

# Oral Treatment with the NADPH Oxidase Antagonist Apocynin Mitigates Clinical and Pathological Features of Parkinsonism in the MPTP marmoset Model

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**Abstract** This study evaluates the therapeutic efficacy of the NADPH oxidase inhibitor apocynin, isolated as principal bioactive component from the medicinal plant *Picrorhiza kurroa*, in a marmoset MPTP model of Parkinson's disease (PD). The methoxy-substituted catechol apocynin has a similar structure as homovanillic acid (HVA), a metabolite of dopamine (DA). Apocynin acquires its selective inhibitory capacity of the reactive oxygen species generating NADPH oxidase via metabolic activation by myeloperoxidase (MPO). As MPO is upregulated in activated brain microglia cells of PD patients and in MPTP animal models, the conditions for metabolic activation of apocynin and inhibition of microglia NADPH oxidase are in place. Marmoset monkeys received oral apocynin (100 mg/kg; p.o.) ( $n=5$ ) or Gum Arabica (controls;  $n=5$ ) three times

daily until the end of the study, starting 1 week before PD induction with MPTP (1 mg/kg s.c. for 8 days). Parkinsonian symptoms, motor function, home-cage activity and body weight were monitored to assess the disease development and severity. Post-mortem numbers of the tyrosine hydroxylase expressing DA neurons in the *substantia nigra* were counted. During the MPTP injections, apocynin limited the body weight loss and relieved parkinsonian symptoms compared to controls (Linear regression,  $P<0.05$ ) indicating a reduction of disease progression. During the last test week, apocynin also improved the hand-eye coordination performance compared with vehicle treatment (resp.  $39.3\pm 4.5\%$  and  $17.7\pm 6.7\%$ ;  $P=0.048$ ) and improved the home cage activity with 32 % ( $P=0.029$ ), indicating anti-Parkinson efficacy. Apocynin also increased the number of surviving DA neurons in MPTP-treated marmosets with 8.5 % ( $P=0.059$ ), indicating a tendency towards a neuroprotective efficacy. In conclusion, compensation for the loss of DA and its metabolite HVA by apocynin mitigates the PD progression and limits the parkinsonian signs and motor-function deterioration.

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## Introduction

Parkinson's disease (PD) is a major progressive neurodegenerative disorder of the central nerve system (CNS). PD is characterized by progressive loss of dopamine (DA) neurons in the nigrostriatal circuitry that governs the control of muscle movement (Fearnley and Lees 1991). Current treatments are still focused on improvement of DA neurotransmission capacity in the brain relieving disease symptoms, but have little effect on the progressive degeneration of DA

cells. Therefore, it would be a better approach to focus on medical intervention in the cell death processes to stop or slow down the progression of the disease and improve the quality of life (Jenner 2008).

A pathological hallmark of PD is the activation of microglia cells associated with degeneration of dopaminergic neurons (Gao and Hong 2008). Accumulating evidence demonstrates the reactive oxygen species (ROS) and pro-inflammatory cytokines produced by microglia are engaged in the induction and perpetuation of the neurodegenerative processes in PD (McGeer and McGeer 2008; McGeer et al. 1988; Hunot et al. 1999). This pathogenic effector mechanism in PD patients is recapitulated in a valid disease model that is elicited by injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into marmoset monkeys (*Callithrix jacchus*). MPTP injection induces selective injury to DA cells in the *substantia nigra pars compacta* (SNpc), a pathogenic event that is immediately followed by the clustering of microglia around injured neurons (Kanaan et al. 2008). Selective inhibitors of microglia ROS production are therefore potentially effective medicines for PD aiming at mitigation or prevention of DA neuron degeneration.

The reaction of microglia myeloperoxidase (MPO) and  $H_2O_2$  with structurally-related ortho-methoxy-substituted catechols, such as eugenol (Thompson et al. 1989), apocynin and vanillic acid (Simons et al. 1990), generates reactive intermediates that bind to free thiol groups. Experiments with apocynin have shown that this principle can be used to prevent the assembly and activation of the ROS generating NADPH oxidase of neutrophilic granulocytes, while leaving beneficial neutrophil functions such as chemotaxis and the phagocytosis and intracellular killing of bacteria intact ('t Hart et al. 1992). Apocynin combines low in vivo cytotoxicity with powerful anti-inflammatory activity in a variety of animal disease models. Apocynin has been tested in various models of ROS-mediated tissue injury. Documented examples of in vivo anti-oxidant activity have been obtained in 1) the WAG/Rij rat model of collagen-induced arthritis (CIA) without affecting humoral and cellular immune parameters ('t Hart et al. 1990), 2) the granulomatous skin reaction to intracutaneous injection of complete Freund's adjuvant ('t Hart et al. 1992), 3) ischemia/reperfusion-induced liver injury in mice (Liu et al. 2008) and 4) multi-organ failure in hemorrhagic shock (Abdelrahman et al. 2005). Furthermore, apocynin has been successfully tested in asthmatic patients for suppression of ozone-induced lung hypersensitivity to metacholine (Peters et al. 2001). Intriguingly, oral apocynin has also been shown to target CNS microglia cells, which release MPO upon activation, and to inhibit the release of neurotoxic ROS in mouse models of amyotrophic lateral sclerosis (ALS) (Boillee and Cleveland 2008; Harraz et al. 2008) and stroke (Tang et al. 2008).

As MPO is upregulated in activated brain microglia cells of PD patients and in the MPTP-induced animal model (Choi et al. 2005), the conditions for metabolic activation of apocynin and inhibition of microglia NADPH oxidase are in place. Directly relevant to PD are the beneficial effects of apocynin on 6-hydroxydopamine (6-OHDA) neurotoxicity (Rodriguez-Pallares et al. 2007) and fMLP induced DA neurotoxicity (Gao et al. 2008). Recent data demonstrate that microglia cell-derived oxiradicals have a critical role in MPTP induced damage to DA neurons, which is abolished by apocynin (Gao et al. 2003).

Strikingly, the apocynin is structurally closely related to vanillic acid (see Fig. 1). The structural similarity between apocynin and vanillic acid suggests that the o-methoxy-substituted catechol homovanillic acid (HVA), which is a metabolite of DA, may be an equally potential antagonist of NADPH oxidase. It is therefore tempting to hypothesize that the shortage of DA and HVA anti-oxidant activity due to the degeneration of DA neurons in the *substantia nigra* of MPTP-injected marmosets can be supplemented by oral administration of apocynin. As apocynin is structurally closely similar to HVA, the metabolite of DA, we anticipated no interference with MPTP (or MPP<sup>+</sup>) or the DA transporter (DAT).

