was expressed as FRS2 over FRS1 (FRS2/FRS1) ratio. For the calculation of resting efflux, the tritium content of the sample collected immediately before the respective stimulation was taken into account. The tissue tritium uptake was determined as the sum release + the tissue content after the experiment and expressed in Bq/g, which reflects the content of radioactivity of the slices after the 60 min washout period, i.e. the radioactivity that is specifically taken up by the tissue. HPLC analyses of the samples (see below) showed that the tritiated effluent represents DA and its metabolites, with a major contribution of [³H]DA. Therefore, although the overall efflux of [³H] can be regarded as an indicator of the release of both [³H]DA and its tritiated metabolites, for the sake of clarity we will refer to tritium release as [³H]DA release.

2.2. HPLC determination of dopamine content, [³H]metabolites and adenine nucleotides

For the simultaneous measurement of biogenic amines and adenine nucleotides a liquid–liquid two-dimensional reversed-phase and ion pair-reversed-phase chromatographic separation was applied, as described earlier (Baranyi et al., 2006). Briefly, a Gilson liquid chromatographic system with 715-operation software (Gilson Medical Electronics Inc., Middleton, WI USA) was used. For the enrichment a “trap-column” (15–25 μm Nucleosil C-18 (20 × 4.0)) was inserted into a loop position. The separation of neurotransmitters was performed on a 3 μm Discovery C18 HS F5 (150 × 4.0 mm) analytical column. The analysis of adenine nucleotides was carried out at the first 10 min with reversed-phase buffer. The flow rate increased from 0.6 to 1.0 ml/min, linearly. The separation of catecholamines was accomplished with ion pair-reversed-phase buffer at constant flow rate 1.0 ml/min. The effluents were monitored by UV, electrochemical and radiochemical detectors. The detectors were connected in a cascade line. The identification of tritium labeled compounds was based upon the known retention times of unlabeled standards.

At the end of release experiments the tissue slices were immediately frozen in liquid nitrogen. The weighed frozen tissue was homogenized in an appropriate volume of ice-cold 0.1 M PCA that contained theophylline (as an internal standard) at 10 nmol/ml concentration and 0.5 mM sodium metabisulphite (antioxidant for biogenic amines). The suspension was centrifuged at 300 g for 10 min at 0–4 °C. The perchloric anion was precipitated by addition of 10 μl of 1 M KOH to 190 μl of the supernatant. The precipitate was then removed by centrifugation. The supernatant was kept at –20 °C until analysis. The pellet was saved for protein measurement according to Lowry et al. (1951). Tissue contents of ATP, ADP, AMP, DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) were expressed in nmol/mg protein.

The perfusion fluid was centrifuged at 300 g for 10 min at 0–4 °C; the supernatant was kept at –20 °C until analysis. For the analysis 545-μl sample volumes were diluted with 5 μl of 10^{–5} mol/l dihydroxybenzyl amine as an internal standard and 500 μl were injected.

2.3. Cell culture

PC12 cells were routinely cultured at 37 °C under an atmosphere of 5% CO₂ in Dulbecco's Modified Eagles's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum. The culture medium was replenished at 3–4 day intervals based on the doubling time of PC12 cells. All experiments were performed with the same stock of cells in the passage number 7–9 to keep inter-experimental variability at a minimum. Cells were seeded 5000 cells per well per 100 μl of fresh medium in 96-well tissue culture plates. All experiments were carried out 24 h after cells were seeded in the 96-well plates. Cells were treated with different concentrations of rotenone (0.5–2 μM) dissolved in equal volumes of DMSO and PEG-300 for 17–48 h and with each antiparkinsonian drug for 1 h before rotenone treatment. Control group was administered with equal amounts of DMSO and PEG-300.

2.4. Assessment of cell viability

Cell viability was assessed using the colorimetric reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). Stock solution of the dye was prepared, filter-sterilized, and stored at -20°C . Cells were incubated with 5 g/l MTT for 4 h at 37°C . After that, the converted dye was solubilized with acidic isopropanol (0.04 M HCl in absolute isopropanol). Reduced MTT was measured at the wavelength of 570 nm.

2.5. Materials

The following materials were used: [^3H]dopamine (specific activity 39 Ci/mmol, from Amersham, Little Chalfont, UK), H_2O_2 (Reanal, Budapest, Hungary), rotenone, L-DOPA, ropinirole, L-deprenyl, DMSO, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Dulbecco's Modified Eagles's Medium, fetal bovine serum (Sigma, St. Louis, MO, USA), GBR12909 (1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine, Tocris Bioscience, Ellisville, MO, USA). Rasagiline mesylate was obtained from TEVA Pharmaceuticals Ltd.

The composition of the Krebs solution was the following: NaCl 113, KCl 4.7, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 25, Na_2EDTA 0.03, ascorbic acid 0.3, and glucose 11.5 mM. All solutions were prepared on the day of use.

2.6. Data analysis

All data were expressed as means \pm S.E.M. of n observations. The statistical analysis was made by ANOVA followed by the Dunnett test (multiple comparisons), or Student's t -test (pair-wise comparisons). P values of less than 0.05 were considered statistically significant.

Adenylate energy charge (EC), introduced by Atkinson (Atkinson, 1968), was calculated accordingly:

$$\text{EC} = ([\text{ATP}] + 0.5[\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$$

The EC value represents the energy resources of a cell as a function of the concentration of nucleotides. A biological system is fully charged when ATP dominates over other adenine nucleotides and the corresponding EC is close to one.

3. Results

3.1. Effects of *in vitro* rotenone pretreatment

When striatal slices were preincubated *in vitro* with the irreversible complex I inhibitor rotenone (10 μM) for 60 min, ATP content of the slices and the energy charge were significantly decreased (Fig. 1A and B). This treatment also depleted striatal DA content (Fig. 1C). After 60 min preperfusion the tissue radioactivity uptake was $7.56 \pm 0.79 \times 10^5$ Bq/g ($n = 8$), and it was reduced to less than the half after rotenone pretreatment (Fig. 1D). The resting [^3H]DA efflux was $1.12 \pm 0.08\%$ ($n = 12$) in rotenone-pretreated striatal slices, significantly higher than in the absence of rotenone ($0.61 \pm 0.03\%$, $n = 12$, $P < 0.01$). These data are similar to that obtained in our previous study (Milusheva et al., 2005) and indicate that *in vitro* rotenone pretreatment mimics the neurochemical changes observed in PD.

Perfusion of the slices with H_2O_2 (100–250 μM) elicited a concentration-dependent elevation of [^3H]DA efflux, which reached its peak 15 min after the beginning of H_2O_2 perfusion (100 μM : $1.45 \pm 0.21\%$, $n = 12$, $P < 0.01$ and 250 μM : $2.63 \pm 0.22\%$, $n = 4$, $P < 0.01$, vs. resting) and then declined and returned to the baseline level (Fig. 2A). Confirming our previous observations (Milusheva et al., 2005), the effect of oxidative stress on [^3H]DA efflux was greatly exacerbated after *in vitro* rotenone pretreatment, and a robust potentiation of H_2O_2 -evoked [^3H]DA release was observed (Fig. 2A).

