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Original Contribution

Increased sensitivity of striatal dopamine release to H_2O_2 upon chronic rotenone treatment

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Abstract

It is believed that both mitochondrial dysfunction and oxidative stress play important roles in the pathogenesis of Parkinson's disease (PD). We studied the effect of chronic systemic exposure to the mitochondrial inhibitor rotenone on the uptake, content, and release of striatal neurotransmitters upon neuronal activity and oxidative stress, the latter simulated by H_2O_2 perfusion. The dopamine content in the rat striatum is decreased simultaneously with the progressive loss of tyrosine hydroxylase (TH) immunoreactivity in response to chronic intravenous rotenone infusion. However, surviving dopaminergic neurons take up and release only a slightly lower amount of dopamine (DA) in response to electrical stimulation. Striatal dopaminergic neurons showed increased susceptibility to oxidative stress by H_2O_2, responding with enhanced release of DA and with formation of an unidentified metabolite, which is most likely the toxic dopamine quinone (DAQ). In contrast, the uptake of [^{3}H]choline and the electrically induced release of acetylcholine increased, in coincidence with a decline in its D_2 receptor-mediated dopaminergic control. Thus, oxidative stress-induced dysregulation of DA release/uptake based on a mitochondrial deficit might underlie the selective vulnerability of dopaminergic transmission in PD, causing a self-amplifying production of reactive oxygen species, and thereby contributing to the progressive degeneration of dopaminergic neurons.

Keywords: Oxidative stress; Parkinson's disease; Dopamine; Striatum; Release; H_2O_2; Acetylcholine

Introduction

Chronic neurodegenerative diseases, which are characterized by a progressive loss of distinct groups of neurons, have a common pathomechanism, since oxidative damage and dysregulation of transmitter release play a central, but not initiative, role in the development of the disease [1]. Oxidative damage is caused by the overproduction of highly reactive oxygen species, which compromise cellular function on multiple target sites [2]. Oxidative stress also contributes to the dysregulation of transmitter release by increasing the extracellular level of glutamate and monoamine transmitters, which by themselves act as triggers of deleterious cellular events leading to neurodegeneration. Whereas glutamate stimulates nitric oxide (NO) production and might initiate peroxynitrite production, monoamines provide an additional source of highly reactive free radicals during their breakdown by monoamine oxidase (MAO) or autooxidation [3], whereby they could reinforce the harmful effect of oxidative stress. In our previous study we showed that H_2O_2 elicits concentration-dependent [^{3}H]noradrenaline release from hippocampal slices, and that this effect is greatly exacerbated if mitochondrial inhibitors such as

Abbreviations: ACh, acetylcholine; [^{3}H]ACh, [^{3}H]acetylcholine; ANOVA, one-way analysis of variance; DA, dopamine; DAQ, dopamine quinone; [^{3}H]DA, [^{3}H]dopamine; DMSO, dimethyl sulfoxide; DOPAC, 3,4-dihydroxyphenylacetic acid; EFS, electrical field stimulation; HVA, homovanillic acid; HPLC, high performance liquid chromatography; L-DOPA, L-(3,4-dihydroxyphenyl)alanine; 6-OHDA, 6-hydroxydopamine; MAO, monoamine oxidase; NA, noradrenaline; PD, Parkinson's disease; PB, phosphate buffer; PEG, polyethylene glycol; PCA, perchloric acid; ROS, reactive oxygen species; TH, tyrosine hydroxylase.

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rotenone or oligomycin are applied at the same time [4]. In line with this observation, dopamine-mediated neurotoxicity in striatal neurons is markedly enhanced if it is combined with minimal mitochondrial inhibition [5].

The major feature of PD is a relatively selective degeneration of the nigrostriatal dopaminergic pathway, leading to progressive motor dysfunction. According to the current hypothesis, it is a multifactorial disease, and both genetic and environmental factors play a role in its aetiology. Among environmental factors, the crucial importance of exposure to pesticides, such as the selective mitochondrial complex I inhibitor rotenone, is emerging. The occurrence of PD in the rural population involved in gardening and agriculture is higher [6,7]. As well, PD patients show a systemic deficit in mitochondrial complex I function [8], which is not restricted to brain dopaminergic neurons but is also manifested in other cells, such as platelets and muscle cells. However, the mechanism whereby systemic complex I dysfunction leads to selective dopaminergic neurotoxicity is unknown.

Recently, Betarbet and co-workers introduced a new model of PD based on subchronic, continuous infusion of rotenone. It reproduced the following features of PD in rats: (1) systemic complex I deficit, (2) motor and behavioural symptoms, (3) progressive degeneration of SN dopaminergic neurones, and (4) formation of ubiquitin and α-synuclein-positive cytoplasmic inclusions in neurons [9]. Therefore, the chronic rotenone-induced Parkinson model may represent an animal model, which more adequately mimics the pathogenesis and progress of the disease than previously used models [10]. Nevertheless, it is not yet determined how the neurochemical parameters of striatal dopaminergic and nondopaminergic transmission are altered in the rotenone-induced Parkinson model. It was reported that acute administration of rotenone increases DA turnover [11], whereas subchronic intermittent treatment (by ip injection) only moderately lowers DA content in the striatum [12,13]. However, these treatments might not reflect the changes with continuous exposure to a moderate dose of rotenone, which probably more adequately mirrors the environmental toxicity. Moreover, it is not known how the presynaptic function of dopaminergic neurons, i.e., the release of DA, is changed, and how vulnerable dopaminergic neurons are to oxidative stress in terms of their ability to release. This latter aspect seems to have particular interest because H_2O_2 releases a higher amount of DA from striatum than noradrenaline (NA) from the cortex [14]. Furthermore, DA is more harmful than other physiologically relevant monoamines [3,15], because its metabolism by MAO produces more H_2O_2 and could also give rise to the formation of toxic quinones and semiquinones [16].

Therefore, this study was designed to explore how striatal neurotransmission is altered in response to conventional neuronal activity and oxidative stress in the rotenone model of PD.

Materials and methods

Implantation of osmotic pumps and animal care

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.

We used male Sprague-Dawley rats (280–320 g). Alzet osmotic minipumps (Alzet Corporation, Palo Alto, CA) were filled with rotenone dissolved in equal volumes of dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG-300) or with the solvent alone. Pumps were kept in sterile 0.9% saline at 37°C overnight before the operation. Ketamine (75 mg/kg), rompum (10 mg/kg), and xylazine (10 mg/kg) were injected intramuscularly as anesthetics. Pumps were implanted under the skin on the back of each animal and were attached to the right jugular vein by a catheter. Control rats received DMSO:PEG (1:1) only. The treated rats received 2–3 mg of rotenone/kg per day (calculation based on weight at the time of surgery). Following surgery, rats were monitored for behavior, weight, and overall health. When rotenone-treated rats showed weight loss, their diet was supplemented with oral administration of Nutrical (Evsco Pharmaceuticals, Buena, NJ). Subcutaneous lactated Ringer's solution (Rindex 5; Reanal, Hungary) injection was given when rats showed signs of dehydration.