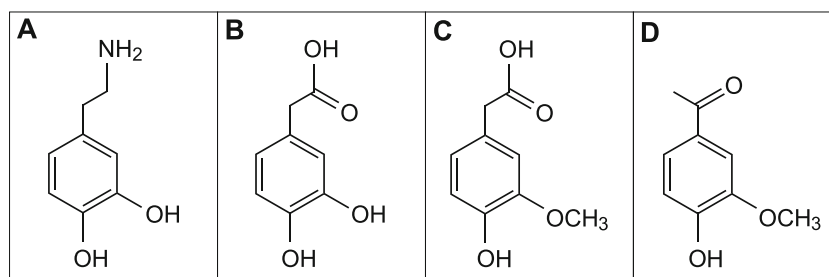
We report protective effects of apocynin on previously documented clinically relevant parameters of the MPTP-treated marmoset model (Philippens et al. 2000; Verhave et al. 2009). This well-validated animal model shows clear and lasting behavioral features, which reflect many aspects of human Parkinsonian symptoms (Jenner and Marsden 1986; van Vliet et al. 2006) and has been recommended as a useful model for the development of novel treatment modalities for PD (Jenner and Marsden 1986; Waters et al. 1987; Colosimo et al. 1992; Fukuda 2001; Philippens 2008).

## Material and methods

### Animals

Ten common marmoset monkeys (*Callithrix jacchus*) of both sexes and between 2 and 3 years of age were purchased from the purpose-bred colony of the Biomedical Primate Research Centre (BPRC) in the Netherlands. Prior to inclusion in the study, the monkeys received a complete clinical, hematological, serological and microbiological examination by the institute's veterinarian; only monkeys declared healthy were included. The monkeys were pair-housed under conventional conditions in spacious cages with a varying cage environment and were under intensive veterinary care throughout the study. The monkey facility was under controlled conditions of humidity (>60 %), temperature (22–26 °C) and lighting (12 h light/dark cycles). Marmosets were

**Fig. 1** Chemical structures of dopamine and discussed o-methoxycatechols. **a** dopamine (DA), **b** DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), **c** DA metabolite homovanillic acid (HVA), **d** apocynin



fed daily with pellet chow for New World monkeys (Special Duit Services, Witham, Essex, UK) enriched with peanuts, biscuits, fruit, vegetables and occasional a mealworm. Water was available ad libitum. According to the Dutch law on animal experimentation, the study protocol and experimental procedure have been reviewed and approved by the Institute's Ethics Committee.

### Drugs

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) was purchased from Sigma Aldrich, St. Louis, USA. MPTP was dissolved in saline and stored in small Eppendorf vials at  $-20^{\circ}\text{C}$  before use. Apocynin (98 %) was purchased from Carl Roth GmbH, Karlsruhe, Germany. Oral apocynin has shown a good safety profile in humans (Peters et al. 2001) and a  $\text{LD}_{50}$  of 9 g/kg in mice (Stolk 1994). We used the same oral dose of 300 mg/kg/day as was found effective in a mouse model of amyotrophic lateral sclerosis (ALS) (Harrasz et al. 2008). Arabic Gum powder from the Acacia tree (Spray Dried) was purchased from Fagron Ltd, UK. Gum Arabic is a supplementary food source for marmoset monkeys.

### Study design

The marmoset monkeys were semi-randomly divided into two groups of five monkeys (3 M/2 F) each, based on their baseline activity level and motor-function performance. The monkeys of the apocynin test group received oral apocynin throughout the experimental period dissolved in Acacia tree Gum Arabic solvent: three times daily 2 ml (100 mg/kg) (apocynin group). The monkeys of the control group received time- and volume-matched vehicle of liquid Gum Arabic instead of apocynin. All other treatments such as PD induction with MPTP and the behavioral tests were identical for both groups. Treatment with apocynin or vehicle started 1 week before the MPTP injections and lasted until the end of the study. After collection of baseline behavioral data, the effects of apocynin were tested to identify possible side effects of this compound on the test systems used. Thereafter, PD was induced all monkeys by 8 daily subcutaneous (s.c.) injections of 1 mg/kg MPTP, dissolved in 0.5 ml of saline, in the abdominal area (total amount of MPTP: 8 mg/kg). Injections

started on Tuesday. No injections were given during the weekend days. This sub-acute PD induction protocol, with daily injections of low doses MPTP, results in a PD stage with slow apoptotic cell death. In this stage the monkeys will not fully be incapacitated and still be able to feed themselves.

The development of clinically evident PD progression was examined by 24-h home cage activity, daily observation in the home cage and weekly behavioral tests (see below). The behavioral tests were focused on the most important motor symptoms that recapitulate motoric deficits in PD patients and therefore have direct human clinical relevance. At the end of the study, the monkeys were humanely euthanized with Euthasol after deep sedation. The predetermined end-point was based on historical data at 2 weeks after the last MPTP injection to be able to quantify effects of apocynin on PD pathology. At necropsy, brains were removed and subsequently processed for examination of PD pathology with quantitative assessment of tyrosine hydroxylase expressing DA neurons in the SNpc.

### Observational tests

The occurrence of typical PD symptoms was recorded by daily inspection using two rating scales, 1) the clinical score for Parkinsonian signs and 2) a scale for scoring abnormal involuntary movements (van Vliet et al. 2006). The observational tests were scored every day before handling of the animals. Items were rated from 0 (=normal/healthy) to 4 (=severely affected).

- 1) Clinical scores included the items apathy (no interest in their environment), immobility, muscle rigidity (assessed as stiffness of legs and tail), tremors and inadequate grooming.
- 2) Scoring of abnormal involuntary movements used the items facial behaviors (jaw, facial muscles, tongue and lips), full body behaviors (upper, lower and trunk), and general severity of involuntary movements and incapacitation due to these movements (Guy 1979; Di Monte et al. 2000).

### Home cage activity

Telemetric acto-meters (REMO 200 transmitters, Remo Technologies Ltd., Salisbury, Wiltshire, UK) were used to

measure the 24-h general home-cage activity to assess whether apocynin reduces disease expression. In human neurodegenerative diseases, such as PD, activity levels and rhythmic patterns are seriously affected. The cumulative activity of 15-s periods was registered. The sum of the 24-h activity was averaged and used as a measure for the change in daily home-cage activity. For the analysis the average of the total activity of three consecutive midweek days were used.

#### Bungalow

The spontaneous explorative loco-motor activity of the marmosets was tested once a week in an automated setup, the so-called Bungalow test system, testing the activity of the marmoset by compartment changes (Wolthuis et al. 1994; Philippens et al. 2000; van Vliet et al. 2006). The monkeys were allowed to move freely for 10 min in a construction consisting of four compartments (23\*23\*23 cm) interconnected by non-transparent tubes, which was placed in a homogeneous environment. A video tracking system registered the movement pattern and the position of the monkey in the apparatus. The number of compartment changes was used as a measure of activity.