HPLC analysis of tritium content showed that DA, and its metabolites: DOPAC, HVA, 3-methyl-4-hydroxyphenyl glycol (MHPG), 3-methyltyramine (3-MT), noradrenaline (NA), as well as trace amines: octopamine (OCT), tyramine (TYR) and beta-phenylethylamine (beta-PEA) were all present in the effluent, with a predominant representation of DA (Fig. 2B). In addition, DAQ, the toxic metabolite of DA, also appeared in the effluent collected during the perfusion with H_2O_2 (Fig. 2B).

When the bath temperature was cooled to 12°C the H_2O_2 -evoked tritium efflux was almost completely abolished (Fig. 2C). Because previous studies showed that at 12°C vesicular transmitter release is maintained, whilst transporter-mediated transmitter release is eliminated (Vizi, 1998), this observation suggests that H_2O_2 -evoked efflux is transporter-mediated and its underlying mechanism might be the reversal of the DA transporter (DAT). Indeed, GBR12909 (100 nM), the selective DAT inhibitor, significantly inhibited [^3H]DA efflux evoked by H_2O_2 (Fig. 2C).

Low frequency EFS (2 Hz, 240 shocks, 25 V) was used to mimic neuronal activity, which elicited a rapid and reproducible efflux of [^3H]DA in untreated slices (FRS1: $1.28 \pm 0.12\%$, $n = 47$) (Fig. 2D) resulting in an FRS2/FRS1 ratio of 0.76 ± 0.09 ($n = 10$) under control conditions. In rotenone-pretreated slices FRS1 was significantly less calculated either in absolute (750 ± 170 Bq/g, $n = 7$ vs. 8740 ± 2050 Bq/g, $n = 10$, $P < 0.001$) or relative terms ($0.39 \pm 0.05\%$, $n = 41$, $P < 0.001$), indicating a functional impairment of dopaminergic transmission (Fig. 2D). The FRS2/FRS1 ratio was 0.79 ± 0.1 ($n = 7$, $P > 0.05$) after rotenone pretreatment, not significantly different from untreated slices.

We have also examined the effect of different concentrations of rotenone pretreatment (0.1–10 μM) on endogenous nucleotide and DA content and on H_2O_2 -evoked [^3H]DA efflux, respectively. Rotenone had a concentration-dependent depleting action on both ATP and DA content of the slices reaching the level of significance at 1 μM for ATP content and 3 μM for DA content (Fig. 3A). However, the decrease of the energy charge became significant only at 10 μM (Fig. 3B). The effect of rotenone on H_2O_2 -evoked [^3H]DA efflux was also concentration-dependent and a robust amplification of the release was already observed at 3 μM (Fig. 3B and C). Therefore, significant energy depletion is not a necessary condition for the potentiation of H_2O_2 -evoked [^3H]DA release in response to rotenone pretreatment.

3.2. Effect of antiparkinsonian drugs on resting and H_2O_2 -evoked [^3H]dopamine efflux after rotenone pretreatment

When slices were preperfused for 60 min with L-DOPA (200 μM), it markedly elevated the resting [^3H]DA efflux (Fig. 4A, Table 1). Nevertheless, H_2O_2 -evoked tritium efflux remained unchanged in the presence of L-DOPA (Fig. 4A, Table 1). HPLC analysis of the effluent showed that the proportion of DA was significantly decreased, whilst its metabolites (MHPG and 3-MT) and trace amines (OCT, TYR) were significantly increased upon L-DOPA perfusion (Fig. 4B), indicating a fast metabolism of DA in the nerve terminals. The proportion of DAQ also increased in the presence of L-DOPA (Fig. 4B).

The selective D2 receptor agonist ropinirole (100 nM), did not elevate the resting [^3H]DA efflux and H_2O_2 -evoked release remained unaffected (Fig. 4C, Table 1). Similarly to L-DOPA treatment, there was a significant decline in the proportion of DA and an increase in the proportion of 3-MT in the effluent in the presence of ropinirole (Fig. 4D). In addition, the proportion of NA was also increased (Fig. 4D). The amount of DAQ, however, did not change in the presence of ropinirole (Fig. 4D).

The MAO-B inhibitor L-deprenyl (0.01–50 μM) concentration-dependently increased the resting [^3H]DA efflux, although this elevation was modest, when compared to the effect of L-DOPA (Fig. 5A, Table 1). By contrast, it had a differential effect on H_2O_2 -evoked [^3H]DA release, depending on the applied concentration. When L-deprenyl was perfused in low concentration (0.01 μM), the H_2O_2 -evoked [^3H]DA efflux was markedly potentiated, whilst it was attenuated by higher concentrations (1–50 μM) of L-deprenyl (Fig. 5A and E; Table 1). In the presence of 50 μM L-deprenyl the proportion of DA and DOPAC was decreased, and a relative increase in the amount of trace amines (TYR, OCT and beta-PEA) was also