Immunohistochemistry

Control, sham-operated, and rotenone-treated animals were decapitated after a short ether stress and the brain was quickly removed. The upper part of the striatum with adjacent cortex was immersed in 4% paraformaldehyde, 0.5% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4, for 6 h. The fixative was changed every half hour. The tissue block was rinsed and washed in 0.1 M PB overnight at 4°C. Forty-micrometer sections were cut by a vibratome in the same buffer. Sections were immersed in 0.05 % Triton X-100 in 0.1 M PB for 1 h at room temperature and, after several washing steps in PB, were blocked for endogenous peroxidase activity with 3 % H_2O_2/PB for 15 min. This was followed by incubation in blocking buffer (5% normal horse serum in PB) with gentle agitation at room temperature for 1 h. Floating sections were then incubated in 1:1000 dilution of the TH antibody (Sigma) overnight at 4°C. One set of sections was incubated in the absence of the antibody as a control. After removal of the solution of TH antibody and several washing steps, sections were incubated at room temperature for 1 h with biotinylated anti-mouse IgG (Vector Laboratories) at a 1:200 dilution. ABC reaction and detection of antigen–antibody complexes were performed according to the Vector Laboratories (Burlingame,

CA) prescription. Stained sections were washed in water, dehydrated in xylene, and mounted in Canada balsam. Staining was investigated with an Olympus CH30 microscope at 10–40 magnifications. Pictures were taken by Olympus digital camera.

Release experiments

Rats were decapitated after a short ether stress and the brain was quickly removed. In some experiments a full cross section of the brain including the striatum and cortex was cut for immunohistochemistry. Then the striatum was dissected out and sliced into 400-μm-thick sections with a McIlwain tissue chopper. Before the slicing, a small piece of striatum was immersed in liquid nitrogen for measurement of DA content. All manipulations were performed on ice. In one set of experiments slices were divided in two and experiments were performed simultaneously on [^{3}H]dopamine ([^{3}H]DA) and [^{3}H]acetylcholine ([^{3}H]ACh) release.

[^{3}H]Dopamine release

Striatal slices were incubated in 2 ml of Krebs solution containing [^{3}H]DA at a concentration of 5 μCi/ml for 45 min and were continuously gassed with a mixture of 95% O_2 and 5% CO_2 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 time to wash out excess radioactivity and to allow tissue equilibration, 3-min samples were collected and assayed for [^{3}H]DA. The samples for HPLC analysis of [^{3}H]metabolites were acidified by 15 μl of 1.9 M perchloric acid (PCA) during the collection.

[^{3}H]Acetylcholine release

Striatal slices were loaded with [*methyl*-^{3}H]choline chloride at a concentration of 4 μCi/ml for 60 min at 37°C. After incubation they were washed with Krebs solution containing 10 μM hemicholinium-3, transferred into a microvolume perfusion system, and superfused at a rate of 0.5 ml/min. After a 60-min preperfusion the effluent was collected in 3-min samples.

Electrical field stimulation (EFS) (20 V, 2 Hz, 1 ms, 240 shocks) was applied during the 3rd (S_1) and the 13th (S_2) fractions. The D_2-receptor antagonist (-)sulpiride was added to the perfusion fluid 20 min before S_2. Its effect on acetylcholine release was characterized by the fractional ratio FRS_2/FRS_1, where FRS_2 and FRS_1 are the amounts of radioactivity released in response to the second and first stimulations, respectively. Oxidative stress was mimicked by addition of H_2O_2 to the Krebs solution starting at the 3rd fraction. In this case no EFS was applied. At the end of the experiment slices were taken off the chambers and 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. In a separate set of experiments slices from untreated rats were kept for 2 h in Krebs solution containing 5 μM rotenone, rinsed with normal Krebs solution, and then loaded with the isotopes.

HPLC determination of dopamine content and [^{3}H] metabolites: Sample preparation

After the preparation of striatum the native tissue was frozen by liquid nitrogen. The weighed frozen tissue was homogenized in an appropriate volume of ice-cold 0.1 mol/L PCA, which contained 10^{-6} mol/L dihydroxybenzylamine (DHBA) as an internal standard. The suspension was centrifuged at 4500 rpm for 10 min at 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. [17].

The perfusion fluid was centrifuged at 300*g* (4500 rpm) for 10 min at 0–4°C; the supernatant was kept at −20°C until analysis. For the analysis 545-μl sample volumes were acidified with 5 μl of 0.1 mol/L PCA, which contained 10^{-5} mol/L DHBA as an internal standard.

The separation was carried out in a Gilson liquid chromatographic system with 715-operation software (Gilson Medical Electronics Inc., Middletown, WI) containing two delivery pumps (Models 305 and 306), and a programmable autoinjector (Model 231-401) with a "trap" column (15–25 μm Nucleosil C-18 (20 × 4.0)), which was connected as a loop. The effluent was monitored with a BAS 400 (BAS Inc., West Lafayette, IN) electrochemical detector. The electrochemical signal was measured by a thin-layer glassy carbon electrode versus Ag/AgCl reference electrode, and the working electrode was maintained at 0.75 V by an Eltron (Budapest, Hungary) potentiostat. The radioactivity of the effluent (1-min collected sample) was determined by liquid scintillation counting. Separation of catecholamines was performed on a 5-μm Discovery C-18 (150 × 4.0 mm) analytical column with a step-gradient working mode at room temperature. The mobile phase "A" was a 0.075 mol/L potassium phosphate buffer, pH 3.45, and contained 0.25 mmol/L EDTA disodium salt. The "B" component of the mobile phase was 0.075 mol/L buffer with 0.25 mmol/L EDTA disodium salt, 5.75 mmol/L octane sulfonic acid sodium salt, as an ion pairing component, and 12 % (v/v) acetonitrile:methanol (3.5:1.0). Buffer "A" was used during the first 10 min of analysis at a 0.6 ml/min flow rate. Then buffer "B" was applied at a 1.0 ml/min flow rate until the end of the analysis. The identification of the tritium-labeled compounds was based upon the known retention times of unlabeled standards of DA (24.5 min), its metabolites, i.e.,

3,4-dihydroxyphenylacetic acid (DOPAC, 16.7 min) and homovanillic acid (HVA, 22.5 min), as well as that of NA (14.4 min). In addition, a new component was detected in the effluent derived from chronically rotenone-treated rats with a retention time of 19 min. We applied the method of oxidative electron transfer of DA by Nappi and Vass [18] using the reductive (-0.1 V polarization potential) detection technique to identify this compound as DAQ by HPLC-EC: chromatograms were obtained by injecting 500-µl samples of reaction mixtures (25 mM of DA in bicarbonate buffer with pH 7.2 after 8 (Model 1) and 55 min (Model 2) of reaction time or 50 mM of DA in water with pH 7.1 at room temperature after a 24-h incubation time (Model 3) giving rise to the generation of two oxidized metabolites of DA during autooxidation at pH 7.2; dopaminechrome with 12.8 min retention time and DAQ with 18.7 min retention time, the latter being identical to the retention time of the unknown compound detected in the tissue effluent. The separation conditions of model solutions were identical to the sample analysis at reductive working electrode potential: -100 mV.