#### Hourglass

The Hourglass test is used to test the marmoset's axial turning ability in a Plexiglas cylinder (11\*27 cm) (Verhave et al. 2009). Healthy marmosets quickly correct their posture in normal direction when placed upside-down. When the motor functions are affected the time to recover the normal posture is prolonged, presenting a measure for akinesia. One trial consisted of one manual 180° turn of the cylinder (like turning an hourglass). A test consisted of ten subsequent trials of 30 s each with inter-trial intervals of 30 s. The time the marmoset needed to get into the upright position after the cylinder turn was measured by means of non-automated video analysis by an observer blinded to the treatment. Maximum time noted was 30 s (also for marmosets which did not turn upright at all). The fastest and slowest turn of each monkey were excluded from the analyses of the average turning time.

#### Hand-eye coordination

The marmosets reward-related hand-eye coordination was tested once a week with an robot-guided automated non-invasive test setup for learned complex behavior (Philippens et al. 2000; van Vliet et al. 2006). Small marshmallow-like rewards, used as a motivating stimulus, were presented by a robot at three different speeds in front of the monkey, challenging the animal to follow and reach out: non-

moving (0.0 m/s for a maximum of 30s) slow (0.04 m/s) and fast (0.08 m/s) moving. All trials started with a brief sound signal to alert the animal. Before the start of the study, all animals were trained until they were able to take 75 % or more of the presented rewards. The percentage of correct hits was used as a criterion to judge the performance of the animal.

#### Dopaminergic cell counts in the substantia nigra

SNpc were analyzed for the presence of DA positive neurons with tyrosine hydroxylase immune reactive (TH-IR) staining. Part A7-A2 (Stephan et al. 1980) of the left hemisphere of the brain containing the *substantia nigra* was isolated at the end of the study and snap frozen on liquid nitrogen. Frozen tissue was stored at  $-80^{\circ}\text{C}$ . Serial sections of 7  $\mu\text{m}$  were cut on a microtome and collected serially on slides and air-dried and frozen at  $-20^{\circ}\text{C}$ . After defrost, the sections were fixated with acetone for 10 min and washed for 5 min with PBS 0.1 M (PH 7.4) and Tween20 0.05 %.

Endogenous peroxidase oxidase was quenched with the DAKO Dual Endogenous Enzyme Block (EnVision™ G/2 Double Stain System, Rabbit/Mouse (DAB+/Permanent Red, code K5361). Then the sections were pre-coated in PBS with 5 % bovine serum albumin (BSA) and 5 % Human AB serum for 10 min. Thereafter, incubation in anti-Tyrosine Hydroxylase (TH) clone TH-2 (Sigma 1:100), in PBS + 1 % BSA + 2 % TritonX100, took place for 10 min at room temperature.

The secondary anti-body Polymer/HRP (anti rabbit/mouse, DAKO, Glostrup, Denmark) was incubated for 10 min. Thereafter, the sections were stained for 10 min with 3'3'-diaminobenzidine (DAB+) Chromogen diluted in substrate buffer containing 0.1 % hydrogen peroxide to visualize bound immunocomplexes. PBS-washes (0.1 M) were applied after each incubation step. After a tap water wash the sections were dehydrated in alcohol series, cleared up in xylol and cover-slipped.

TH-IR positive neurons were counted in six systematically sampled sections, with a distance between two sections of approximately 210  $\mu\text{m}$ , throughout the entire SNpc of each marmoset per group. First, the SNpc was delineated at low magnification (10 $\times$ ) using a computer-assisted morphometry system consisting of an Olympus Stream Digital Imaging Solutions with a color video camera. Thereafter, all TH-IR neurons in the SNpc were counted manually within each section in duplo at a magnification of 200 $\times$  using an Olympus light microscope. TH-IR neurons were counted if the nucleus came into focus. Analysis was performed starting with the first anterior appearance of TH-IR neurons (Bregma  $-2.5$ ) and extending to the caudal parts of the SNpc (Bregma  $-4$ ). Every 30th section was taken for cell counting. In total 6 sections were taken from each animal.

Within the six sections, the total number of TH-IR positive neurons was expressed as a percentage of the number of cells found in healthy untreated control brains of marmoset monkeys.

Statistics

Statistical analysis was performed using GraphPad Prism® version 5.0d. All parameters after MPTP treatment were tested for statistical significance with an ANOVA, followed by Newman-Keuls Multiple Comparison test. For column analyses a paired *t*-test was used and a Mann–Whitney procedure for two unrelated groups. Daily parkinsonian symptoms scores, body weight and 24-h home cage activity were analyzed by Linear regression. All *P* values <0.05 were considered as significant effects.

Results

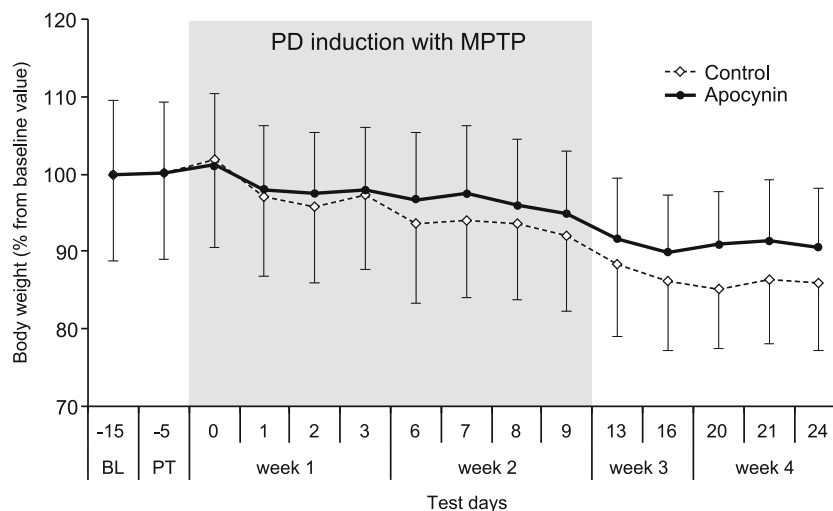
Body weight

Figure 2 depicts body weight loss relative to the start of the study. The body weight was measured once a week during the pre-study phase (baseline) and at least twice a week during the test weeks. Apocynin pretreatment had no detectable effect on the bodyweight in this phase (Fig. 2, PT). During PD induction the body weight was measured on the days of MPTP injection. In the 4 weeks after the start of MPTP injections the body weight declined approximately with 10 %. Initially no effect of apocynin was detected, but

after week 1 control animals lost significantly more bodyweight than the apocynin-treated group (apocynin:  $R^2=0.8187$ , control:  $R^2=0.8563$ ); Linear regression analysis of the curves indicates that the loss of body weight proceeded faster in the vehicle-treated monkeys compared with the apocynin-treated monkeys (difference between the slopes:  $P=0.0233$ ,  $F=5.8119$ ,  $DFn=26$ ).