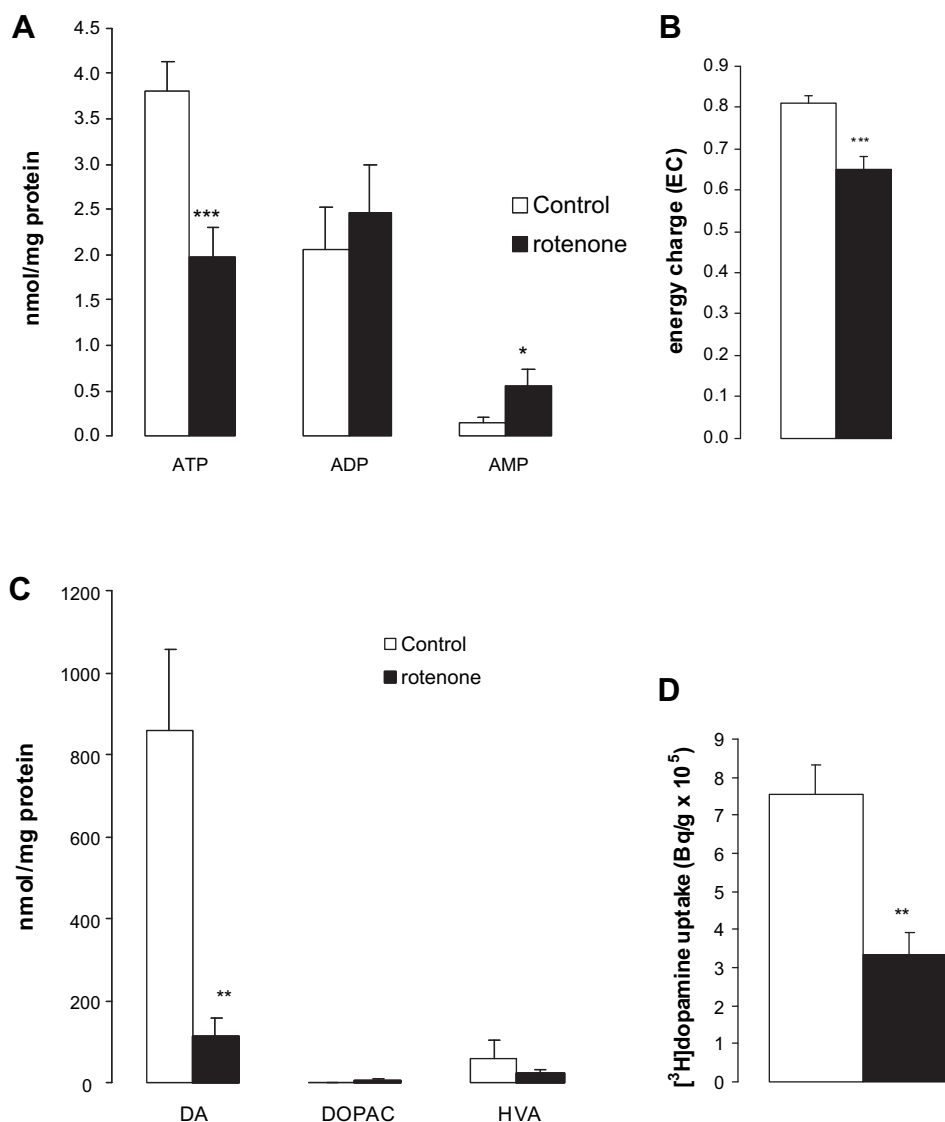


Fig. 1. Acute rotenone pretreatment (1 h at 37 °C, continuous oxygenation) before incubation with the isotope decreases ATP content (A) the energy charge (B) endogenous DA content (C) and uptake of [³H]DA into rat striatal slices (D). Values are means ± SEM in 6 (control) and 7 (rotenone) identical experiments. The tissue content of ATP, ADP, AMP, DA and its metabolites (DOPAC, HVA) are expressed in nmol/mg protein. For the calculation of energy charge (EC) see [Materials and methods](#). [³H]DA uptake is calculated as the sum release + the tissue content after the experiment and expressed in Bq/g. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significance vs. rotenone-free controls.

observed (Fig. 5B). However, the proportion of DAQ did not change (Fig. 5B).

The recently introduced antiparkinsonian drug rasagiline (0.01–50 μM) did not change significantly the resting [³H]DA efflux, except at 1 μM concentration, where it slightly elevated the release (Fig. 5C, Table 1). A mild, but significant decrease in H₂O₂-evoked [³H]DA efflux was observed at 100 nM concentration, although this effect disappeared in higher concentration (Fig. 5C and E; Table 1). Similarly to L-deprenyl, rasagiline (50 μM) decreased the proportion of DA in the effluent and increased the proportion of OCT and beta-PEA (Fig. 5D). Importantly, DAQ production was completely inhibited in the presence of rasagiline (Fig. 5D).

3.3. Effect of antiparkinsonian drugs on EFS-evoked [³H]DA efflux in rat striatal slices

The tritium uptake and resting [³H]DA efflux in these experiments were $5.06 \pm 0.64 \times 10^5$ Bq/g and $0.57 \pm 0.03\%$, respectively

(*n* = 47). Antiparkinsonian drugs, used in previous experiments, were perfused here between the 1st and 2nd stimulation period and their effect on EFS-evoked [³H]DA efflux was expressed as FRS2/FRS1 ratios compared to the FRS2/FRS1 ratios in the absence of the drug.

When EFS-evoked [³H]DA efflux was compared to control slices it did not significantly change in response to L-DOPA either in the absence or presence of rotenone (Fig. 6). However, the FRS2/FRS1 ratio was significantly higher in the slices which had been exposed to rotenone (10 μM). Ropinirole (100 nM) significantly attenuated electrically evoked [³H]DA efflux in untreated striatal slices (Fig. 6). This effect was abolished in rotenone-pretreated slices (Fig. 6). The MAO-B inhibitor L-deprenyl (0.01–50 μM) did not change EFS-evoked [³H]DA efflux in untreated and rotenone-treated striatal slices (Fig. 6). Rasagiline (50 μM) was also without significant effect on electrically evoked [³H]DA efflux, irrespectively from rotenone pretreatment (Fig. 6). Similar data were obtained using a lower concentration of rasagiline (0.01 μM) (data not shown).