Drugs

The following materials were used: [^{3}H]dopamine (specific activity 52 Ci/mmol) and [*methyl*-^{3}H]choline chloride (specific activity 82 Ci/mmol, both from Amersham, Little Chalfont, UK), H_2O_2 (Reanal, Budapest, Hungary), rotenone, (-)sulpiride, DMSO, and PEG-300 (Sigma, St. Louis, MO).

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.

Data analysis

All data were expressed as means ± SE of *n* observations. The statistical analysis was made by one-way analysis of variance (ANOVA) followed by the Bonferroni test (multiple comparisons), or Student's *t* test (pairwise comparisons). *P* values of less than 0.05 were considered statistically significant.

Results

TH immunohistochemistry

Whereas no difference was observed in TH immunoreactivity in the striatum between sham-operated and untreated rats (Figs. 1A and B), the staining intensity was progressively decreased after 2 (Fig. 1C) and 4 weeks (Fig. 1D) of chronic iv rotenone (2– 3 mg/kg per day) infusion, indicating the loss of dopamine-synthesizing

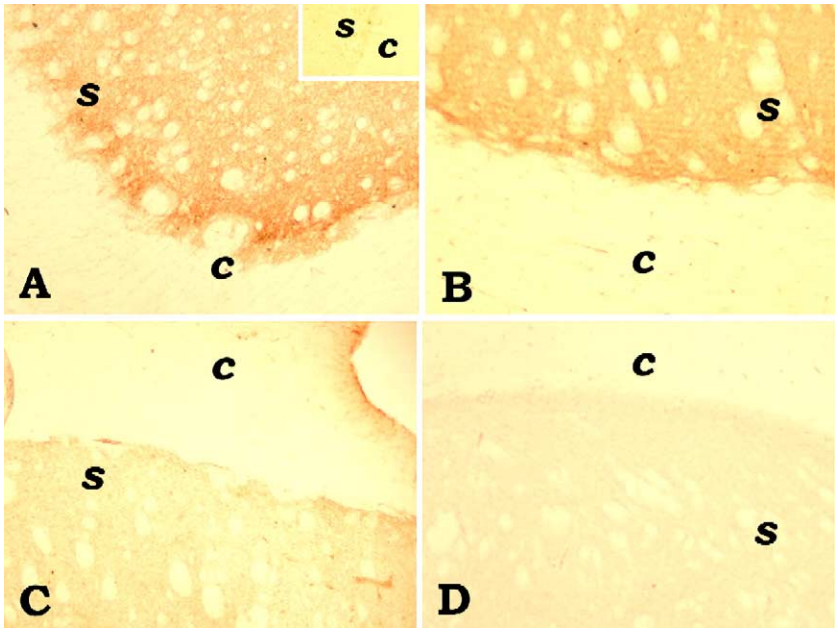

Fig. 1. Immunostaining for TH in the brain section of Sprague-Dawley rats. Staining was performed on 40-µm vibratome sections of the brain tissue blocks containing the upper part of the striatum with adjacent cortex. Fixation was done by immersion (4% paraformaldehyde, 0.5% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4). Dilution of applied TH antibody (Sigma) was 1:1000. Detection of antigen–antibody complexes was performed by DAB as chromogen. Original magnification: 10×. S, striatum; c, cortex. (A) Brain section of nontreated animal. Strong immunoreactivity for TH in the striatum. (Insert) In the absence of the antibody, there is no staining. (B) Rat was treated with the vehicle of rotenone. Immunoreactivity for TH is similar to the staining found in the control animal. (C) Short-term (2 weeks) rotenone treatment. Staining for TH is significantly weaker in the striatum than in control. (D) After long-term (4 weeks) treatment with rotenone, immunoreactivity for TH is only in traces.

capacity of dopaminergic nerve terminals. These observations are in good agreement with the findings of a previous study using the identical treatment regime of rotenone [9] and indicate that chronic exposure to rotenone elicits progressive degeneration of dopaminergic terminals in the striatum.

Dopamine content and [^{3}H]dopamine release

The endogenous DA content in the striatum of sham-operated, control rats was similar to the content in untreated rats (613.9 ± 50.5 pmol/mg protein, $n = 4$ versus 606.3 ± 71.5 pmol/mg protein, $n = 6$, $P>0.05$, respectively). The DA content of rotenone-treated rats gradually decreased during the period of chronic infusion (Fig. 2). The amount of DOPAC and HVA, metabolites of DA, on the other hand, showed a tendency toward gradual increase (Fig. 2), and the ratios of DOPAC/DA and HVA/DA significantly increased with the time of rotenone infusion (Table 1), showing an increase of metabolic activity and turnover in the rotenone-treated rats.

[^{3}H]DA release was measured under basal conditions and in response to electrical stimulation in striatal slices from control, short-term (2 weeks) and long-term (4 weeks) rotenone-treated rats, and also from slices preincubated in vitro with 5 μM rotenone. Whereas the resting release of [^{3}H]DA did not change between untreated rats and those that received chronic treatment, the uptake of tritium showed a tendency toward decrease in parallel with the length of the rotenone treatment, which become significant in the long-treated group (Table 2). The [^{3}H]uptake was the lowest when the slices were preincubated in vitro for 2 h with 5 μM rotenone (Table 2), and as a consequence of this change the resting release was higher in this group. Similarly, the electrical field stimulation-evoked release of [^{3}H]DA, which showed a tendency toward decrease upon rotenone treatment, was the lowest in this group (Table 2). On the other hand, when electrical stimulation-evoked release was expressed as a percentage of the actual tritium content there was no obvious difference between the experimental groups, indicating that those terminals, in which the DA uptake system was intact, preserve their ability to release DA upon neuronal activity (Fig. 3). Supporting this idea, the FRS_2/FRS_1 ratio was also similar in all four experimental groups (Table 2).

