Neuroprotective effects of modafinil in a marmoset Parkinson model: behavioral and neurochemical aspects

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The vigilance-enhancing agent modafinil has neuroprotective properties: it prevents striatal ischemic injury, nigrostriatal pathway deterioration after partial transsection and intoxication with 1-methyl-1,2,3,6-tetrahydropyridine. The present study determines the protective effects of modafinil in the marmoset 1-methyl-1,2,3,6-tetrahydropyridine Parkinson model on behavior and on monoamine levels. Twelve marmoset monkeys were treated with a total dose of 6 mg/kg 1-methyl-1,2,3,6-tetrahydropyridine. Simultaneously, six animals received a daily oral dose of modafinil (100 mg/kg) and six animals received vehicle for 27 days. Behavior was observed daily and the locomotor activity, hand–eye coordination, small fast movements, anxiety-related behavior and startle response of the animals were tested twice a week for 3 weeks. Modafinil largely prevented the 1-methyl-1,2,3,6-tetrahydropyridine-induced change in observed behavior, locomotor activity, hand–eye coordination and small fast movements, whereas the vehicle could not prevent the devastating effects of 1-methyl-1,2,3,6-tetrahydropyridine. Dopamine levels in the striatum of the vehicle + 1-methyl-1,2,3,6-tetrahydropyridine-treated animals were reduced to 5% of control levels, whereas the dopamine levels of the modafinil + 1-methyl-1,2,3,6-tetrahydropyridine-treated animals were reduced to 41% of control levels. The present data suggest that modafinil prevents decrease of movement-related behavior and dopamine levels after 1-methyl-1,2,3,6-tetrahydropyridine intoxication and can be an efficaceous pharmacological intervention in the treatment of Parkinson's disease. *Behavioural Pharmacology* 17:453–462 © 2006 Lippincott Williams & Wilkins.

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Introduction

In Parkinson's disease (PD), the output of the basal ganglia is irreversibly affected owing to degeneration of the neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta. This results in the manifestation of symptoms including akinesia, postural instability, rigidity and resting tremors (Dauer and Przedborski, 2003).

PD is incurable, because present medications (predominantly with L-dopa) do not counteract progression of the disease and long-term medication is associated with declining efficacy and increased side effects (Clarke, 2004). Therefore, a better strategy aims to focus on prevention of the neuronal loss in an attempt to stop or slow down the progression of the disease. One way to achieve neuroprotection is via pharmacological interference aimed at crucial steps in the neuronal cell death process to promote neuronal survival. Although some potential drug candidates were tested in clinical trails there is as yet no proven neuroprotective treatment (Clarke, 2004).

The actual cause of PD is unknown. Evidence exists suggesting that factors like mitochondrial dysfunction, oxidative stress, excitotoxicity and inflammatory processes, either separately or cooperatively, are involved in the neurodegenerative process causing PD (Alexi *et al.*, 2000).

Modafinil (Modiodal) is a vigilance-stimulating compound and marketed for the treatment of narcolepsy (Bastuji and Jouvet, 1988). The mechanism of action of modafinil is not clear, although it is suggested to increase indirectly wakefulness via α-1 noradrenergic neurotransmission (Duteil *et al.*, 1990) but it also decreases γ-amino-*n*-butyric acid (GABA) release in sleep-related areas and striatum (Ferraro *et al.*, 1996, 1998). Modafinil also influences dopamine (DA) release, whereas knocking out the DA transporter prevents the stimulative properties of modafinil (Wisor *et al.*, 2001).

Modafinil could also be very promising as a neuroprotective compound. Modafinil in cultured cortical cells prevented glutamate toxicity (Antonelli *et al.*, 1998), it

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prevented increases in toxic aspartate and glutamate levels after striatal ischemic injury caused by endothelin-1 in rats (Ueki *et al.*, 1993a) and it prevented development of lesions in the hippocampus induced by the neurotoxic nerve gas soman (Lallement *et al.*, 1997). After partial transsection of the DA pathway (Ueki *et al.*, 1993b) and also in 1-methyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD models in mice (Fuxe *et al.*, 1992) and marmosets (Jenner *et al.*, 2000), modafinil protected dopaminergic neurons from degeneration. The latter two studies suggest that modafinil could be a candidate drug for neuroprotection in PD at a behavioral and a neuronal level. More insight into the effects of modafinil on different dopamine and nondopamine-related symptoms and the relation to neuronal function is, however, needed. Therefore, the present study focuses on putative neuroprotective effects of modafinil in the marmoset MPTP Parkinson model, with extensive behavioral tests and biochemical measurements, which were not included in earlier studies.

The present MPTP Parkinson model is the most used experimental model for PD (Dauer and Przedborski, 2003). The neurotoxic agent MPTP selectively damages neurons in the substantia nigra by blocking the electron transport chain of the mitochondria leading to a loss in mitochondrial function resulting in a depletion of adenosine triphosphate and finally cell death. MPTP is effective in mice and marmosets. The mouse MPTP model is, however, not suitable for behavioral studies because parkinsonian symptoms do not develop clearly and disappear within a few days (Schmidt and Ferger, 2001). The marmoset model is more suitable for behavioral studies because marmosets show clear and lasting behavioral features after MPTP treatment, which reflect many aspects of human Parkinson symptoms (Jenner and Marsden, 1986). Even a clinically used observational scale for involuntary movements (abnormal involuntary movement scale, AIMS) can be applied to the marmoset without adaptation (Di Monte *et al.*, 2000).