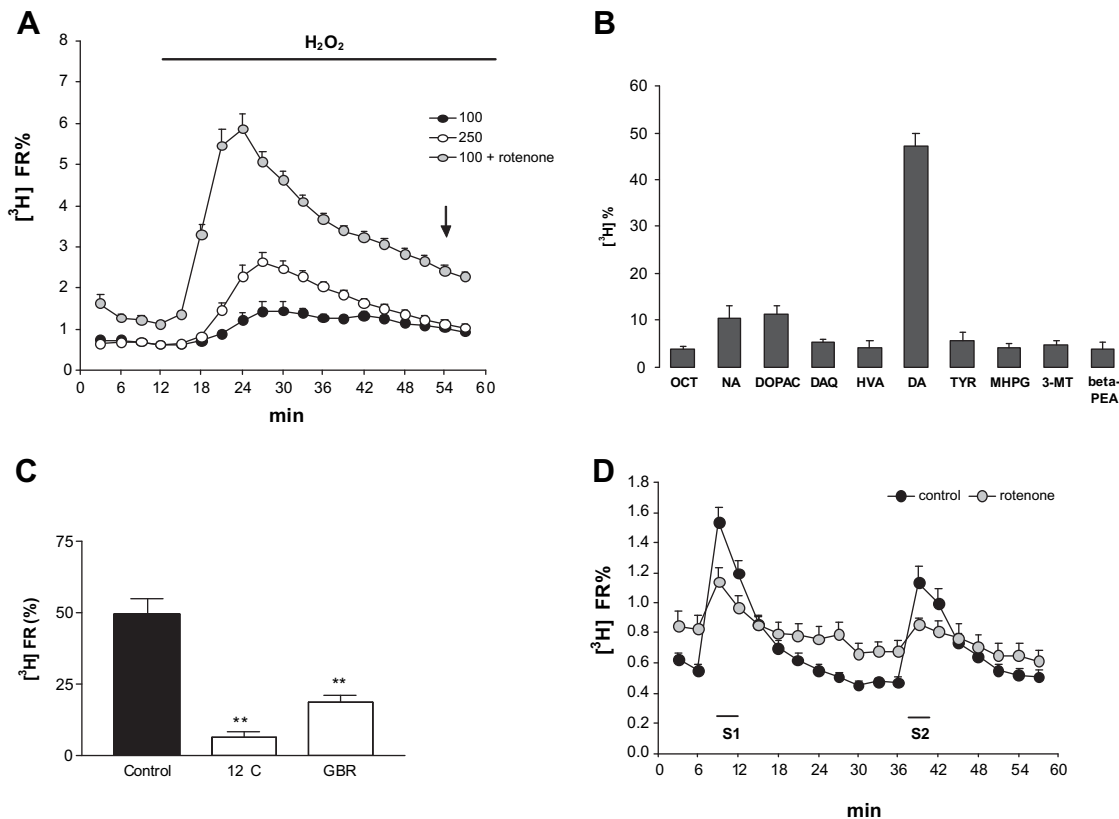


Fig. 2. Pretreatment of striatal slices by rotenone increases the sensitivity of dopaminergic neurotransmission to oxidative stress and decreases [³H]DA release evoked by electrical field stimulation. **A.** H₂O₂ was added to the perfusion fluid at the 3rd fraction in 100–250 μM concentration, as indicated by the legend. [³H]DA release is expressed as fractional release (FR%, for calculation see *Materials and methods*), as a function of time. Means ± SEM of 12 (100, 100 + rotenone) and 4 (250) observations are presented. **B.** Tritium composition of the sample indicated by arrow on Fig. 2A. Samples were analyzed by HPLC and the amount of tritiated octopamine (OCT), noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine quinone (DAQ), homovanilic acid (HVA), DA, tyramine (TYR) 3-methyl-4-hydroxyphenyl glycol (MHPG), 3-methyltyramine (3-MT), beta-phenylethylamine (beta-PEA) is expressed as a percentage of total tritium label (*n* = 4). **C.** Effect of cooling the bath temperature to 12 °C (12C), and the DA transporter inhibitor GBR12909 (GBR, 100 nM) on [³H] release evoked by 100 μM H₂O₂. The total [³H] release evoked by H₂O₂ was calculated by the area-under-the-curve (AUC) method. GBR12909 was administered 18 min before H₂O₂ application, 12 °C was applied 30 min before the beginning of the sample collection period and onwards. **D.** EFS-evoked [³H] release. Slices were perfused with Krebs solution and subjected to electrical field stimulation (25 V, 1 ms, 2 Hz, 240 shocks) at the 3rd and 13th sample collection period (S1, S2) after 60 min pre-incubation with rotenone (10 μM) or Krebs' solution (control). [³H] release is expressed as fractional release (FR%, for calculation see *Materials and methods*), as a function of time. Means ± SEM of 10 (control) and 7 (rotenone) observations are presented. ***P* < 0.01, significance vs. controls.

3.4. Effect of antiparkinsonian drugs on rotenone-induced inhibition of mitochondrial respiration in PC12 cells

The mitochondrial respiration was evaluated as a measure of cell viability in cultured PC12 cells. The irreversible complex I inhibitor rotenone had a concentration and time-dependent inhibitory effect on the reduced MTT of cultures, decreasing to $75.4 \pm 5.4\%$ of the control after 20 h treatment with 1 μM concentration (*n* = 40, *P* < 0.01, Fig. 7A and B). L-DOPA, although slightly restored respiration at lower concentrations (10–20 μM), profoundly inhibited mitochondrial function at higher concentrations (100–200 μM) (Fig. 7C). By contrast, ropinirole (0.01–10 μM) had no substantial effect on cell viability. L-deprenyl (0.01–10 μM) and rasagiline (0.01–10 μM) concentration-dependently protected against the effect of rotenone, however, the effect of L-deprenyl reached the threshold of significance only at 10 μM (Fig. 7C), whilst that of rasagiline was already significant at 0.01 μM (Fig. 7C).

4. Discussion

The main objective of this study was to get insight into the mechanism of oxidative stress induced [³H]DA release in the rotenone-treated rat striatum and to examine how current

antiparkinsonian drugs affect the exacerbated, pathological DA efflux and the formation of the toxic DA metabolite DAQ. We have elaborated a useful simple method to test new antiparkinsonian drug candidates, which (1) confirms observations already known from the therapy and (2) at the same time gives a more detailed insight into their mode of action. In accordance with the findings established in our previous studies (Milusheva et al., 2003, 2005, 2008; Baranyi et al., 2006) *in vitro* pretreatment with the irreversible complex I inhibitor rotenone elicited substantial energy deprivation, depleted endogenous DA from the striatum and decreased [³H]DA uptake into the slices. Moreover, it simulated the neurochemical changes seen after *in vivo* subchronic pretreatment with low dose rotenone, i.e. in rotenone-induced Parkinson model (Sherer et al., 2003; Milusheva et al., 2005), which is closer to the ethiopathogenetic conditions presumed in human PD. Thus, H₂O₂-evoked [³H]DA release exhibited a robust potentiation after rotenone pretreatment, and the released tritiated DA was partly converted to DAQ. In addition, in agreement with previous findings, trace amines such as radiolabeled TYR, OCT and beta-PEA were formed from [³H]DA, which can be generated either intra- or extracellularly, and may be involved in a buffer-like mechanism regulating the brain availability of monoamines (Dyck et al., 1983). Contrary to H₂O₂-evoked release, the electrically evoked [³H]DA