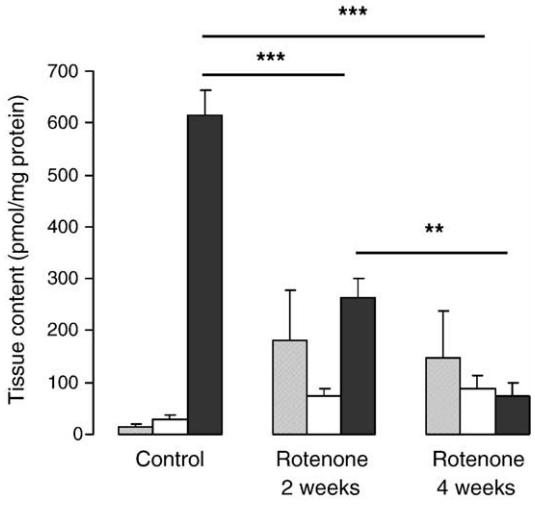

Fig. 2. Intravenous infusion of rotenone by implanted Alzet minipump decreases striatal content of DA. Gray bars show the content of DOPAC; open bars, HVA; and filled bars, DA. Control rats received only DMSO:PEG (1:1) for 4 weeks (control, $n = 6$). The treated rats received 2–3 mg of rotenone /kg per day (calculation based on weight at the time of surgery) for 2 weeks (rotenone 2 weeks, $n = 7$) or for 4 weeks (rotenone 4 weeks, $n = 7$). Results are expressed as pmol/mg protein. Means ± SE of n observations are presented. Significance: $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1
Changes in dopamine metabolite ratios corresponding to the time of rotenone infusion

Ratio	Experimental group		
	Control	Rotenone 2 weeks	Rotenone 4 weeks
DOPAC/DA	0.02 ± 0.004 (6)	1.21 ± 0.71 (7)	3.04 ± 1.17* (7)
HVA/DA	0.05 ± 0.02 (6)	0.35 ± 0.1 (7)	4.19 ± 2.19 *** (7)

Control, continuous intravenous infusion of DMSO:PEG (1:1) for 4 weeks; rotenone 2 weeks, continuous intravenous infusion of 2–3 mg rotenone /kg per day for 2 weeks; rotenone 4 weeks, continuous intravenous infusion of 2–3 mg rotenone /kg per day for 4 weeks. Means ± SE of n observations are presented. The number of experiments is given in parenthesis. Significance versus the control: $^{*}P < 0.05$, $^{***}P < 0.001$.

H_2O_2 (0.1–0.25 mM) elicited dose-dependent elevation of [^{3}H]DA release (Fig. 4). The effect of oxidative stress was significantly enhanced in rats chronically treated with rotenone as compared with the sham-operated controls. The effect of H_2O_2 was proportionate to the dose of rotenone and the length of treatment. When H_2O_2 (0.1 mM) was applied to in vitro rotenone (5 μM)-pretreated slices, the maximum increase of resting release was comparable to the maximum effect of 0.25 mM H_2O_2 obtained in slices from rats chronically treated for 4 weeks (Fig. 4).

HPLC analysis of tritiated metabolites demonstrated the dynamics of the changes in their composition under resting conditions (Fig. 5A) and in response to 0.25 mM H_2O_2 application for 15 min (corresponding to the maximum release) (Fig. 5B) or for 30 min (Fig. 5C). Under normal conditions, the effluent contained tritiated DA, DOPAC, HVA, and NA. The proportion of DA was higher, whereas that of DOPAC was lower, in the effluent collected during H_2O_2 treatment, but there was no significant change in the composition of these metabolites in response to chronic rotenone treatment. Interestingly, however, an unidentified peak appeared in the effluent, under conditions of oxidative stress, but only in the chronically treated rats, which is most likely dopamine quinone (DAQ) (Figs. 5B and C).

[^{3}H]Acetylcholine release

In a set of experiments striatal slices from control and chronically treated (4 weeks) rats were divided into two parts to measure [^{3}H]ACh and [^{3}H]DA release simulta-

Table 2
Changes in the parameters of [^{3}H]DA release depending on the time of treatment and concentration of rotenone

Experimental group	[^{3}H] uptake (Bq $\times 10^4$/g)	Resting release (%)	Evoked release (Bq/g)	FRS_2/FRS_1
Control	121 ± 6.9 (26)	0.63 ± 0.02 (52)	21108 ± 2101 (9)	0.74 ± 0.06 (9)
Rotenone 2 weeks	109 ± 8.4 (28)	0.68 ± 0.01* (60)	11603 ± 2338* (12)	0.70 ± 0.03 (12)
Rotenone 4 weeks	101 ± 3.8* (39)	0.65 ± 0.01 (64)	15282 ± 1685 (15)	0.75 ± 0.04 (15)
In vitro incubated slice	51.4 ± 4.9** (18)	1.31 ± 0.04** (32)	8066 ± 1961** (5)	0.67 ± 0.06 (5)

Control, continuous intravenous infusion of DMSO:PEG (1:1) for 4 weeks; rotenone 2 weeks, continuous intravenous infusion of 2–3 mg rotenone /kg per day for 2 weeks; rotenone 4 weeks, continuous intravenous infusion of 2–3 mg rotenone /kg per day for 4 weeks; in vitro incubated slice, slices were kept in Krebs containing 5 μM rotenone for 2 h at 37°C and continuous oxygenation, before incubation with the isotope. EFS (2 Hz, 1 ms, 20 V, 240 shocks) was applied during the 3rd (S_1) and the 13th (S_2) fractions. Means ± SE of n observations are presented. The number of experiments is given in parenthesis. Significance versus control: *$P < 0.05$, **$P < 0.01$.

neously. The uptake of tritiated choline and the release of radioactivity in response to EFS (S_1) were significantly enhanced in rotenone-treated rats (Fig. 6).

The effectiveness of dopaminergic control on EFS-evoked ACh efflux was tested by application of the D_2-receptor antagonist (-)sulpiride (10 μM) before the second stimulation. In both control and rotenone-treated rats sulpiride enhanced the evoked [^{3}H]ACh release (Fig. 7). However, the ratio S_2/S_1 was decreased in rotenone-treated rats (2.47 ± 0.39; $n = 4$ vs 1.68 ± 0.11; $n = 9$, $P < 0.05$), suggesting a lower efficiency of dopaminergic inhibition in chronically treated animals. The effect of sulpiride was further attenuated in slices pretreated in vitro with rotenone (5 μM) for 2 h (Fig. 7), resulting in an S_2/S_1 ratio of 0.98 ± 0.125, $n = 8$. The resting [^{3}H]ACh release was also higher in pretreated slices compared to chronically treated rats (Fig. 7).