In the present study, the protective effects of modafinil on behavior were tested using two extensive behavioral observation scales for PD and functional tests measuring locomotor activity, hand–eye coordination (HEC), small fast movements and the startle response. The human threat test (HTT) was applied to measure whether anxiety-related behavior is sensitive to changes induced by MPTP and could be changed after a neuroprotective intervention with modafinil.

Another important marker for neuroprotection is the protection of monoaminergic neurotransmission in the brain. In PD and the MPTP model, DA levels in the striatum, the main area receiving dopaminergic output from the substantia nigra, are most heavily affected, owing to reduction of the substantia nigra neurons. Metabolites and other monoamines, noradrenaline (NA) and serotonin (5-HT), can also be used as markers for neuronal damage and neuroprotection, and were studied in brains of vehicle and modafinil-treated PD animals and control brains.

In an extension of the work of Jenner *et al.* (2000), the neuroprotective effects of modafinil against PD induction on the functional outcome, with an extensive battery of behavioral tests, and on neurotransmitter levels are described in this paper. The results of measurements with magnetic resonance imaging and spectroscopy and with immunohistochemistry will be covered in a subsequent publication.

Methods

Subjects

Adult male and female marmoset monkeys *(Callithrix jacchus)*, aged 2–6 years, with initial body weights between 350 and 550 g, were obtained from the primate center BPRC, The Netherlands and Harlan, UK. The ambient temperature in the housing room was regulated at $25 \pm 2^{\circ}C$ and the relative humidity was always $> 60\%$. A 12-h light–dark cycle was maintained, lights on from 07.00 to 19.00 h. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The Netherlands' organisation for applied scientific research (TNO) committee on Animal Care and Use approved all protocols for the animal experiments.

Study design

Twelve naïve marmosets were treated in total with 6 mg/kg MPTP subcutaneously over 9 days (day 1: 2 mg/kg and days 2, 3, 6 and 9: 1 mg/kg). Six of these animals (four males; two females) received a daily oral dose of 100 mg/kg modafinil from experimental day 1 until day 27. The remaining six animals (three males; three females) received a daily oral dose of the vehicle (10% sucrose solution). The dose of modafinil was based on the lowest effective dose in naïve marmosets (Van Vliet *et al.*, 2006), which was in accordance with an MPTP study in mice (Fuxe *et al.*, 1992) and a comparable study in marmoset monkeys (Jenner *et al.*, 2000). By using this dose, the parallels in the behavioral aspects with the study of Jenner *et al.* (2000) can be used to increase the comparability and therefore increase the value of the study as a research contribution. The oral modafinil or vehicle treatment was given directly after the subcutaneous MPTP injections. One animal in the vehicle group died during the anesthesia procedure before the magnetic resonance imaging scan, therefore the data of this animal are omitted.

The occurrence of parkinsonian symptoms was observed daily before and after administration of the treatment

using two rating scales: clinical score and AIMS. On days 13, 17, 20, 24 and 27 the behavioral tests, namely the HEC, locomotor activity, startle response and small fast movements were tested in noninvasive test systems. The HTT was executed on these days simultaneously with the behavioral observations. Before disease induction, the animals were trained on the HEC task and baseline values of all test systems were obtained. The after-administration behavioral observations and tests were started 2 h after administration. This time span reflects the peak activity of modafinil in marmoset monkeys on the basis of the pharmacokinetic results of modafinil in our institute (data not published), the study of Jenner *et al.* (2000), and the t_{max} of modafinil in humans (Robertson and Hellriegel, 2003).

As modafinil is a vigilance-enhancing agent, temporary symptomatic effects on motor function could be present besides the neuroprotective effects of modafinil. Therefore, a distinction is made between before and after administration: the behavioral observations were made twice a day and most tests were performed either before the daily administration on days 13, 20 and 27 or 2 h after administration on days 17 and 24. Only the small fast movements test was tested in the opposite order. In the figures, this distinction is indicated with solid and striped bars.

Behavioral assessment

Observation of signs and symptoms

For the observation of signs and symptoms, two rating scales were used.

(1) A general clinical scoring list in which the condition of the animal was rated. The following symptoms were registered: appetite, inadequacy of grooming by inspection of the fur; apathy by testing the responsiveness of the animal to its surrounding; immobility; rigidity and presence of tremors. The rates of severity were coded from 0 (normal) to 4 (severe).
(2) The AIMS is a 9-item rating scale, designed to record in detail the occurrence of involuntary movements (Guy, 1976). The AIMS is widely used clinically for qualification of involuntary movements, occurring in PD (Katzenschlager *et al.*, 2004). These scales have successfully been applied for more than 10 years in the monkey research in our institute. The AIMS includes facial, mouth (lips, peri-oral area, jaw and tongue), extremity and trunk movements. The global judgment of the severity and the incapacitation owing to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Movements that occur upon stimulation by the observer were rated one step lower than those observed spontaneously.

Spontaneous exploratory behavior (Bungalow test)

The levels of activity and exploratory behavior can play an important role in practically all measurements of animal behavior. A device called the 'Bungalow test' automatically and quantitatively assesses these parameters and is extensively described and validated (Wolthuis *et al.*, 1994; Philippens *et al.*, 2000). The apparatus consists of four horizontally placed nontransparent boxes ($23 \times 23 \times 23\,cm^3$), all interconnected by six PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. The animals could freely move and change from one compartment to another during the 20-min session. A video tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the number of compartment changes during the session.