Clinical signs of Parkinsonism

Gross Parkinsonian signs were recorded on a daily basis by visual inspection of the monkeys in their home cage. In Fig. 3 the average cumulative results are shown of clinical scores (Fig. 3a) and involuntary movements (Fig. 3b). Although the maximum level of the involuntary movements and clinical score was lower in the apocynin-treated group than in the vehicle-treated group, there was no significant difference based on ANOVA analysis between the two groups. During the first week of MPTP injections the development of involuntary movements was identical in both groups. In the second week, the apocynin-treated monkeys displayed less severe involuntary movements than the vehicle-treated control monkeys. In the two following weeks after the MPTP injections the apocynin-treated group displayed less severe clinical scores and involuntary movements than the vehicle-treated monkeys. Linear regression analysis on the clinical score curves indicates a significant difference on the elevation ( $P=0.0005$ ,  $F=17.1201$ ,  $DFn=1$ ,  $DFd=21$ ). This was mainly caused by less muscle rigidity and apathy in the apocynin-treated monkeys (average score compared to the vehicle-treated group is resp.  $71 \pm 5$  and  $81$



**Fig. 2** Effect of oral apocynin treatment on bodyweight. Average body weight decline during disease progression as a percentage of the baseline body weight before treatment (BL) of control parkinsonian monkeys (dotted line, *n*=5) and of apocynin-treated parkinsonian monkeys (solid line, *n*=5): Baseline (BL), pretreatment before PD induction (PT), PD induction with MPTP injections (box, week 1

and week 2) and 2 weeks after PD induction (week 3 and week 4). The indicated days in weeks 1 and 2 are the days of the MPTP injections. The loss of bodyweight was significantly more pronounced in the vehicle-treated control group compared with the apocynin-treated group (Linear regression analysis of the slope of the curves,  $P=0.0233$ )

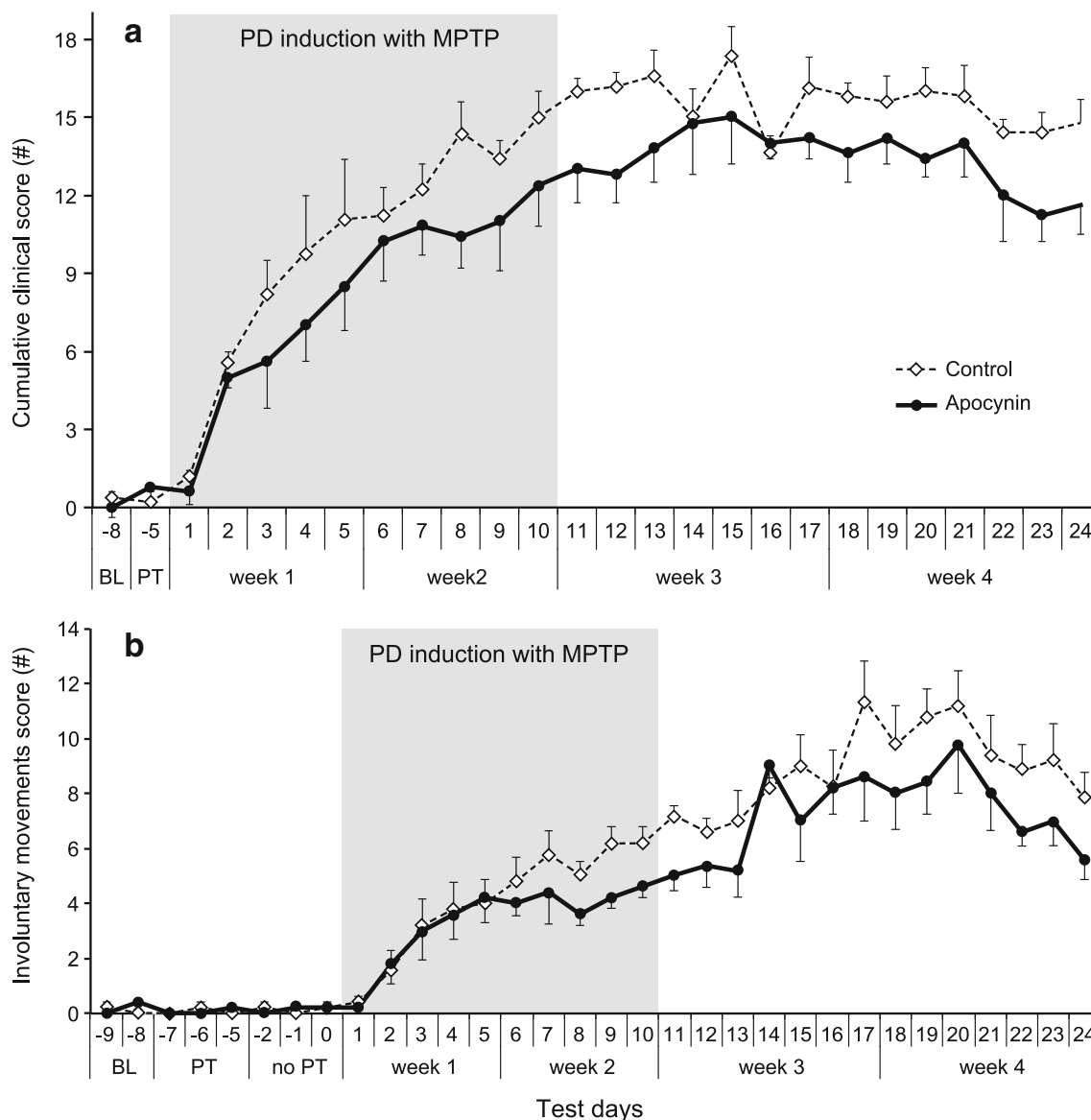


$\pm 5\%$ ). Also immobility and tremors were less observed (resp.  $86\pm 4$  and  $85\pm 4\%$ ). Only grooming was not positively affected by apocynin ( $101\pm 2\%$  compared to the vehicle-treated monkeys). On the slope of the clinical score (Fig. 3a, apocynin:  $R^2=0.9047$ , control:  $R^2=0.9182$ ) no differences were found ( $P=0.1704$ ). On the curves of the involuntary movement scale (Fig. 3b, apocynin:  $R^2=0.7733$ , control:  $R^2=0.9363$ ) the slope as well the elevation differ significantly between the two treatment groups; the curve of the apocynin-treated group was significantly lower compared

with the curve of the control group (slope:  $P=0.0183$ ,  $F=6.6052$ ,  $DFn=1$ ,  $DFd=20$ ; elevation:  $P=0.0068$ ,  $F=9.024$ ,  $DFn=1$ ,  $DFd=21$ ).