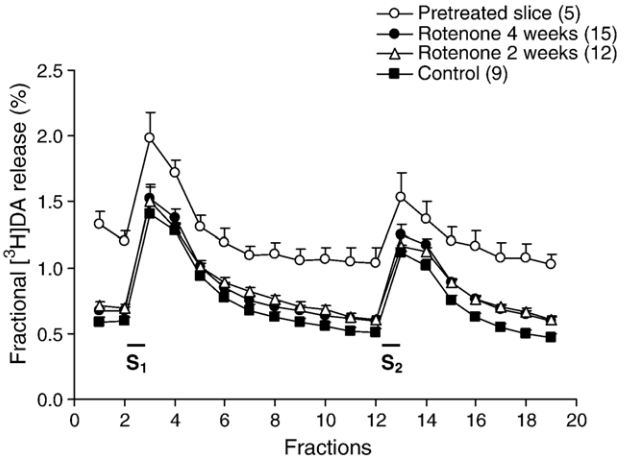

Fig. 3. Fractional [^{3}H]DA release from striatal slices of rats that had undergone different treatments: Control (squares), continuous intravenous infusion of DMSO:PEG (1:1) for 4 weeks, $n = 9$; rotenone 2 weeks (triangles), continuous intravenous infusion of 2–3 mg rotenone /kg per day for 2 weeks, $n = 12$; rotenone 4 weeks (filled circles), continuous intravenous infusion of 2–3 mg rotenone /kg per day for 4 weeks, $n = 15$; pretreated slice (open circles), slices were kept in Krebs solution containing 5 μM rotenone for 2 h at 37°C and continuous oxygenation, before incubation with the isotope, $n = 5$. EFS (2 Hz, 1 ms, 20 V, 240 shocks) was applied during the 3rd (S_1) and the 13th (S_2) fractions. Results are expressed as the percentage of the actual tritium content in the slices (fractional release). Means ± SE. of n observations are presented.

Discussion

The recently introduced Parkinson model achieved by chronic, intravenous [9], or subcutaneous [19] infusion of rotenone was reported to reproduce the major pathological and behavioral hallmarks of the disease: selective degeneration of substantia nigra dopaminergic neurons, appearance of cytoplasmic inclusions reminiscent of Lewy bodies in degenerating neurons, and motor and postural deficits. Furthermore, dopaminergic brain regions that had undergone such treatment exhibit oxidative damage [20] and intensive microglial activation [21], substantiating the common pathomechanism of rotenone-induced toxicity and PD. Conversely, other recent studies found that the neurodegeneration caused by continuous rotenone infusion is not restricted to dopaminergic neurones, and a serious systemic toxicity also appears, which is not characteristic of the disease [22,23]. Moreover, other studies [10,24] highlighted the high interindividual variability in the effectiveness of rotenone to cause nigrostriatal degeneration in rats, questioning the usefulness of this model in the assessment of potential drugs against PD. Indeed, continuous rotenone infusion in our experiments also elicited weight loss and signs of severe systemic toxicity, which could be, however, at least partly counterbalanced by alert monitoring of the weight loss and feeding the experimental animals with high energy, palatable nutrients and vitamins. Clearly, 28-day treatment with a relatively low, but toxic, dose of rotenone is still not enough to precisely follow the progress of the human disease, which presumably develops slowly after years or decades of continuous or intermittent exposure to subtoxic doses of environmental poisons. Further refinement is necessary in the treatment regime of this model. On the other hand, one should also consider that although the toxic systemic effect of rotenone might be averted by direct central administration, this route of administration hardly imitates the environmental pesticide exposure presumed in the human disease.

We also observed a rather high variance in the susceptibility of rats to systemic toxicity and in the degree of severity of striatal dopaminergic degeneration between individual animals, even if the animals that did not exactly meet the experimental conditions were excluded from the analysis [22]. However, this might mirror the different

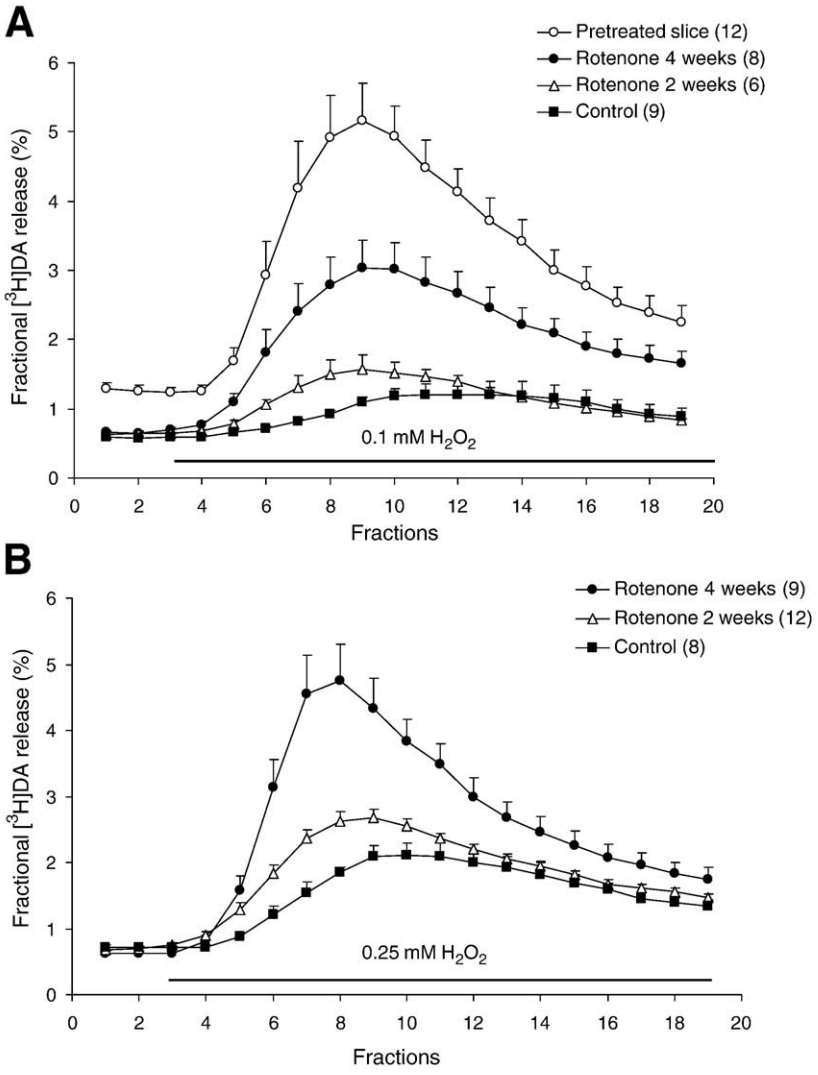

Fig. 4. Rotenone treatment increases the sensitivity of dopaminergic neurotransmission in rat striatum to oxidative stress. (A) H_2O_2 0.1 mM was added to the perfusion fluid at the 3rd fraction; (B) H_2O_2 0.25 mM. Control (squares), infusion of DMSO:PEG (1:1) for 4 weeks; rotenone 2 weeks (triangles), infusion of 2–3 mg rotenone /kg per day for 2 weeks; rotenone 4 weeks (filled circles), infusion of 2–3 mg rotenone /kg per day for 4 weeks; pretreated slice (open circles), slices, kept in Krebs solution containing 5 μM rotenone for 2 h at 37°C and continuous oxygenation, before incubation with the isotope. Results are expressed as the percentage of the actual tritium content in the slices (fractional release). Means ± SE of *n* observations are presented. The number of experiments is indicated in the legend.