Hand–eye coordination task

An automated robot-guided apparatus with positive reinforcement as a motivating stimulus (small pieces of marshmallow) was used to assess HEC (Philippens *et al.*, 2000). The marmoset was placed in front of a test panel provided with a window (8×5 cm). A robot arm presented a reward behind the window. With this system, three types of trials were performed: one using a nonmoving reward in the middle of the window, one using a slow horizontally moving reward (0.04 m/s) and one using a fast horizontally moving reward (0.08 m/s). The animal was allowed 1 min to grasp the nonmoving reward. Each type of trial was presented 14 times in one session. At the beginning of each trial, a sound signal was presented, intended to alert the animal. A pressure detector in the robot arm and infrared detectors in the window registered hits and attempts and speed of performance. A 'hit' was registered when the animal successfully retrieved the reward from the robot arm. The percentage of correct hits was used as a criterion to judge the animal's performance.

Small fast movements test

Small fast movements are very hard to detect by observation. Therefore, an automated test system, which makes use of a capacitive transducer, was used. Changes in the transducer capacitance resulted in a signal, which was constructed of different behavioral components. Gross movements with the extremities were filtered out. Only small fast movements were detected. A transparent plastic tube (diameter: 18 cm and height: 26 cm) was placed in a homogeneous electrical field, created by an electrical potential difference across two vertically placed metal plates. As the animal, situated in the plastic tube between the plates, was a conducting medium, any change in posture of the animal would lead to a change of plate capacitance. Both plates were driven, via a buffer amplifier, at the same potential as the detection plate. These signals were amplified, filtered

(5–20 Hz) and fed into the AD converter. Crosses above the noise level were used as an indication for the small fast movements.

Human threat test

The HTT is a non-human primate putative model of anxiety. It is based on findings that marmosets will exhibit fear-related behavior in the presence of a human observer in front of the cage (Carey *et al.*, 1992; Van Vliet *et al.*, 2005). Most pronounced behavior would be retreating to the back of the cage and showing characteristic postures. The behavior was assessed in the home cage ($40 \times 60 \times 60$ cm) with a hanging basket in the back of the cage, a wooden board (20×10 cm, 30 cm above cage floor) on the left side in the back and on the other side a perch, at the same height, positioned from the back to the front of the cage. To assess the behavior, the observer stood approximately 30–100 cm from the cage front and made eye contact with the marmoset throughout a 2-min test period. During this period, the movements, behavior and position of the marmoset in the cage were recorded by video registration. A range of parameters was obtained according to Carey *et al.* (1992), based on Stevenson and Poole (1976): (1) the number of characteristic postures exhibited: tail posture (tail raise to present the genital region), scent marking (the anal and genital area is pressed against the substrate to be marked with excretion of the glands), arched pilo-erection (arched back posture with full body piloerection), slit stare (stare with the eyes half closed in combination with tufts flattened and exposure of the teeth), rearing (upright position with flexed paws), twisting (head and torso movement from side to side); (2) the time spent in the front of the cage and (3) the number of position changes in the cage.

Auditory startle response

The auditory startle reflex is a motor response following an intense sound stimulus. The startle response is a sensitive method to determine how different neurotransmitter systems or drugs modulate sensorimotor activities. The apparatus for marmoset monkeys has been described earlier and validated by Philippens *et al.* (2000). The animals were placed in a transparent plastic tube on a pressure transducer in an illuminated sound attenuated box. Twenty startle stimuli (20 ms, 120 dB; white noise) were delivered in random order every 14 ± 4 s. For the duration of 200 ms, the force exerted by the animal upon presentation of the stimulus was registered. The startle reflex was represented by the amplitude of the response.

High-pressure liquid chromatography analysis

For determination of brain monoamine levels, four MPTP-treated animals from the modafinil group and five animals from the vehicle group were used. Furthermore, six brains of naïve animals were used to establish control values of the monoamines. At day 37, 10 days after the last modafinil administration, brains were removed after decapitation of the sedated animals. The striatum of one hemisphere was isolated after termination and was directly frozen in liquid nitrogen. The tissue (100–500 mg) was weighed and homogenized in 10 ml 0.4 mol/l perchloric acid containing 20 ng/ml 3,4-dihydroxybenzylamine hydrobromide (Sigma Chemical Co, St Louis, Missouri, USA) and 20 ng/ml (±)-isoproterenol hemisulfate salt (Sigma chemical Co, St Louis, Missouri, USA) as internal standards. The homogenate was centrifuged at 22 000 *g* for 30 min at 4°C and 1 ml of supernatant was adjusted to about pH 4.0 with 250 µl 2 mol/l sodium acetate. The homogenate samples were stored at −70°C for a maximum of 6 weeks. The monoamines NA, DA and 5-HT, and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) were determined by ion-pair reversed-phase liquid chromatography. A 2–50 µl sample was injected on a RP18 LiChrosfer 100 column (125×4 mm internal diameter, 5 µm particle size; Merck, Darmstadt, Germany) connected to a Thermo Separations Products (San Jose, California, USA) model P100 solvent delivery pump and AS300 autosampler and a Coulochem II Model 5011 electrochemical detector (ESA, Bedford, Massachusetts, USA). The mobile phase consisted of a 30 mmol/l citrate/40 mmol/l phosphate buffer, pH 4.0, containing 0.27 mmol/l Na_2 ethylene diaminetetraacetic acid, 1.8 mmol/l heptanesulfonic acid and 5% methanol. The potential of the electrode was set at 420 mV. External standards were determined in each assay run. Calibration plots were linear from 2 to 500 ng/ml for each compound. The lower limit of detection was 2 ng/ml. The intra-assay coefficient of variation amounted to 2%.