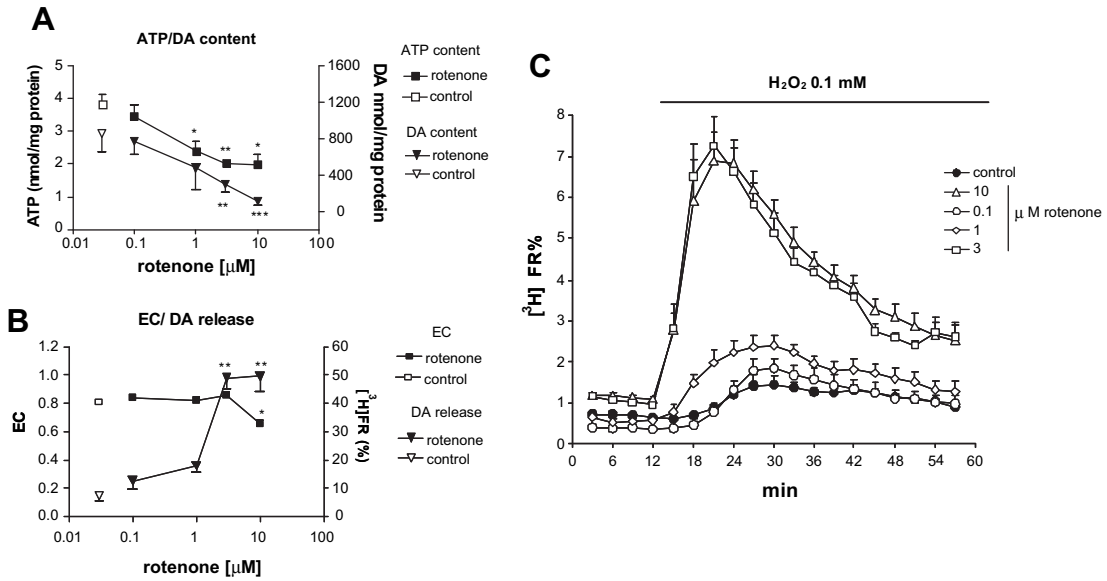


Fig. 3. Effect of different concentrations of rotenone pretreatment on the tissue content of ATP and DA (A) energy charge (B) and H₂O₂-evoked [³H]DA release (B, C). Slices were incubated for 60 min with different concentrations of rotenone, indicated on the abscissa or the legend, loaded with isotope and then perfused with Krebs solution and subjected to H₂O₂ (100 μ M) as indicated by the horizontal bar in panel C. The tissue contents of ATP and DA are expressed in nmol/mg protein. For the calculation of energy charge (EC) see Materials and methods. The total [³H] release evoked by H₂O₂ was calculated by the area-under-the-curve (AUC) method (B). In panel C [³H] release is expressed as fractional release. Values are means \pm SEM in 4–8 identical experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significance vs. controls.

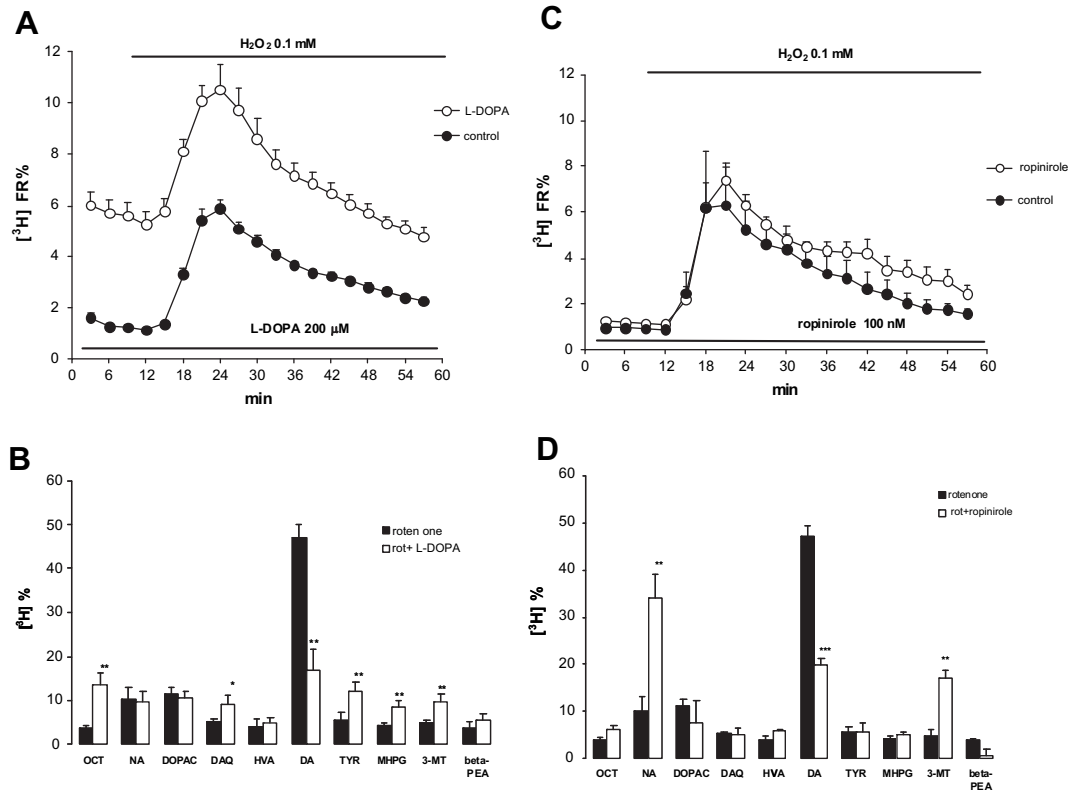


Fig. 4. Effect of L-DOPA (200 μ M) and ropinirole (100 nM) on H₂O₂-evoked [³H]DA release in the rotenone (10 μ M)-pretreated rat striatal slices. Slices were preperfused with L-DOPA for 60 min (A) or with ropinirole for 18 min (C) and then challenged with H₂O₂ (100 μ M) perfusion, as indicated by the horizontal bar. Note that L-DOPA substantially elevated the basal tritium efflux. [³H] release is expressed as fractional release (FR%), for calculation see Materials and methods, as a function of time. Means \pm SEM of 6 (control), 8 (L-DOPA) and 8 (ropinirole) observations are presented. (B, D) Tritium composition of the sample indicated by arrow on Fig. 2A from control (*n* = 4), L-DOPA treated (*n* = 8, B), and ropinirole treated (*n* = 8, D) slices. Samples were analyzed by HPLC and the amount of tritiated octopamine (OCT), noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine quinone (DAQ), homovanillic acid (HVA), DA, tyramine (TYR) 3-methyl-4-hydroxyphenyl glycol (MHPG), 3-methyltyramine (3-MT), beta-phenylethylamine (beta-PEA) is expressed as a percentage of total tritium label. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 significance vs. controls.

Table 1
Effect of antiparkinsonian drugs on the resting and H₂O₂-evoked [³H]DA efflux from striatal slices pretreated with rotenone (10 μM).

Drug	Resting (FR%)	H ₂ O ₂ -evoked (FR%)
l-DOPA		
–	1.12 ± 0.08	36.34 ± 2.11
200 μM	5.26 ± 0.48***	28.87 ± 10.18
Ropinirole		
–	0.90 ± 0.03	38.2 ± 12.18
100 nM	1.12 ± 0.1	48.33 ± 4.16
l-deprenyl		
–	0.96 ± 0.13	42.45 ± 4.97
0.01 μM	0.83 ± 0.06	133.11 ± 10.73**
0.1 μM	1.74 ± 0.07**	45.98 ± 8.15
1 μM	0.96 ± 0.10	27.69 ± 1.89
50 μM	2.10 ± 0.15**	20.57 ± 4.32*
Rasagiline		
–	1.14 ± 0.08	49.56 ± 5.44
0.01 μM	1.01 ± 0.05	34.09 ± 7.77
0.1 μM	1.00 ± 0.13	23.71 ± 2.77*
1 μM	1.59 ± 0.19*	34.22 ± 5.26
50 μM	0.82 ± 0.08	50.23 ± 5.02

Rat striatal slices were preincubated with 10 μM rotenone for 60 min; then loaded with [³H]DA for 45 min, and subsequently superfused with Krebs solution for 60 min. H₂O₂ (100 μM) perfusion was applied from the 3rd sample collection period and onwards. All drugs were applied 18 min before the H₂O₂ application, except l-DOPA, which was preperfused for 60 min. Resting [³H]DA efflux was expressed as a percentage of the amount of radioactivity in the tissue at the time of sample collection (fractional release, %), and the tritium content of the sample collected immediately before the H₂O₂ application was taken into account. H₂O₂-evoked [³H]DA efflux was expressed by calculating the net release in response to H₂O₂ perfusion by the area-under-the-curve method – that is, by subtracting the release before H₂O₂ application from the values measured after H₂O₂ application. Values are means ± SEM in 4–12 identical experiments. *P < 0.05, **P < 0.01, ***P < 0.001, significance vs. drug-free respective controls.

release was substantially reduced in rotenone-pretreated slices, consistently with the functional impairment of dopaminergic terminals.