genetic predisposition of individuals to rotenone toxicity, which could again be valid also in the human population. As for the neurochemical correlates of the disease, in our experiments chronic rotenone treatment reproduced the changes that are expected in the human disease, as striatal DA content was significantly decreased, correlating with the length of the treatment and the development of motor deficits in animals. The change in DA content after 28 days (more than 80%) even surpasses the level of reduction in the recent observations of Alam and Schmidt [13], who found approx 50% reduction of endogenous DA content after a chronic 2.5 mg/kg per day ip treatment of rotenone. Notably, in this latter study rotenone-induced behavioral changes could be normalized by combined benserazide and L-(3,4-dihydroxyphenyl)alanine (L-DOPA) administration, indicating that the effect of rotenone on the motor system is indeed related to the malfunction of dopaminergic neurons. However, we could observe only a moderate decline in the uptake of [^{3}H]DA into striatal slices even after long-term treatment, whereas an acute, direct preincubation of slices with a higher dose of rotenone elicited a more profound decrease. Taken into account that striatal endogenous DA content and TH immunoreactivity are substantially reduced, our findings could be explained by the hyperfunction of surviving dopaminergic terminals, and increased DA turnover. This assumption is supported by the increased DOPAC/DA and HVA/DA ratios found in our experiments and is in line with the data of Thiffault et al. [11]. They also found increased DA turnover after acute treatment with a moderate dose of rotenone. One should consider here that

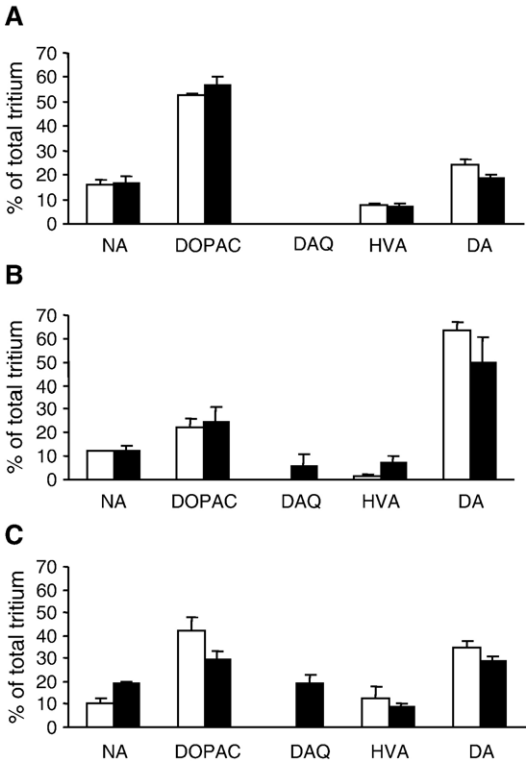

Fig. 5. Distribution of tritiated DA metabolites under resting conditions (A) and after application of 0.25 mM H_2O_2 for 15 min (B) or for 30 min (C) in release experiments from striatal slices of rats that had undergone different chronic treatment. Note that in slices treated with H_2O_2, an unidentified peak appeared in the effluent, which is most likely DAQ, as tentatively identified by the method of Nappi and Vass [18]. Open columns, control rats, i.e., infusion of DMSO:PEG (1:1) for 4 weeks, n = 4; filled columns, infusion of 2–3 mg rotenone /kg per day for 4 weeks, n = 5. Results are expressed as the percentage of total tritium content in the effluent. Means ± SE of n observations are presented. NA, noradrenaline; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DAQ, dopamine quinone; DA, dopamine.

rotenone treatment applied in our study does not cause an immediate and complete degeneration of striatal dopaminergic terminals like, e.g., 6-hydroxydopamine (6-OHDA) pretreatment, and [^{3}H]DA can be taken up by dopaminergic terminals even if its own DA-synthesizing machinery is already destroyed. Consequently, functional dopaminergic terminals released only a slightly lower amount of [^{3}H]DA during neuronal activity in response to field electrical depolarization.

Another matter of recent debate is whether neurodegeneration induced by chronic systemic treatment with rotenone selectively targets the brain dopaminergic system or represents a more extended widespread neurodegeneration, in which other neurons such as serotonergic neurons or cholinergic interneurons are also damaged. The latter scenario could be implicated in atypical parkinsonian syndrome, rather then idiopathic sporadic PD [21], albeit the late clinical stages of PD are also characterized by extended nondopaminergic lesions [10]. In our experiments cholinergic neurons did not show any sign of hypofunction, at least in functional terms, although we used a different rat strain (Sprague-Dawley but not Lewis rats) from the study of Hoglinger et al. [22]. Instead, the uptake of [^{3}H]choline into striatal slices and the amount of electrical stimulation-induced [^{3}H]ACh release were markedly enhanced. These changes might be due to the high sensitivity of cholinergic neurons to the denervation of dopaminergic neurons and the loss of the D_2-receptor-mediated modulation of striatal cholinergic transmission by endogenous dopamine. In line with this assumption, the facilitatory effect of the D_2 receptor antagonist sulpiride was attenuated in parallel with the rotenone treatment, and completely abolished in in vitro-treated slices, representing the most deleterious treatment on dopaminergic neurons. Taken together, our data indicate that rotenone elicits serious dysregulation of neurotransmitter release with hypofunction of dopaminergic neurons and consequent hyperfunction of cholinergic interneurons, rather than nonselective, extended impairment of neurotransmission.