Drug

Modafinil (Modiodal: D,1-2-[(diphenylmethyl)sulfinyl] acetamide) was used in ground tablet form (Laboratoire L., Lafon, France). One tablet contains 100 mg modafinil and filling compounds: lactose, cornstarch, magnesium monosilicate $2H_2O$, sodium croscarmellose, polyvidon, talc and magnesium stearate. Before usage the ground tablets were homogenized freshly in a 10% sucrose solution in a dose volume of 1.5 ml/kg.

Statistics

The results of this study are presented as mean ± SEM and parametric statistical analysis was applied with a significance level of $P < 0.05$. The scores of the behavioral observation scales were analyzed with an independent *t*-test to reveal differences between the two treatment groups. The results of the behavioral tests were analyzed in two ways. First, the difference between the two treatment groups was obtained. Therefore, an overall repeated-measure analysis was applied on the results of days 13, 20 and 27 and of days 17 and 24 (see

below). When relevant, an independent *t*-test was applied. Second, the difference between baseline and test day results of each treatment values was tested with a paired *t*-test. Temporary symptomatic effects of modafinil were tested via comparison of observational data before administration (see Fig. 1) and after administration (data not shown) with a paired *t*-test. These effects in the behavioral tests were analyzed with a comparison between pooled data of days 13 and 20 vs. day 17, and pooled data of days 20 and 27 vs. day 24 with independent *t*-tests. As a result of the alternate test schedule, the comparison of the small fast movements was made between the data of day 13 vs. day 17 and day 24 vs. day 27 and pooled data of days 17 and 24 vs. day 20 with independent *t*-tests. The difference between the monoamine levels of each treatment was tested with a one-way ANOVA followed by a *t*-test when relevant.

Results

Behavioral observation

During the first three experimental days, after receiving the first two MPTP injections, all animals developed similar symptoms (see Fig. 1). Hereafter, a discrepancy between the groups emerged. The vehicle-treated PD animals showed a mild parkinsonian symptomatology, whereas the modafinil-treated PD animals (from day 3) were not affected by the last MPTP injections and ended with rather weak parkinsonian symptoms. In the modafinil-treated PD group, all parameters of the clinical score were present until day 17. After day 17, decreased appetite, rigidity and tremors were the most pronounced symptoms, whereas scores for the inadequacy of grooming, apathy and immobility returned to normal values. In the vehicle-treated group, all parameters were apparent during the whole experiment.

Hand–eye coordination

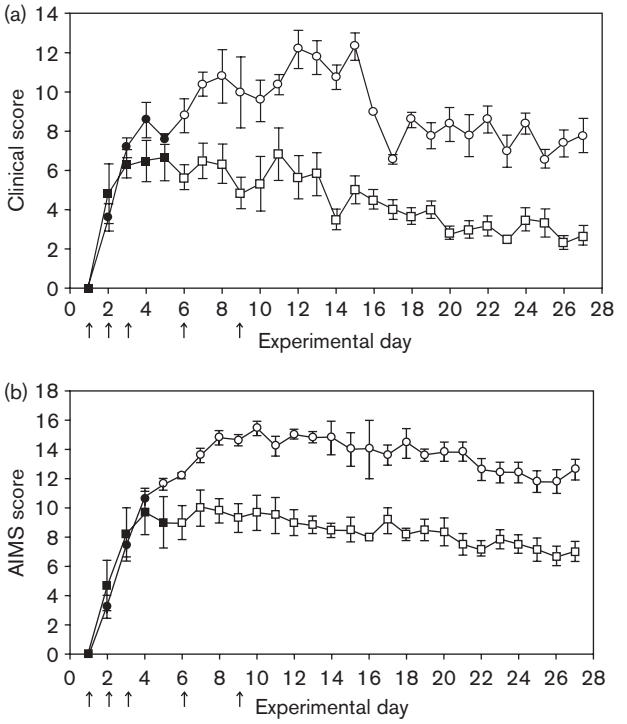
On all five test days, the performance of the modafinil-treated PD animals on the HEC task was clearly better than the performance of the vehicle-treated PD animals [see Fig. 2, $F(1,9) = 17.8$, $P = 0.002$, before administration (BA); $F(1,9) = 14.4$, $P < 0.005$, after administration (AA); *t*-test, $P < 0.05$], though an improvement over time of the performance of the vehicle-treated PD animals was also observed [$F(3,2) = 38.2$, $P < 0.025$ (BA)].

The HEC of the modafinil-treated PD animals was comparable to the performance before disease induction; only at days 13 and 20 was the performance slightly lower (paired *t*-test, $P < 0.05$). The HEC of the vehicle-treated PD animals was worse than at baseline level on all test days (paired *t*-test, $P < 0.05$).