Cooling the bath temperature to 12 °C, which inhibits transporter-mediated processes, or the DAT inhibitor GBR12909 inhibited H₂O₂-evoked [³H]DA efflux, indicating that its underlying mechanism is the reversal of DA transporter. This finding corroborates both with our previous findings on H₂O₂ induced noradrenaline release (Milusheva et al., 2003), and with the view that oxidative stress compromises the vesicular storage of DA and accumulates DA in the cytoplasm (Fahn and Sulzer, 2004; Sai et al., 2008), which might be able to elicit the reversal of the transporter.

Because striatal slices appeared to be less sensitive to the action of rotenone than cultured cells (see also below) we have chosen relatively high concentration of rotenone, whereby we could reproduce the effects of *in vivo* subchronic exposure of low dose rotenone. Nevertheless, we also explored how permanent inhibition of mitochondrial respiration obtained by pretreatment with different concentrations of rotenone, could reinforce this pathological scenario. Sai et al. (2008) has recently observed that rotenone downregulates the vesicular monoamine transporters leading to a cytoplasmic accumulation of dopamine in PC12 cells. Accordingly, we found that potentiation of H₂O₂-evoked [³H]DA release could be observed even in those concentrations, where significant drop in the energy charge was not seen yet. This fact implies that the effect is rather due to rotenone-induced oxidative stress (Bao et al., 2005; Milusheva et al., 2008) than to energy depletion caused by complex I inhibition.

In PD, l-DOPA is usually used in combination with peripherally acting DOPA decarboxylase inhibitors in order to enhance central dopaminergic tone. l-DOPA is taken up by the nerve terminals and

glia, where it is converted to DA by aromatic amino acid decarboxylase. This way l-DOPA is assumed to increase the turnover, content and releasable pool of DA in residual, functionally intact striatal nerve terminals (Lloyd et al., 1975). In our experiments an acute treatment with l-DOPA substantially elevated the resting [³H]DA efflux in rotenone-pretreated slices. This effect is probably due to the incorporation of l-DOPA to DA pools and subsequent mobilization of the tritiated DA. A relative increase in electrical stimulation-evoked [³H]DA efflux was also observed after rotenone pretreatment, indicating the compensatory hyperactivity of residual terminals. Similar adaptive changes in the releasable pool of DA were observed in striatal slices after 6-OHDA pretreatment (Snyder et al., 1990; Ogawa et al., 2000; Juranyi et al., 2004). However, H₂O₂-evoked, pathological [³H] release did not attenuate upon l-DOPA application, and tritium composition was unfavorably changed leading to a decreased amount of DA and increased formation of DAQ and trace amines. These findings are concordant with the potential of l-DOPA to elicit oxidative damage in cell culture (Pardo et al., 1995; Walkinshaw and Waters, 1995) and its inability to slow down the progression of neuronal loss in PD (Ogawa, 1994; Fahn et al., 2004). Accordingly, we observed a pronounced decline in mitochondrial respiration of PC12 cells co-treated with rotenone and l-DOPA in similar concentrations.

Ropinirole, the D2 receptor agonist, is applied as a replacement of endogenous DA alone or in combination with l-DOPA in the pharmacotherapy of PD. Therefore, it is not expected to release DA by its own. In our experiments, ropinirole did not affect pathological, H₂O₂-evoked tritium efflux and the formation of DAQ either. However, ropinirole elicited a shift in the composition of tritiated effluent towards the formation of metabolites on the expense of DA, which resulted in an actual decrease in the amount of [³H]DA. Although we have no ready explanation, why the metabolism of tritiated dopamine is changed after exogenous l-DOPA and ropinirole addition, these changes might reflect an altered responsiveness of striatal dopaminergic metabolic pathways after rotenone treatment. On the other hand, ropinirole also inhibited electrical field stimulation-induced [³H]DA efflux. This inhibitory effect is most likely due to the activation of inhibitory D2 autoreceptors, in line with previous observations (Eden et al., 1991; Limberger et al., 1991; Vizi, 2000). However, like with l-DOPA, the FRS2/FRS1 ratio is increased in rotenone-treated slices. These findings indicate that the D2 receptor-mediated presynaptic inhibition (1) is restricted to action-potential-dependent vesicular release of DA and does not extend to pathological, cytoplasmic efflux of DA evoked by oxidative stress, and (2) it is alleviated in parallel with the rotenone-induced degeneration of the dopaminergic terminals.

The reason for the use of MAO-B inhibitors l-deprenyl and rasagiline in PD is that they enhance the endogenous dopaminergic tone by inhibition of the metabolism of endogenous DA or exogenous l-DOPA. In addition, both drugs possess some, although not identical neuroprotective properties (Finberg et al., 1996; Youdim and Riederer, 2004; Blandini, 2005). Interestingly, l-deprenyl at low nanomolar concentration profoundly facilitated H₂O₂-evoked, but not the electrically evoked [³H]DA efflux. Electrical field stimulation and H₂O₂ release catecholamines from different pools: the electrically-induced release is vesicular, whilst H₂O₂-evoked release is found to be transporter-mediated. Therefore, one can assume that l-deprenyl affects the cytoplasmic pool of DA directly or indirectly either via its conversion to the indirect catecholamine releasers TYR and PEA, or by the inhibition of reuptake of DA (Zsilla et al., 1986; Lamensdorf et al., 1999). However, rasagiline, another MAO-B inhibitor, which has only weak effect on striatal DA uptake (Lamensdorf et al., 1999), was devoid of DA releasing effect, which favors the latter explanation. l-deprenyl diminished pathological H₂O₂-evoked DA efflux, but only in the high micromolar