#### 24-h telemetric home cage activity

A frequently observed symptom in Parkinson patients is apathy. To test the presence of this feature in MPTP marmoset PD model, the home cage activity of individual monkeys was recorded continuously by telemetric data



**Fig. 3** Effect of oral apocynin treatment on gross parkinsonian signs. **a** Clinical score, and **B**. Involuntary movements score. The parkinsonian signs were recorded on daily basis of control parkinsonian monkeys (dotted line,  $n=5$ ) and of apocynin-treated parkinsonian monkeys (solid line,  $n=5$ ). Observations were made before the start of the study (baseline, BL), during treatment with apocynin or vehicle prior to PD induction (PT) and after the pretreatment (no PT), during PD induction

with eight MPTP injections over 2 weeks (box, week 1 and week 2) and during the 2 weeks after PD induction (week 3 and week 4). The numbers on the x-axis indicates the time in days. Both scoring scales show significant differences between the curves of the treatment groups (A. Clinical score: elevation  $P=0.0005$ , **b** Involuntary movements: slope  $P=0.0183$  and elevation:  $P=0.0068$ )

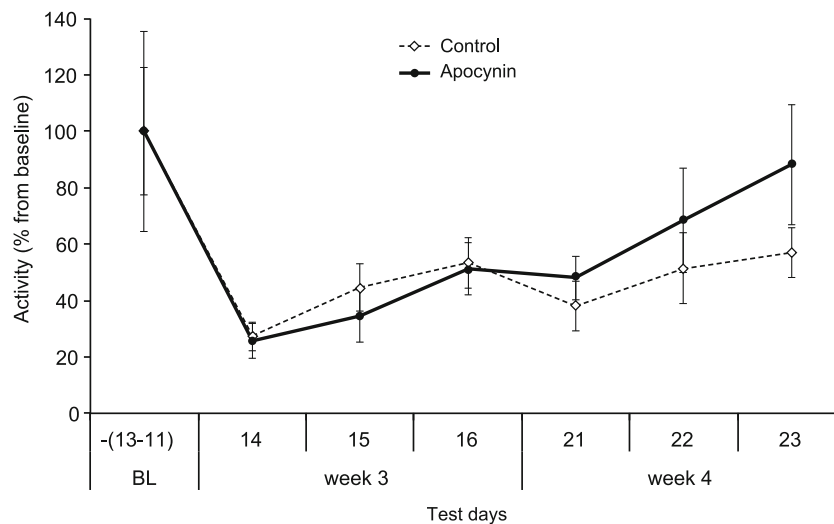
acquisition. Data were collected during the following episodes: 1) The week before the start of apocynin treatment (baseline), and 2) The first and second week after the MPTP injections. Only data obtained at days that were not disturbed by animal handling and behavioral measurements were used for the analyses. Therefore, we only used the Tuesday, Wednesday and Thursday of the post MPTP injection episode. The activity is given as arbitrary units relative to baseline. The average percentages home cage activity of the first week and second week after PD induction was for the control group respectively  $44.28 \pm 15$  and  $51.70 \pm 16$  % and for the apocynin-treated group respectively  $33.87 \pm 8$  and  $61.33 \pm 13$  %. The activity of the apocynin-treated group over the 2 weeks was significantly improved ( $P=0.0285$ , Repeated Measures ANOVA followed by a paired  $t$ -test,  $t=3.354$ ,  $df=4$ ). The activity of the control monkeys was not improved ( $P=0.2865$ ). In Fig. 4 the activity of these 2 weeks are presented separately for each tested day to indicate the progression of the improvement in time. Both curves show a significant change in time (apocynin:  $P=0.0061$  and control:  $P=0.0108$ , Repeated Measures ANOVA). However, the apocynin-treated monkeys show an improvement in activity compared to the vehicle-treated monkeys during the 2 weeks after PD induction. Linear regression analysis of the curves (apocynin:  $R^2=0.9320$ , control:  $R^2=0.5476$ ) indicates a significant difference on the slope ( $P=0.0206$ ,  $F=8.28695$ ,  $DFn=1$ ,  $DFd=8$ ).

#### Loco-motor activity (Bungalow task)

Parkinson patients display seriously disturbed motoric functions. The presence of this aspect in the MPTP marmoset PD model is recorded in the bungalow test, which quantifies spontaneous exploratory loco-motor behavior (see Fig. 5). The baseline activity (no apocynin, no MPTP) measured over 10 min was very high in the two groups (control group:  $46.4 \pm 5.8$ ; apocynin group:  $43.6 \pm 13.0$  compartment changes). The normal average activity in marmoset monkeys in the Bungalow is 40 compartment changes during a 20-min session. The oral pretreatment with apocynin and with vehicle resulted in a reduction of the activity of all monkeys to this normal level (control:  $26.0 \pm 8.0$  and apocynin:  $27.6 \pm 10.7$  compartment changes). After the first dose of MPTP (1 mg/kg) only a slight activity reduction was found, whereas after 6 MPTP injections (total dose 6 mg/kg MPTP) the activity was reduced to almost zero (control:  $1.0 \pm 0.3$  and apocynin:  $0.6 \pm 0.4$  compartment changes;  $P<0.05$ ). The reduced locomotor activity persisted until the end of the study. Only in week 4 after the start of MPTP injections a slight but not significant recovery was noticed (control:  $5.0 \pm 2.0$  and apocynin:  $5.8 \pm 1.5$  compartment changes).