Locomotor activity

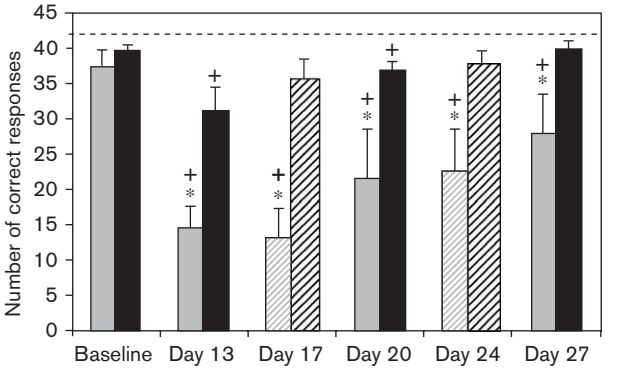
The modafinil-treated PD animals were more active in the Bungalow test than the vehicle-treated PD animals

Fig. 1



Mean ± SEM of the clinical (a) and abnormal involuntary movement scale (AIMS) (b) score before daily administration. Circles indicate the vehicle-treated Parkinson's disease (PD) animals ($n = 5$) and squares modafinil-treated PD animals ($n = 6$). On experimental days 14–16, only $n = 2$–4 were scored, owing to magnetic resonance imaging and magnetic resonance spectroscopy scans of some of the animals (data not shown in this paper). Arrow indicates day of 1-methyl-1,2,3,6-tetrahydropyridine injection. Modafinil or vehicle was given from day 1 to 27. Open data point indicates significant difference between vehicle-treated and modafinil-treated PD animals (*t*-test, $P < 0.05$).

Fig. 2



Mean + SEM of performance in the hand–eye coordination (HEC) task of the vehicle-treated Parkinson's disease (PD) animals ($n = 5$, gray bars) and modafinil-treated PD animals ($n = 6$, black bars). Solid bars indicate the results before administration of modafinil. Striped bars indicate results 2 h after administration. Dashed line indicates maximum number of trials. *$P < 0.05$ vehicle-treated PD animals vs. modafinil-treated PD animals. +$P < 0.05$ before vs. after disease induction.

[see Fig. 3, $F(1,9) = 9.9$, $P < 0.02$ (BA); $F(1,9) = 14.1$, $P < 0.005$ (AA)]. More specific, the locomotor activity of the modafinil-treated group was significantly higher on experimental days 17, 24 and 27 (t-test, $P < 0.05$), but a difference between the two treatments was also present on experimental day 13 (t-test, $P = 0.09$).

The activity of the modafinil-treated PD animals was comparable to the baseline activity before PD induction. The activity of the vehicle-treated PD animals was clearly reduced compared with their baseline values on all test days (paired t-test, $P < 0.05$, except day 27).

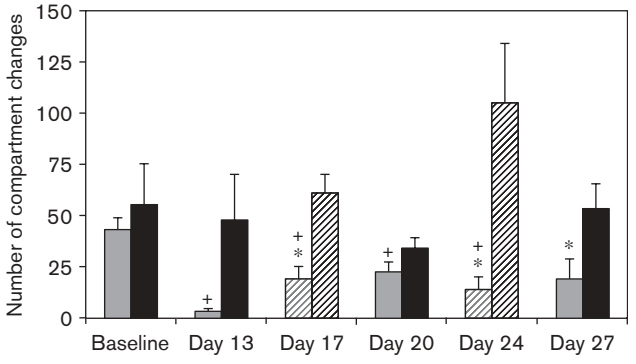
Small fast movements test

The small fast movements were tested to establish whether modafinil was able to restore the lack of these fine motor movements after MPTP. Vehicle-treated PD animals showed fewer small fast movements than before the disease induction (Fig. 4). This was clear after experimental days 13, 17 and 20 (paired t-test, $P < 0.05$). The small fast movements of the modafinil-treated PD animals were at the same level as before disease induction. Therefore, a difference between the experiment groups was also found [$F(1,9) = 16.7$, $P < 0.01$ (AA); $F(1,9) = 9.2$, $P < 0.02$ (BA)].

Human threat test

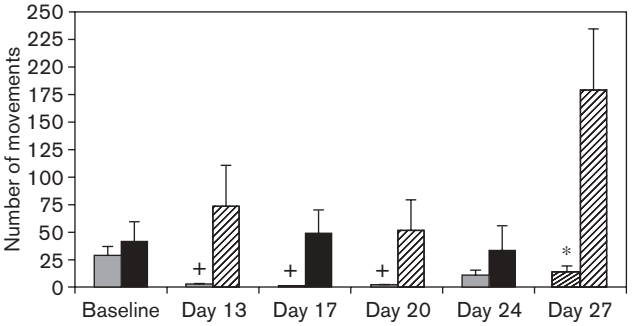
The two anxiety-related parameters of the HTT, namely the 'number of body postures' and 'time spent in front', did not differ between the two treatment groups [data not shown; body postures: $F(1,8) = 0.006$ (BA), $F(1,8) = 0.14$ (AA), NS; front: $F(1,9) = 2.1$ (BA), $F(1,9) = 0.12$ (AA), NS]. No changes were found in both treatment groups compared with baseline.

Fig. 3



Mean + SEM of locomotor activity in the Bungalow test of the vehicle-treated Parkinson's disease (PD) animals ($n = 5$, gray bars) and modafinil-treated PD animals ($n = 6$, black bars). Solid bars indicate the results before administration. Striped bars indicate results two hours after administration. *$P < 0.05$ vehicle-treated PD animals vs. modafinil-treated PD animals. + $P < 0.05$ before vs. after disease induction.

Fig. 4



The number of small fast movements (mean + SEM) of vehicle-treated Parkinson's disease (PD) animals ($n = 5$, gray bars) and modafinil-treated PD animals ($n = 6$, black bars). Solid bars indicate the results before administration. Striped bars indicate results 2 h after administration. An overall difference was found between the experimental groups. *$P < 0.05$ vehicle-treated PD animals vs. modafinil-treated PD animals. + $P < 0.05$ before vs. after disease induction.