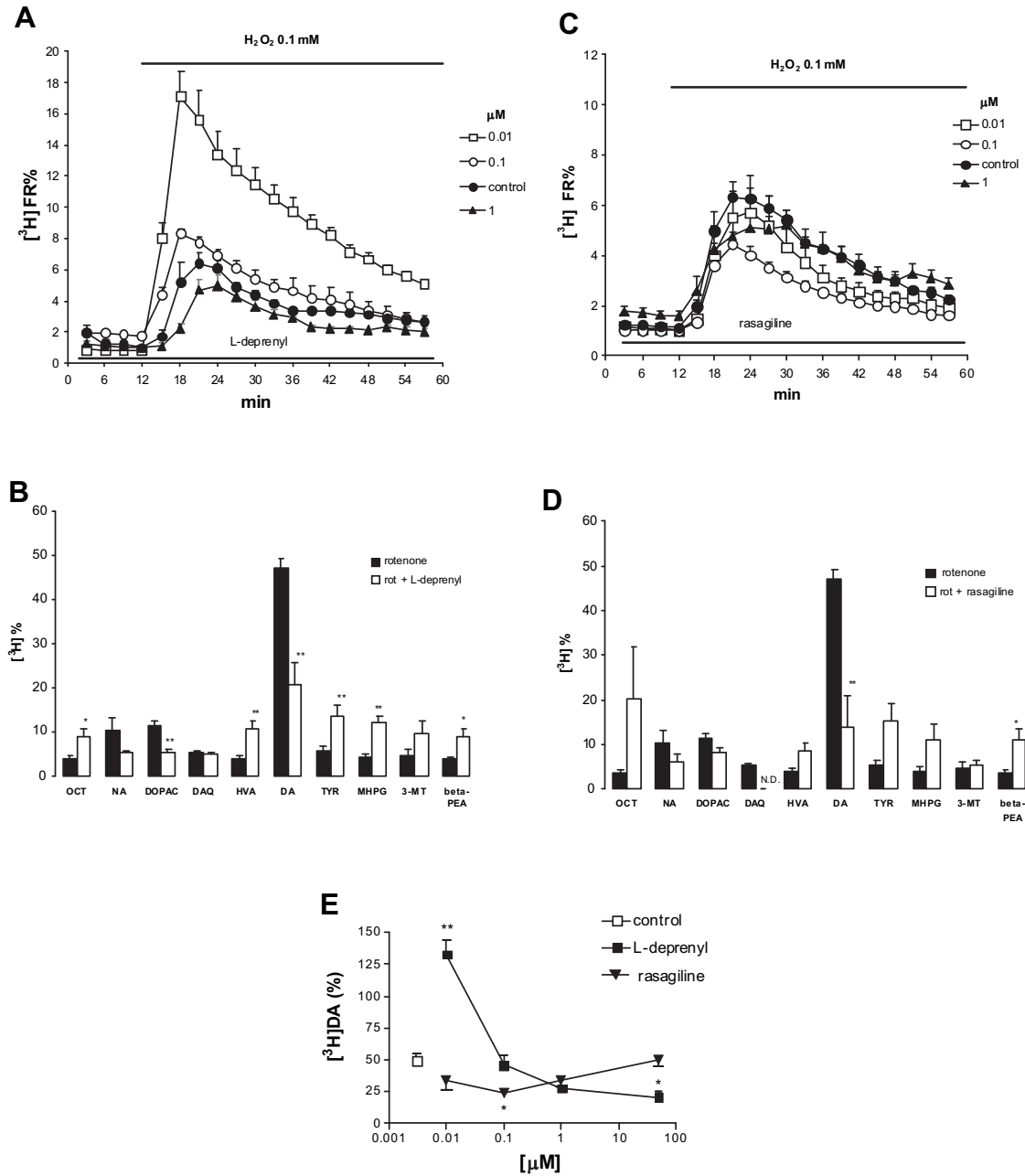


Fig. 5. Effect of l-deprenyl (0.01–50 μM) and rasagiline (0.01–50 μM) preperfusion on H₂O₂-evoked [³H]DA release in the rotenone (10 μM)-pretreated rat striatal slices. Slices were preperfused for 18 min with l-deprenyl (A) or rasagiline (C) in concentrations indicated in the legend, and then challenged with H₂O₂ (100 μM) perfusion, as indicated by the horizontal bar. [³H] release is expressed as fractional release (FR%, for calculation see Materials and methods), as a function of time. Means ± SEM of 8 (control), 8 (l-deprenyl) and 8 (rasagiline) observations are presented. (B, D) Tritium composition of the sample indicated by arrow in Fig. 2A from control (n = 4), l-deprenyl (50 μM, n = 4, B) and rasagiline (50 μM, n = 6, D) treated slices. Samples were analyzed by HPLC and the amount of tritiated octopamine (OCT), noradrenaline, (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine quinone (DAQ), homovanilic acid (HVA), DA, tyramine (TYR) 3-methyl-4-hydroxyphenyl glycol (MHPG), 3-methyltyramine (3-MT), beta-phenylethylamine (beta-PEA) is expressed as a percentage of total tritium label. E. Concentration-dependence of the effect of l-deprenyl and rasagiline on H₂O₂-evoked [³H]DA release in the rotenone (10 μM)-pretreated rat striatal slices. The total [³H] release evoked by H₂O₂ was calculated by the area-under-the-curve (AUC) method in the absence and presence of different concentrations of l-deprenyl and rasagiline, indicated on the abscissa. *P < 0.05, **P < 0.01, significance vs. controls. N.D., not detectable.

concentration, and it was not able to prevent the formation of DAQ. By contrast, rasagiline in 100 nM concentration, which is close to therapeutic levels, exhibited a significant inhibitory effect on H₂O₂-evoked DA efflux.

When the collected samples were analyzed by HPLC, a decreased proportion of tritiated DA and DOPAC and an increase in OCT, HVA and beta-PEA were detected in the presence of l-deprenyl. This pattern is characteristic to a preferential MAO-B inhibition as PEA is

a preferred substrate of MAO-B, while DA is primarily metabolized by MAO-A in the rat striatum (Yang and Neff, 1974; Green et al., 1977). Rasagiline elicited a similar change in the composition of metabolites, with a remarkable exception: unlike l-deprenyl, it completely prevented the formation of toxic DAQ, which provides an additional explanation to its neuroprotective, disease-modifying potential. Since DAQ is thought to be formed by both enzymatic and non-enzymatic conversion of DA (Graham, 1978), rasagiline, which