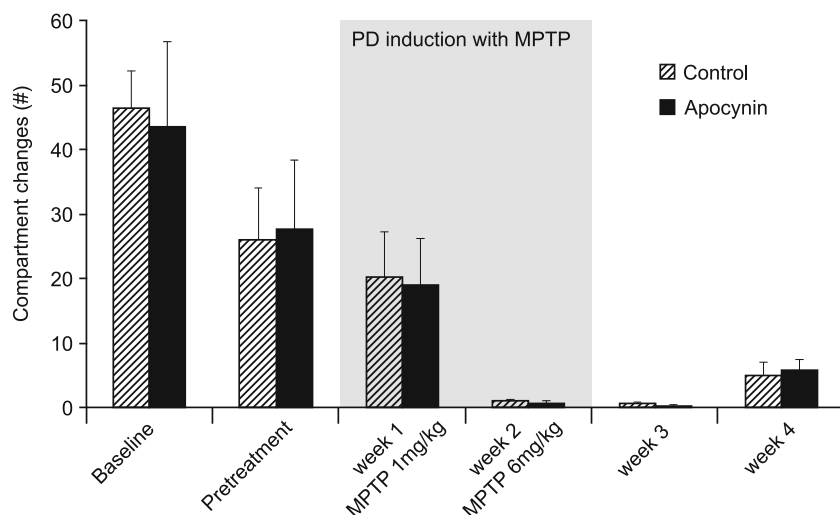
#### Righting reflex (Hourglass test)

A frequently observed symptom in Parkinson patients is impairment of the righting reflex. The presence of this symptom in MPTP PD marmosets is recorded in the



**Fig. 4** Effect of oral apocynin treatment on 24 h telemetric homecage activity. Averaged 24-h telemetric home cage activity as a percentage of the baseline activity before treatment (BL) of control parkinsonian monkeys (dotted line,  $n=5$ ) and of apocynin-treated parkinsonian monkeys (solid line,  $n=5$ ). Baseline data was averaged over three consecutive days (midweek). The numbers on the x-axis indicates the time in days. The cumulative activity as a percentage of the baseline

activity of the Tuesday, Wednesday and Thursday of the first and second week after PD induction with MPTP are shown (week 3 and week 4). The recovery of home cage activity was significantly more pronounced in the apocynin-treated monkeys compared with the vehicle-treated control monkeys (Linear regression analysis on the slope of the curves,  $P=0.0206$ )

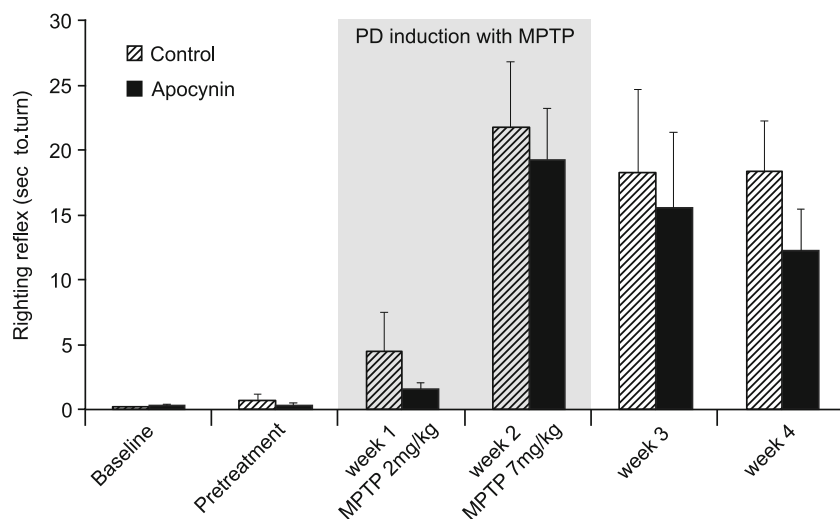


**Fig. 5** Effect of oral apocynin treatment on locomotor activity in bungalow test. Average number of compartment changes in the Bungalow test, as a measure for loco-motor activity, measured before treatment (*baseline*), during pretreatment with vehicle (*hatched bars*,  $n=5$ ) or apocynin (*solid bars*,  $n=5$ ), during PD induction with MPTP

(box, after a total dose of resp. 1 and 6 mg/kg) and 1 and 2 weeks after PD induction (week 3 and week 4). The reduction, due to the MPTP injections, is significantly lower compared to baseline and pretreatment values for both groups ( $P<0.05$ ). Significant differences between the groups were not observed

hourglass test. In brief, a monkey is placed in a Perspex cylinder, which is turned 180°. The time the monkeys needed to turn into the normal upright position is shown in Fig. 6. Testing of this parameter before apocynin treatment and MPTP injected shows that all monkeys needed less than 1 s to regain their normal upright position (control group:  $0.16\pm 0.06$  s. and apocynin group:  $0.25\pm 0.10$  s.). The oral treatment with apocynin or vehicle did not affect this parameter as all monkeys still needed less than 1 s. Already after two injections of MPTP (total dose of 2 mg/kg) a delayed righting reflex can be measured.

The delay was less in apocynin-treated monkeys (control monkeys:  $4.47\pm 3.02$  s.; apocynin-treated monkeys:  $1.53\pm 0.49$  s.), but the difference was not significant. After the last MPTP injections and the following 2 weeks the capacity of both groups to turn within the maximum test period of 30 s. was strongly reduced compared to baseline and pretreatment (control monkeys: resp.  $21.73\pm 5.07$ ,  $18.26\pm 6.41$ , and  $18.33\pm 3.95$  s.; apocynin-treated monkeys: resp.  $19.26\pm 3.90$ ,  $15.50\pm 5.88$ , and  $12.19\pm 3.23$  s.,  $P<0.05$ , Newman-Keuls Multiple Comparison post test). Although the



**Fig. 6** Effect of oral apocynin treatment on righting reflex in the hourglass test. Average time to turn in the Hourglass test for righting reflex and akinesia of control parkinsonian monkeys (*hatched bars*,  $n=5$ ) and of apocynin-treated parkinsonian monkeys (*solid bars*,  $n=5$ ) during baseline, pretreatment before PD induction, PD induction with

MPTP (box, after a total dose of resp. 2 and 7 mg/kg) and one (week 3) and two (week 4) weeks after PD induction with MPTP. Although the apocynin group showed a tendency towards a shorter righting reflex time, differences between the two groups were not significant



ability to resume normal position recovered more quickly in the apocynin-treated monkeys, the difference with the vehicle-treated control monkeys was not significant.

### Hand-eye coordination

The hand-eye coordination test measures the fine motor skills of MPTP PD marmosets, which is a prevalent motoric impairment of PD patients. In Fig. 7 the performance is shown as the percentage correct hits per session. The performance in the hand-eye coordination task was reduced by approximately 50 % after MPTP (as well after 1 mg/kg as 7 mg/kg) and week 3 after PD induction (in total 8 mg/kg MPTP) by approx. 75 % in both groups (Repeated Measures ANOVA followed by a Newman-Keuls Multiple Comparison post test,  $P < 0.05$ ). In week 4 after the termination of MPTP injections the apocynin-treated monkeys showed a significant recovery in their performance compared with the monkeys of the control group ( $P = 0.0476$ , Mann–Whitney test).