The activity parameter of the HTT: 'the number of position changes' showed a significant difference between the vehicle and modafinil-treated PD animals [see Fig. 5; $F(1,9) = 17.5$, $P = 0.002$ (BA); $F(1,9) = 13.0$, $P = 0.001$ (AA)]. The difference in activity between the two treatment groups was observed on all test days (t-test, $P < 0.05$; except day 27, $P = 0.08$). The activity of the modafinil-treated PD animals was comparable to baseline values (except days 24 and 27). The activity of the vehicle-treated PD animals was clearly reduced compared with baseline values (paired t-test, $P < 0.05$; except day 27).

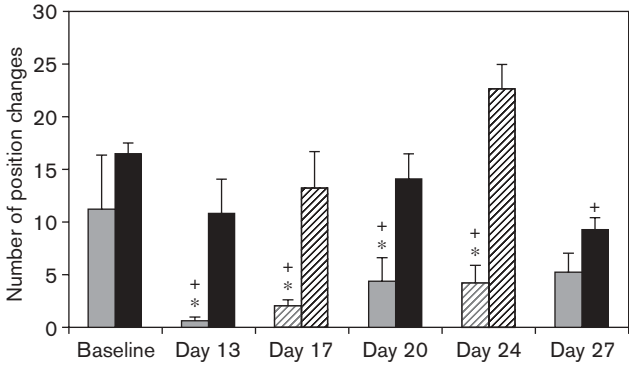
Startle response

Neither the shape of the curve nor the timing of the startle response of both groups was affected after the induction of PD by MPTP (see Fig. 6). One animal of the vehicle group was considered an outlier at baseline, because of its extreme startle response and was therefore omitted in the analysis. A decrease of the amplitude was observed over time [days 13, 20 and 27; vehicle: $F(2,6) = 4.6$ $P = 0.06$; modafinil: $F(2,10) = 5.3$ $P < 0.05$], but this habituation of the startle response was also seen in naïve marmosets.

Difference symptomatic and neuroprotective effects of modafinil

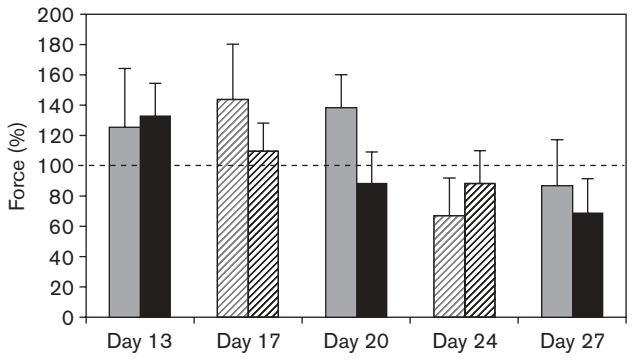
A shift in time of observation was included in the protocol, to rule out any temporary symptomatic effects of modafinil on motor function. The animals were observed daily before and after administration and the tests were executed on days 13, 20 and 27 before administration and on days 17 and 24 2 h after administration (the small fast movements test in opposite order).

Fig. 5



Mean + SEM of position changes in the human threat test (HTT) of the vehicle-treated Parkinson's disease (PD) animals ($n=5$, gray bars) and modafinil-treated PD animals ($n=6$, black bars). Solid bars indicate the results before administration. Striped bars indicate results 2 h after administration. $*P<0.05$ vehicle-treated PD animals vs. modafinil-treated PD animals. $+P<0.05$ before vs. after disease induction.

Fig. 6



The startle response (mean + SEM) as a percentage of the baseline values of the vehicle-treated Parkinson's disease (PD) animals ($n=4$, gray bars) and modafinil-treated PD animals ($n=6$, black bars). Solid bars indicate the results before administration. Striped bars indicate results 2 h after administration. Dashed line indicates baseline level (100%).

No difference was found between the clinical and AIMS score obtained before and 2 h after the treatment. On most behavioral tests, no difference was found between the moments of execution of the tests. Only in the two activity tests (locomotor activity and number of position changes of the HTT), activity on day 24 (AA) was higher than the activity on days 20 and 27 (BA; see Figs 3 and 5; *t*-test, $P < 0.05$). More small fast movements were present on day 27 (AA) than on day 24 (BA; see Fig. 4; *t*-test, $P < 0.05$).

Monoamine levels

DA levels in the striatum of modafinil-treated PD animals were decreased to 41% of control DA level, whereas vehicle-treated PD animals showed a reduction of 95% (see Table 1). The DA metabolites, HVA and DOPAC, were decreased in the vehicle-treated PD animals compared with control values. In the modafinil-treated PD animals, HVA content was lower than control values, but higher than the level of vehicle-treated PD animals. Therefore, a change in the DA turnover, the ratio between degradation and synthesis of DA [(DOPAC + HVA)/DA] was found. DA turnover of the modafinil-treated PD animals was comparable to the turnover of control animals. Vehicle-treated PD animals had a higher DA turnover ratio than modafinil-treated PD animals and naïve animals. The 5-HT level was decreased in both MPTP-treated groups. The 5-HT metabolite, 5-HIAA, was slightly decreased in the vehicle-treated group; however, the modafinil-treated PD animals were not different from controls. Therefore, an increase in the turnover ratio between 5-HIAA and 5-HT was found in modafinil-treated PD animals but not in vehicle-treated PD animals. The NA levels of the three groups were comparable, because MPTP had no effect on these levels.