The most impressing finding of the present study from a pathophysiological point of view is that H_2O_2 exerted a greatly exacerbated effect on the release of DA in chronic rotenone-treated rats as compared to the sham-operated controls and this effect is positively correlated to the dose of rotenone and also the length of treatment. This finding is concordant with our previous observations that rotenone and H_2O_2 have a supraadditive impact on the release of monoamine transmitters and that even a minimal mitochondrial deficit could predispose neurons to the harmful effect of subsequent oxidative stress [4]. Since both mitochondrial inhibitors and H_2O_2 cause energy deficit, it can be assumed that vesicular DA storage is also compromised and DA accumulates in the cytoplasm, from which it could flow out by diffusion or by the reversal of the DA transporters. The

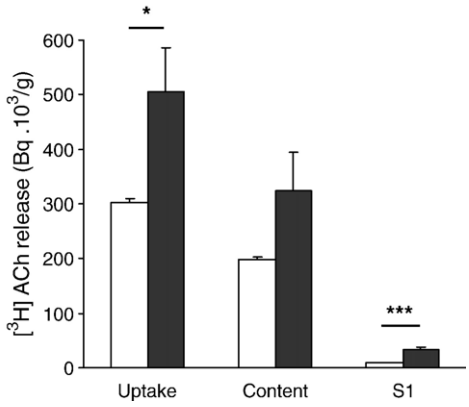

Fig. 6. Effect of chronic rotenone treatment on the parameters of [^{3}H]ACh release from rat striatal slices. Open columns, infusion of DMSO:PEG (1:1) for 4 weeks, n = 5; filled columns, infusion of 2–3 mg rotenone /kg per day for 4 weeks, n = 9. Uptake, the sum of release + the radioactivity remaining in the slices at the end of perfusion; content, the radioactivity remaining in the slices at the end of perfusion; S1, the amount of radioactivity released in response to EFS (2 Hz, 1 ms, 20 V, 240 shocks) applied during the 3rd fraction. Results are expressed as Bq $\times 10^3$/g. Means ± SE of n observations are presented. Significance: $^*P < 0.05$, $^{***}P < 0.001$.

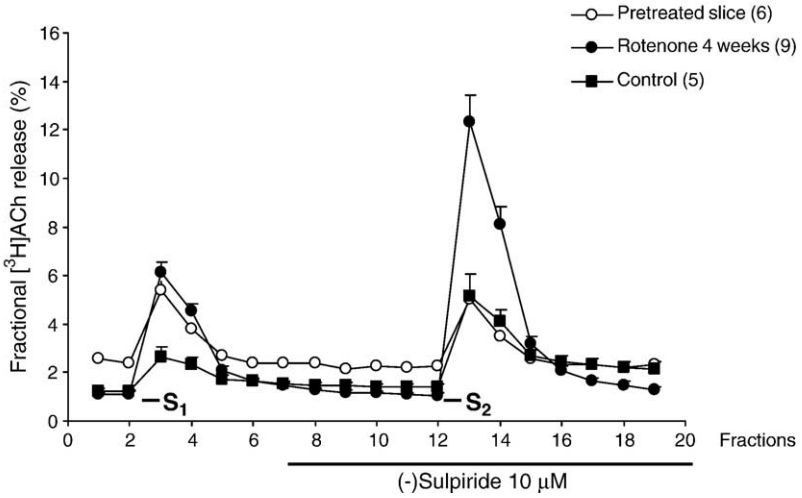

Fig. 7. Effect of the D_2-receptor antagonist (-)sulpiride (10 μM) on evoked [^{3}H]ACh release from striatal slices of rats that had undergone different treatments: Control (squares), infusion of DMSO:PEG (1:1) for 4 weeks; rotenone 4 week (filled circles), infusion of 2–3 mg rotenone /kg per day for 4 weeks; pretreated slice (open circles), slices kept in Krebs solution containing 5 μM rotenone for 2 h at 37°C and continuous oxygenation, before incubation with the isotope. EFS (2 Hz, 1 ms, 20 V, 240 shocks) was applied during the 3rd (S_1) and the 13th (S_2) fractions. Sulpiride was added 20 min before the second stimulation, as indicated by the horizontal bar. Results are expressed as the percentage of the actual tritium content in the slices (fractional release). Means ± SE of *n* observations are presented. The number of experiments is indicated in the legend.

cytoplasmic accumulation of DA could also give rise to the formation of toxic products, which could reinforce the effect of the combined challenge of mitochondrial dysfunction and oxidative stress in dopaminergic neurons. This may cause relatively selective dopaminergic neurodegeneration, as indicated by the progressive decrease in striatal TH immunoreactivity and endogenous DA content. Such a concept is supported by the results of the HPLC analysis showing that the composition of tritium in the effluent after H_2O_2 perfusion was different in sham-operated controls and rotenone-treated rats, and that in the latter an unidentified peak appeared in the effluent, which is most likely DAQ. Dopamine quinones have been demonstrated to be formed in the presence of H_2O_2 and to be toxic to dopaminergic neurons [16]. In addition, peroxynitrite, which is generated in excess after identical rotenone treatment in striatal NADPH diaphorase-containing neurons [25], may also directly oxidize DA [26]. The central role of DA in rotenone-induced neurodegeneration is underlined by the finding that the blockade of its synthesis by α-methylparatyrosine protects against rotenone-induced neuronal death of dopaminergic cells in a primary mixed mesencephalic culture [27]. The question arises what is the functional consequence of increased release of DA and its metabolites in response to oxidative challenge. Whereas the toxic effect of intracellular DA is limited to the neuron where it is generated, extracellular DA and its metabolites could damage adjacent or even remote terminals and contribute to the spread of the degeneration along the dopaminergic pathway. This assumption serves as an explanation of why a higher percentage of striatal dopaminergic fibers than SN dopaminergic neurons degenerate after a given rotenone treatment [22] and why the loss of striatal DA is generally greater than the percentage loss of dopaminergic neurons [28,29] in PD. Moreover, this non-synaptic DA release could serve as a source of further self-amplifying ROS production imposing an extra oxidative burden to dopaminergic neurons [30] and sensitizing them to a relatively mild mitochondrial deficit caused by continuous subtoxic rotenone exposure. Nevertheless, alternative ROS-producing pathways not selective to the dopaminergic neurons, such as direct mitochondrial or microglial superoxide production, may also contribute to the oxidative damage of dopaminergic and nondopaminergic terminals after rotenone treatment [31,32]. Increased extracellular DA, on the other hand, could compensate for the function of degenerating neurons and thereby contribute to the late onset of clinical symptoms, which is a major factor limiting the effectiveness of current treatment used in clinical practice.

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References

[1] Ischiropoulos, H.; Beckman, J. S. Oxidative stress and nitration in neurodegeneration: cause, effect, or association? *J. Clin. Invest.* **111:**163–169; 2003.

[2] Halliwell, B. Reactive oxygen species and the central nervous system. *J. Neurochem.* **59:**1609–1623; 1992.

[3] Cadet, J. L.; Brannock, C. Free radicals and the pathobiology of brain dopamine systems. *Neurochem. Int.* **32:**117–131; 1998.

[4] Milusheva, E.; Sperlagh, B.; Shikova, L.; Baranyi, M.; Tretter, L.; Adam-Vizi, V.; Vizi, E. S. Non-synaptic release of [^{3}H]noradrenaline in response to oxidative stress combined with mitochondrial dysfunction in rat hippocampal slices. *Neuroscience* **120:**771–781; 2003.