### Pathology

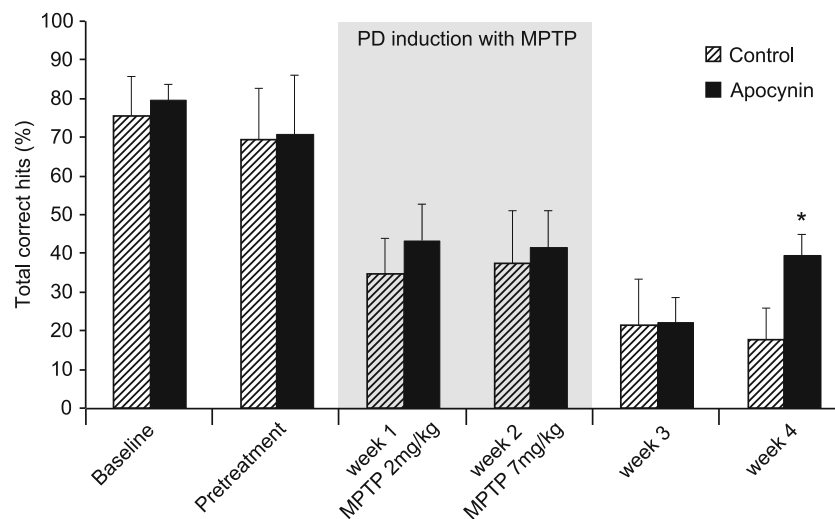
Staining of the SNpc with tyrosine hydroxylase (TH), both treatment groups shows the huge loss of TH-IR positive neurons in MPTP-injected monkeys in comparison to non-injected controls ( $P < 0.05$ ) (Table 1). Figure 8 shows that in the apocynin-treated group less damage had occurred than in the vehicle-treated group. The quantification given in

Table 1 revealed 12.31 % surviving TH<sup>+</sup> cells in the apocynin vs 3.78 % in the vehicle group ( $P = 0.0592$ ). In most MPTP treated monkeys also a strong reduction was found of TH-IR positive fibers as visible in control brain and partly in brains of apocynine treated monkeys (Fig. 8a and b). In the apocynin-treated monkeys this reduction was less than in the vehicle-treated monkeys.

### Discussion

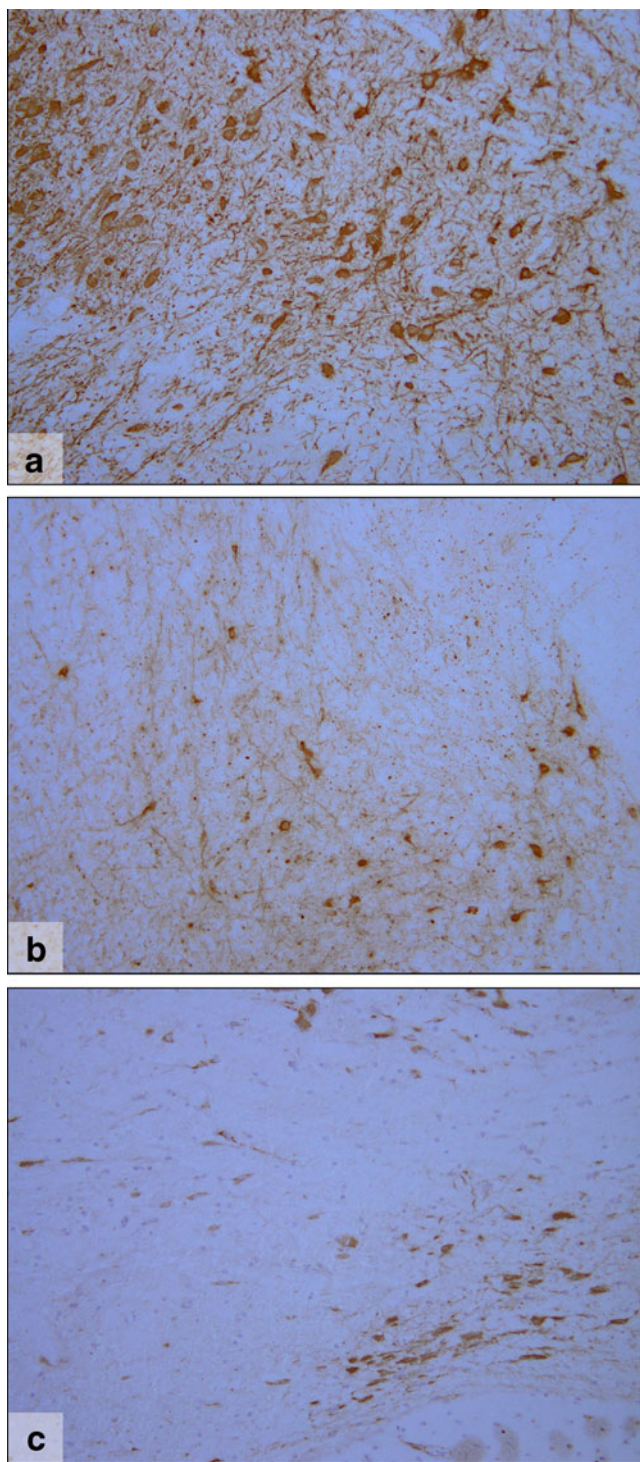
The microglia cell is considered as a CNS-resident immune cell with a key pathogenic function PD. Microglia cells exert their pathogenic function in PD animal models in part by the secretion of neurotoxic ROS, as illustrated by the protective effect of inhibitors of microglial activation that mitigate the neurotoxic effects of MPTP in mice (Wu et al. 2002; Gao et al. 2003). The microglial reaction in PD is presumably associated with long-term neurodegeneration and is therefore held responsible for the perpetuation of the degenerative process (McGeer and McGeer 2008). In neurodegenerative diseases activated microglia produce a number of substances, such as cytokines, that sustain their activation by an autocrine loop and keep the neurodegenerative process ongoing (Pocock and Liddle 2001).