Discussion

The results of this study confirm previous findings with modafinil in MPTP-treated marmosets (Jenner *et al.*, 2000), and extend those findings with more extensive behavioral and neurochemical evaluations. The study confirms the protective effects of modafinil against parkinsonian symptoms induced by MPTP in marmoset monkeys on various behavioral aspects and monoamine levels. It also generates information on the sensitivity of behavioral tests for the effects of MPTP, which has not been extensively studied in the marmoset. Clinical and abnormal involuntary movement scores showed a clear difference between the modafinil-treated PD animals and vehicle-treated PD animals. Locomotor activity, HEC and small fast movements of the modafinil-treated PD animals were comparable to values before disease induction and were clearly better than in vehicle-treated PD animals. DA levels in the striatum showed similar results, although the levels of the modafinil-treated group were lower than control values.

As the balance of neurotransmitters in the basal ganglia is disturbed by MPTP, tests measuring movement-related behavior are the most sensitive. Locomotor activity is an often used and well-validated parameter in MPTP-marmoset studies (Jenner *et al.*, 2000; Kupsch *et al.*, 2001). In the present study, two other tests, namely HEC and small fast movements, were also included, and these proved to be highly sensitive to MPTP-induced deficits. The effect of MPTP on the startle response was also tested. Our results show that neither the shape of the curve nor the timing of the startle response was changed by MPTP. This is in accordance with the study of Leng *et al.* (2004) in the MPTP-mouse model. Both

Table 1 Monoamine and metabolite levels (mean ± SEM) in μg/g tissue in the striatum of the vehicle ($n=5$) and modafinil ($n=4$)-treated PD animals compared with untreated control ($n=6$) levels

	Control	MPTP + vehicle	MPTP + modafinil
Dopamine	5.47 ± 0.85	0.27 ± 0.08***	2.23 ± 0.05*/+ +
DOPAC	0.73 ± 0.06	0.16 ± 0.06***	1.14 ± 0.29 + +
HVA	5.37 ± 0.59	0.34 ± 0.09***	2.82 ± 0.48*/+ +
Dopamine turnover	1.24 ± 0.18	2.10 ± 0.29*	1.89 ± 0.31 +
Noradrenaline	0.49 ± 0.12	0.19 ± 0.04	0.35 ± 0.06
Serotonin	0.36 ± 0.05	0.18 ± 0.04*	0.15 ± 0.01*
5-HIAA	1.20 ± 0.11	0.76 ± 0.30	1.27 ± 0.08
Serotonin turnover	3.48 ± 0.4	4.42 ± 1.37	8.34 ± 0.27***/+

PD, Parkinson's disease; MPTP, 1-methyl-1,2,3,6-tetrahydropyridine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, 3-methoxy-4-hydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindole-3-acetic acid.
Dopamine turnover: [(DOPAC + HVA)/ DA]; serotonin turnover: (5-HIAA/5-HT); *** vs. control levels $P<0.001$; * vs. control levels $P<0.05$; + + vs. vehicle-treated PD animals $P<0.01$; + vs. vehicle-treated PD animals $P<0.05$ (ANOVA followed by *t*-test).

studies are in contrast with the delayed startle response found in Parkinson patients and reduced adjustment of the gait during startle stimuli (Vidailhet *et al.*, 1992; Nieuwenhuijzen *et al.*, 2005). An explanation for the different outcome lies in the changed noradrenergic neurotransmission in the Parkinson patients (Braak *et al.*, 2003) in contrast to the unchanged NA system in the marmoset MPTP model (our data; Waters *et al.*, 1987). The central noradrenergic neurotransmission controls the startle response, as demonstrated by reduced startle response after 6-OHDA lesions in the locus coerulus (Adams and Geyer, 1981) and in the Parkin null mouse, which has a clear loss of locus coerulus neurons, but not of the nigrostriatal DA system (von Coelln *et al.*, 2004). Anxiety-related behavior, as measured with the HTT, was not changed after the devastating effects of MPTP. It can be concluded that, despite deprived movements of vehicle-treated PD animals, anxiety-related behavior was unchanged as compared with before disease induction.

In this study, vehicle-treated PD animals showed an improvement of symptoms over time in clinical score and the three tests reflecting movement. This is a general outcome in marmoset monkeys, which is due to compensatory mechanisms to improve DA function, such as higher DA turnover, reflecting neuronal activity or an increase in susceptibility or amount of DA receptors (Bezard and Grossman, 1998). Recovery takes place during the first weeks after the MPTP induction and, depending upon the severity of the lesion, finally resulting in residual parkinsonian symptoms (Rose *et al.*, 1993).

Modafinil may act as a symptom-controlling drug by temporary short-lived effects, and as a neuroprotective drug. Therefore, in this study, behavior was observed twice a day (before and after administration) and an alternation in testing before or after administration was included in the behavioral tests (see Methods section). Measurement 2 h after administration showed increased locomotor activity (Bungalow test and the number of position changes in the HTT), which was not present when measured before administration. This psychostimulating property of modafinil is also apparent in naïve animals (van Vliet *et al.*, 2006). The small fast movements test showed a clear increase after modafinil treatment on the last test day, when tested after administration. When modafinil was given to naïve animals, the small fast movements were not changed (data not shown). As the small fast movements of the modafinil-treated PD animals were comparable to baseline, also after modafinil administration, this extreme behavior on this particular day is probably due to external factors.