[5] McLaughlin, B. A.; Nelson, D.; Erecinska, M.; Chesselet, M. F. Toxicity of dopamine to striatal neurons in vitro and potentiation of cell death by a mitochondrial inhibitor. *J. Neurochem.* **70:**2406–2415; 1998.

[6] Gorell, J. M.; Johnson, C. C.; Rybicki, B. A.; Peterson, E. I.; Richardson, R. J. The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurology* **50:**1346–1350; 1998.

[7] Menegon, A.; Board, P. G.; Blackburn, A. C.; Mellick, G. D.; Le Couteur, D. G. Parkinson's disease, pesticides, and glutathione transferase polymorphisms. *Lancet* **352:**1344–1346; 1998.

[8] Schapira, A. H.; Cooper, J. M.; Dexter, D.; Jenner, P.; Clark, J. B.; Marsden, C. D. Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* **1:**1269; 1989.

[9] Betarbet, R.; Sherer, T. B.; MacKenzie, G.; Garcia-Osuna, M.; Panov, A. V.; Greenamyre, J. T. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.* **3:**1301–1306; 2000.

[10] Hirsch, E. C.; Hoglinger, G.; Rousselet, E.; Breidert, T.; Parain, K.; Feger, J.; Ruberg, M.; Prigent, A.; Cohen-Salmon, C.; Launay, J. M. Animal models of Parkinson's disease in rodents induced by toxins: an update. *J. Neural Transm. Suppl.* **65:**89–100; 2003.

[11] Thiffault, C.; Langston, J. W.; Di Monte, D. A. Increased striatal dopamine turnover following acute administration of rotenone to mice. *Brain Res.* **885:**283–288; 2000.

[12] Alam, M.; Schmidt, W. J. Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. *Behav. Brain Res.* **136:**317–324; 2002.

[13] Alam, M.; Schmidt, W. J. L-DOPA reverses the hypokinetic behaviour and rigidity in rotenone treated rats. *Behav. Brain Res.* **153:**439–446; 2004.

[14] Langeveld, C. H.; Schepens, E.; Stoof, J. C.; Bast, A.; Drukarch, B. Differential sensitivity to hydrogen peroxide of dopaminergic and noradrenergic neurotransmission in rat brain slices. *Free Radic. Biol. Med.* **19:**209–217; 1995.

[15] Maker, H. S.; Weiss, C.; Brannan, T. S. Amine-mediated toxicity. The effects of dopamine, norepinephrine, 5-hydroxytryptamine, 6-hydroxydopamine, ascorbate, glutathione and peroxide on the in vitro activities of creatine and adenylate kinases in the brain of the rat. *Neuropharmacology* **25:**25–32; 1986.

[16] Segura-Aguilar, J.; Metodiewa, D.; Welch, C. J. Metabolic activation of dopamine o-quinones to o-semiquinones by NADPH cytochrome P450 reductase may play an important role in oxidative stress and apoptotic effects. *Biochim. Biophys. Acta* **1381:**1–6; 1998.

[17] Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement wiht the folin phenol reagent. *J. Biol. Chem.* **193:**265–275; 1951.

[18] Nappi, A. J.; Vass, E. The effect of nitric oxide on the oxidations of l-Dopa and dopamine mediated by tyrosinase and peroxidase. *J. Biol. Chem.* **276:**11214–11222; 2001.

[19] Sherer, T. B.; Kim, J. H.; Betarbet, R.; Greenamyre, J. T. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp. Neurol.* **179:**9–16; 2003.

[20] Sherer, T.B.; Betarbet, R.; Testa, C. M.; Seo, B. B.; Richardson, J. R.; Kim, J. H.; Miller, G. W.; Yagi, T.; Matsuno-Yagi, A.; Greenamyre, J. T. Mechanism of toxicity in rotenone models of Parkinson's disease. *J. Neurosci.* **23:**10756–10764; 2003.

[21] Sherer, T. B.; Betarbet, R.; Kim, J. H.; Greenamyre, J. T. Selective microglial activation in the rat rotenone model of Parkinson's disease. *Neurosci. Lett.* **341:**87–90; 2003.

[22] Hoglinger, G. U.; Feger, J.; Prigent, A.; Michel, P. P.; Parain, K.; Champy, P.; Ruberg, M.; Oertel, W. H.; Hirsch, E. C. Chronic systemic complex I inhibition induces a hypokinetic multisystem degeneration in rats. *J. Neurochem.* **84:**491–502; 2003.

[23] Lapointe, N.; St-Hilaire, M.; Martinoli, M. G.; Blanchet, J.; Gould, P.; Rouillard, C.; Cicchetti, F. Rotenone induces non-specific central nervous system and systemic toxicity. *FASEB J.* **18:**717–719; 2004.

[24] Perier, C.; Bove, J.; Vila, M.; Przedborski, S. The rotenone model of Parkinson's disease. *Trends Neurosci.* **26:**345–346; 2003.

[25] He, Y.; Imam, S. Z.; Dong, Z.; Jankovic, J.; Ali, S. F.; Appel, S. H.; Le, W. Role of nitric oxide in rotenone-induced nigro-striatal injury. *J. Neurochem.* **86:**1338–1345; 2003.

[26] LaVoie, M. J.; Hastings, T. G. Peroxynitrite- and nitrite-induced oxidation of dopamine: implications for nitric oxide in dopaminergic cell loss. *J. Neurochem.* **73:**2546–2554; 1999.

[27] Sakka, N.; Sawada, H.; Izumi, Y.; Kume, T.; Katsuki, H.; Kaneko, S.; Shimohama, S.; Akaike, A. Dopamine is involved in selectivity of dopaminergic neuronal death by rotenone. *Neuroreport* **14:**2425–2428; 2003.

[28] Bernheimer, H.; Birkmayer, W.; Hornykiewicz, O.; Jellinger, K.; Seitelberger, F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J. Neurol. Sci.* **20:**415–455; 1973.

[29] Riederer, P.; Wuketich, S. Time course of nigrostriatal degeneration in Parkinson's disease. A detailed study of influential factors in human brain amine analysis. *J. Neural Transm.* **38:**277–301; 1976.

[30] Tretter, L.; Sipos, I.; Adam-Vizi, V. Initiation of neuronal damage by complex I deficiency and oxidative stress in Parkinson's disease. *Neurochem. Res.* **29:**569–577; 2004.

[31] Sipos, I.; Tretter, L.; Adam-Vizi, V. The production of reactive oxygen species in intact isolated nerve terminals is independent of the mitochondrial membrane potential. *Neurochem. Res.* **28:**1575–1581; 2004.

[32] Gao, H. M.; Hong, J. S.; Zhang, W.; Liu, B. Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. *J. Neurosci.* **22:**782–783; 2002.