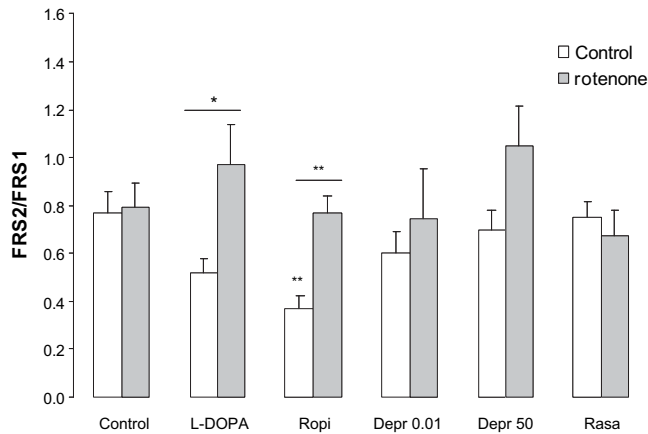


Fig. 6. Effect of L-DOPA (200 μ M), ropinirole (Ropi, 100 nM), L-deprenyl (Depr, 0.01–50 μ M) and rasagiline (Rasa, 50 μ M) on EFS-evoked [3 H]DA release in rotenone-pretreated (rotenone) and untreated (control) rat striatal slices. Slices were perfused with Krebs solution and subjected to electrical field stimulation (25 V, 1 ms, 2 Hz, 240 shocks) at the 3rd and 13th sample (S1, S2). Drug perfusion started after the first stimulation period. [3 H]DA release is expressed as FRS2/FRS1 ratios measured in the presence and absence of each drug. Results show the means \pm SEM of 4–8 identical experiments. Asterisks indicate significant differences between drug treatment and respective controls, or between untreated and rotenone-treated animals, as indicated (* P < 0.05, ** P < 0.01).

is an irreversible, approx. 10-times more potent MAO-B inhibitor than L-deprenyl (Blandini, 2005), might be able to prevent abnormal formation of oxidative metabolites by inhibition of MAO-B, or independently from that. We note, however, that in contrast to human brain, where MAO-B accounts for about 80% of MAO activity (Youdim and Riederer, 2004), MAO-A is the predominant MAO isoform in rat (Azzaro et al., 1985). Therefore, the protective effect of rasagiline might be more pronounced in human.

In our experiments L-deprenyl and rasagiline modestly elevated the resting [3 H]DA efflux in rotenone-pretreated slices but failed to affect EFS-evoked release. Previous studies showed that MAO-B inhibitors significantly enhance striatal DA efflux only upon long-term or *in vivo* chronic application (Dluzen and McDermott, 1991; Lamensdorf et al., 1996), whereas acute treatment do not affect basal or depolarization-evoked DA efflux (Butcher et al., 1990; Lamensdorf et al., 1996). Our results corroborate with the latter observation and indicate that long-term treatment with MAO inhibitors is essential in order to detect a facilitation of depolarization-evoked DA release.

Cultured PC 12 cells, which are purely catecholaminergic, proved to be more sensitive to the action of rotenone than striatal slices, which are heterogeneous in origin. Thus, in line with other observations (Ren et al., 2005; Sai et al., 2008; Wang et al., 2008) and with the putative role of altered metabolism of DA (Dukes et al., 2005; Sai et al., 2008) rotenone elicited more than 50% reduction of mitochondrial respiration even in 2 μ M concentration in PC 12 cells. Nevertheless, the findings with antiparkinsonian drugs in PC 12 cells correlated with those obtained in striatal slices and the most convincing protective effect was obtained with rasagiline, followed by L-deprenyl and ropinirole, whereas L-DOPA worsened the survival of the cells. This order correlates with the ability of these compounds to prevent or promote the formation of DAQ and/or to decrease pathological oxidative stress induced [3 H]DA efflux in slice experiments.

In conclusion, oxidative stress induced, pathological DA release and the formation of toxic metabolites of DA are distinctly modified by the tested antiparkinsonian drugs and our data add further insight on their site of action. We suggest that future concepts of antiparkinsonian drug-design should incorporate differential effects on physiological and oxidative stress-induced, pathological DA release and the inhibition of the formation of oxidative metabolites from the released DA.

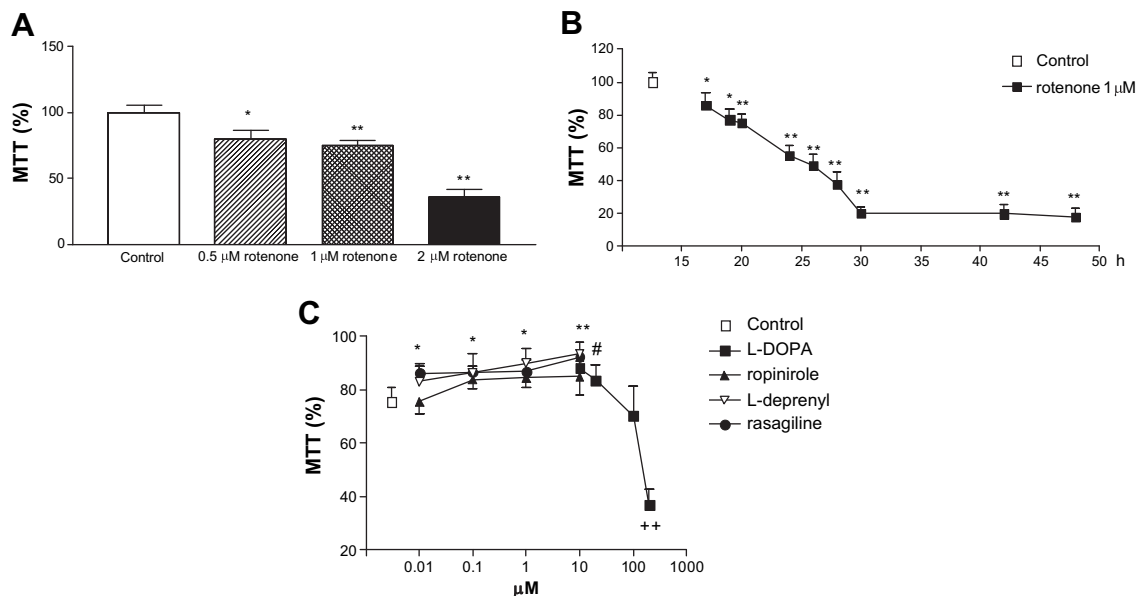


Fig. 7. Effects of L-DOPA, ropinirole, L-deprenyl and rasagiline on rotenone-induced neurotoxicity in PC12 cells. A. Concentration-dependence of the effect of rotenone. Cells were treated with rotenone in the concentrations indicated or with DMSO:PEG-300 (control) for 20 h * P < 0.05, ** P < 0.01, significance vs. controls. B. Time-dependence of the effect of rotenone. Cells were treated with rotenone (1 μ M) or with DMSO:PEG-300 (control) for various periods of time, indicated on the abscissa. * P < 0.05, ** P < 0.01, significance vs. controls. C. Cells were pretreated with the L-DOPA, ropinirole, L-deprenyl and rasagiline for 1 h, in concentrations indicated in the abscissa before treatment with 1 μ M rotenone for 20 h. Data are expressed as percent of values in DMSO:PEG-300 treated control cultures, and are means \pm S.E.M. of four experiments. #, * P < 0.05 and **, ** P < 0.01 compared to rotenone treatment using ANOVA followed by the Dunnett test.

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