The neuroprotective properties of modafinil have already been shown at a neuronal level in both mice and marmoset MPTP models, whereas tyrosine hydroxylase immunoreactivity, a marker of viable dopaminergic neurons, and DA uptake, were higher in protected animals than in vehicle-treated PD animals (Fuxe *et al.*, 1992; Jenner *et al.*, 2000). The mechanism of protection of modafinil has not yet been clarified. It is clear that modafinil does not act as an MAO-B inhibitor, to obstruct conversion of MPTP into MPP + , the actual damaging compound, as this is ineffective if administered 5 min after MPTP (Sundstrom and Jonsson, 1986). Fuxe *et al.* (1992) showed that the neuroprotective properties of modafinil against MPTP are independent of the time of administration (15 min before until 3 h after administration). Therefore, modafinil does not act as a DA uptake blocker, because a blocker cannot prevent damage when administrated 2 h after MPTP (Sundstrom and Jonsson, 1986).

The effects of modafinil on GABA and glutamate release in distinct areas can play a role in the protection of the neurons. Modafinil inhibits GABA release in areas involved in the direct and indirect pathways of the basal ganglia–thalamus–cortex loop (Ferraro *et al.*, 1997). The prevention of the inhibitory effects of GABA by modafinil can result in a normalization of the MPTP-induced disturbed balance of the basal ganglia-thalamo-cortical circuitry, especially in the indirect pathway between the striatum and the external globus pallidus (Wichmann and DeLong, 1998). The stimulation of glutamate release in the ventrolateral and ventromedial thalamus (Ferraro *et al.*, 1997) can result in an increased excitatory output towards the cortex and therefore restore the dysfunctional motor loop (Wichmann and DeLong, 1998). The improved function of the dysfunctional motor loop is reflected in the present study, in which the motor behavior of the modafinil-treated PD animals was nearly normal.

In this study, two things became apparent during the period of disease induction. First, the first three

subsequent MPTP injections resulted in comparable observational scores, both the clinical score and the involuntary movements scale, in both treatment groups. During the following days, the scores of modafinil-treated PD animals stayed at the same level, whereas scores of vehicle-treated PD animals continued to worsen. Second, the last two MPTP injections on days 6 and 9 did not affect the modafinil-treated PD animals. In the marmoset study of Jenner *et al.* (2000), a comparable picture was shown: MPTP injections were given on five subsequent days and after this period the difference between the modafinil and vehicle treatments became apparent, owing to an improvement of symptoms of the modafinil-treated group over time, whereas in our study a stable level of motor deficits was reached. An explanation of the delayed protective effects of modafinil can be the reduction of excitotoxicity, as discussed in other studies of the neuroprotective effects of modafinil (see Introduction). Modafinil is able to increase the glutamine synthase activity in glial cells resulting in a reduction of glutamate (Touret *et al.*, 1994). The number of glial cells is increased after MPTP in mouse and marmoset (Mackenzie *et al.*, 1997; Kurosaki *et al.*, 2004) and this activation occurs within a time frame that enables these glial cells to participate in the dopaminergic demise (Teismann *et al.*, 2003). The more the glial cells that are present due to MPTP, the more the glutamate is removed due to modafinil administration, and the less excitotoxicity will take place and therefore more cells are protected. In the vehicle-treated group the excitotoxicity will continue to result in more damage. The insensitivity of the modafinil-treated PD animals against the last two MPTP injections could on the one hand result from the above-described processes, but on the other hand could also result from a change in neurotransmitter balance in the basal ganglia after repeated modafinil treatment.

Monoamine levels in the striatum of MPTP-treated animals were in line with the behavioral observations. DA levels in the modafinil-treated PD animals were lower than control values, although behavior of these animals was nearly normal. The reason for this deviation in the parameters might be that, owing to compensatory mechanisms, more than 60% of the DA neurons have to be lost before manifestation of the parkinsonian symptoms (Dauer and Przedborski, 2003). The higher DA turnover observed in vehicle-treated PD animals has also been reported in other marmoset MPTP studies and parkinsonian patients (Scatton *et al.*, 1983; Rose *et al.*, 1989). The observed changes in 5-HT levels of the vehicle-treated PD animals are comparable to chronic and more severe MPTP studies (Perez-Otano *et al.*, 1991; Russ *et al.*, 1991). Modafinil-treated PD animals showed a remarkable change in 5-HT turnover, owing to reduced 5-HT levels but normal metabolite production. As the animals were 10 days off treatment before decapitation, a direct influence of modafinil can be excluded. Presumably, the direct or indirect protective effects or the sustained administration of modafinil could have increased the activity of the remaining serotonergic neurons in the striatum, as modafinil does affect 5-HT levels in the brain (Ferraro *et al.*, 2002).

In conclusion, this study confirms that modafinil has protective properties against MPTP damage of the substantia nigra neurons on functional outcome, as seen in clinical and abnormal involuntary movement scores and behavioral tests concerning movements and coordination, and on monoamine levels in the striatum. The focus on the functionality of neurons is an extension of earlier studies of neuroprotective effects of modafinil in PD models. It is as yet unclear what is the actual protective mechanism of modafinil, although it is likely to be a multifactorial drug effect interfering with acute cellular processes within the first hours after the intoxication, tempering the excitotoxicity and changing the neurotransmitter balance, resulting in a reduction of neuronal sensitivity and restoration of the basal ganglia-thalamocortical loop.

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