Just like in peripheral phagocytic cells, microglia produce ROS by NADPH oxido-reductase (NADPH oxidase), which is assembled after cell activation from membrane-bound and cytosolic proteins. Moreover, just like neutrophilic granulocytes



**Fig. 7** Effect of oral apocynin treatment on fine motor skills in the hand-eye coordination test. The total number of correct hits in the hand-eye coordination task of control parkinsonian monkeys (hatched bars,  $n = 5$ ) and of apocynin-treated parkinsonian monkeys (solid bars,  $n = 5$ ) during baseline, pretreatment before PD induction, PD induction with MPTP (box, after a total dose of resp. 2 and 7 mg/kg) and one (week 3) and two (week 4) weeks after PD induction with MPTP. For both groups there was a significant decline in performance (Repeated

Measures ANOVA,  $P < 0.001$ ). As well the PD induction weeks (weeks 1 and 2) as the post MPTP weeks are significantly lower compared to baseline and pretreatment ( $P < 0.05$ , Newman-Keuls Multiple Comparison post test). \*Two weeks after termination of the MPTP injections (week 4) the performance on the hand-eye coordination task was significantly better in the apocynin-treated monkeys compared with the control monkeys ( $P = 0.0476$ , Mann–Whitney test)



**Fig. 8** TH-IR positive neurons. Images of dopaminergic neurons in the SNpc (magnification: 200 $\times$ ) of a healthy control monkey (**a**), apocynin-treated parkinsonian monkey (**b**) and a vehicle-treated parkinsonian monkey (**c**)

microglia cells release MPO upon activation. Microglia cells are therefore potential targets of the antioxidant activity of o-methoxycatechols. These observations led us to hypothesize that activation of the microglia oxidative burst might be

**Table 1** Percentage of dopaminergic neurons in the SNpc compared to control healthy marmoset brain, visualized with stained with TH immunohistochemistry staining

Treatment group	Vehicle control	Apocynine
% TH positive cells compared to controls	3.78 $\pm$ 1.60	12.31 $\pm$ 3.28*

\*Student *t*-test with Welch correction:  $P=0.0592$

suppressed with apocynin, just like was demonstrated for neutrophils in different animal disease models.

Intriguingly, apocynin indeed attenuated MPTP-induced dopaminergic neurodegeneration in cell culture from mice in the presence of glia cells, (Gao et al. 2003). Additionally, dopaminergic neurons from mice lacking NADPH oxidase are significantly more resistant to MPTP neurotoxicity than those from the wild-type controls (Gao et al. 2003).

The pharmacological potential of apocynin as specific inhibitor of the phagocyte oxidative burst was discovered in a screening of the roots of *Picrorhiza kurroa*, a plant used in Ayurvedic medicine, for anti-oxidant principles (Simons et al. 1990). The mechanism underlying the highly specific mode of action was resolved in subsequent years demonstrating that apocynin is converted by MPO into a symmetrical quinone methide dimer through the formation of a 5,5' carbon-carbon bond (Thompson et al. 1989; Thompson et al. 1993). Compounds with free thiol (–SH) groups, such as glutathione, L-cysteine, cellular proteins or subunits of NADPH oxidase inhibit this dimerization process (Johnson et al. 2002) by trapping quinone methide into stable conjugates. Thus “metabolically activated” apocynin effectively inhibits the assembly of NADPH dependent oxidoreductase complexes in activated phagocytes via covalent binding to the thiol-groups (Simons et al. 1990; Stolk et al. 1994) that link cytosolic subunits with membrane-bound flavoprotein-cytochrome complexes (Bedard and Krause 2007). Catechols without the o-methoxy-substituted group, such as (gallo-) catechins, show promising effects in neurodegenerative disease models (Mandel and Youdim 2004), but lack the high specificity for MPO-containing cells, as these do not undergo MPO-dependent metabolic activation. The highly specific metabolic activation by MPO that converts the non-toxic apocynin into a potent thiol-blocker makes apocynin an almost ideal metabolically stable oral pro-drug that becomes active only in brain regions where microglia are activated (‘t Hart and Simons 1992).

The primary aim of the current research was to test the protective properties of apocynin on the development and progression of PD related motor deterioration in the elected PD model, the MPTP model in common marmosets. In MPTP treated monkeys, the numbers of activated microglia cells in the SNpc were found significantly higher compared

to non-treated monkeys one year post PD induction with MPTP (Barcia et al. 2004). On the other hand, microglial activation may also be involved in the sequence of pathological changes that lead to dopaminergic neuronal damage after MPTP intoxication as microglial activation was found prior to DA depletion in mice (Czlonkowska et al. 1996).

We report here that oral apocynin treatment in healthy marmoset monkeys, did not affect the motor functions of healthy monkeys, which is in line with previous findings about the safety profile of apocynin in human (Peters et al. 2001). Only in the Bungalow task, testing loco-motor activity, a reduction in compartment changes was found during the pretreatment. However, it is unlikely that this effect is caused by apocynin as the same reduction was observed in control monkeys receiving only vehicle. Shortly after the start of MPTP injections, a progressive decline of body weight was observed, which was significantly less in the apocynin-treated monkeys compared to the vehicle-treated monkeys. The expressed typical parkinsonian signs were less severe in the apocynin-treated monkeys. With regard to motor functions we observed in the apocynin-treated group a significantly quicker recovery of fine motor skills, assayed by the hand-eye coordination task, after MPTP injections were stopped and direct toxic effects of MPTP had washed out. A longer follow-up period could have proven whether parkinsonian signs improved further under the influence of apocynin; not only for the hand-eye coordination task but also for the tendency towards an improvement on the righting reflex in the apocynin-treated monkeys. The brain-pathology analysis showed a huge decline of TH positive neurons in both groups. Although the percentage of damage compared to healthy control brain was more than 85 % in both groups, the apocynin-treated monkeys showed less damage in the SNpc compared to the vehicle control monkeys (remaining neurons in the apocynin group:  $12.31 \pm 3.28$  % and in the vehicle control group:  $3.78 \pm 1.60$  %). The injury attenuation by apocynin treatment showed a strong tendency compared to the vehicle treatment ( $p=0.0592$ ) but was not significantly different. A less severe disease induction protocol might have resulted in a more pronounced difference on neuron damage.

In conclusion, oral administration of apocynin exerts a clearly detectable beneficial effect on multiple disease parameters in MPTP-induced PD marmoset monkeys, including the parkinsonian signs, loss of body weight, fine motor skills and the home-cage activity. The beneficial effect of apocynin was most obvious after the MPTP injections were stopped. The likely explanation is that MPTP has a direct neurotoxic effect, which may mask neurotoxic effects of the microglia oxidative burst. After the stop of MPTP injections, the direct neurotoxic effect of MPTP washed out and the model is more sensitive to the modulation of other neurotoxic mechanisms. Together, the presented data

warrants the conclusion that daily oral apocynin treatment slows down and limit the disease development in the model.

It is intriguing to speculate whether one of the consequences of DA neuronal death is reduced availability of HVA, an o-methoxycatechol that just like vanillic acid qualifies as a potential inhibitor of the microglia oxidative burst. The possibility to compensate this deficit with oral apocynin offers a new treatment concept for the progressive neurodegeneration in PD patients. Oral apocynin has shown a good safety profile in humans (Peters et al. 2001). In vitro studies with human neutrophils show that metabolically activated apocynin affects only cytotoxic mechanisms of the phagocytes, but leave important homeostatic functions intact, such as the chemotactic migration and intracellular killing of bacteria, illustrating the selective mechanism of action.

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**Conflict of interest** There is no conflict of interest.

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