Chapter 6

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

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Abstract

The psychostimulant 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 (MPTP). The present study determines the protective effects of modafinil in the marmoset MPTP Parkinson model on behavior and on monoamine levels.

Twelve marmoset monkeys were treated with a total dose of 6 mg/kg MPTP. 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 three weeks. Modafinil largely prevented the MPTP-induced change in observed behavior, locomotor activity, hand-eye coordination and small fast movements, whereas the vehicle could not prevent the devastating effects of MPTP. Dopamine (DA) levels in the striatum of the vehicle + MPTP treated animals were reduced to 5% of control levels, whereas the DA levels of the modafinil + MPTP treated animals were reduced to 41% of control levels.

The present data suggest that modafinil prevents decrease of movement-related behavior and DA levels after MPTP intoxication and can be a potent pharmacological intervention in the treatment of Parkinson's disease (PD).

Introduction

In PD the output of the basal ganglia is irreversibly affected due to degeneration of the neuromelanin-containing DAergic neurons in the substantia nigra pars compacta (SNpc). This results in manifestation of symptoms including akinesia, postural instability, rigidity and resting tremors (Dauer and Przedborski 2003). PD is incurable, since present medications (predominantly with levodopa) do not counteract progression of the disease and long-term medication is associated with declined 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 no proven neuroprotective treatment yet (Clarke 2004).

The actual cause of PD is unknown. There is evidence 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 is a vigilance-stimulating compound and marketed for treatment of narcolepsy (Bastuji and Jouvet 1988). The mechanism of modafinil is not clear, although it is suggested to increase indirectly wakefulness via α1-noradrenergic neurotransmission (Duteil et al. 1990) but also γ-aminobutyric acid (GABA) release is reduced by it in sleep-related areas and striatum (Ferraro et al. 1996, 1998). Modafinil influences 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 prevented increases in toxic aspartate and glutamate levels after striatal ischemic injury caused by endothelin-1 in rats (Ueki et al. 1993b) and it prevented development of lesions in the hippocampus induced by the neurotoxic nerve gas soman (Lallemant et al. 1997). After partial transsection of the DA pathway (Ueki et al. 1993a) and also in 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 suggest that modafinil could be a candidate drug for neuroprotection in PD at behavioral and neuronal level. However, more insight into the effects of modafinil on different DA and non-DA-related symptoms and the relation to neuronal function is 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. Earlier studies did not include these parameters.

This MPTP Parkinson model is the most used experimental model for PD (Dauer and Przedborski 2003). The neurotoxic agent MPTP selectively damages neurons in the SNpc by blocking the electron transport chain of the mitochondria

leading to a loss in mitochondrial function resulting in a depletion of ATP and eventually cell death. MPTP is effective in mice and marmosets. However, the mouse MPTP model is 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 after MPTP treatment clear and lasting behavioral features, which reflect many aspects of human Parkinson symptoms (Jenner and Marsden 1986). Even a clinically used observational scale for involuntary movements (AIMS) can be applied to the marmoset without adaptation (Di Monte et al. 2000).

In the present study, the protective effects of modafinil are tested on behavior using two extensive behavioral observation scales for PD and functional tests measuring locomotor activity, hand-eye coordination, small fast movements and the startle response. The human threat test was applied to measure whether anxiety-related behavior is sensitive for changes induced by MPTP and is possibly 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 DAergic output from the SNpc, are most heavily affected due to reduction of the neurons in the SNpc. Metabolites and other monoamines, noradrenaline (NA) and serotonin (5-HT) can also be as markers for neuronal damage and neuroprotection and are studied in brains of vehicle and modafinil treated parkinsonian animals and control brains.

In addition to 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. Results of the measurements with magnetic resonance imaging (MRI) and spectroscopy (MRS) and with immunohistochemistry will be covered in chapter 7.

Materials and methods

Animals

Adult male and female marmoset monkeys *(Callithrix jacchus)*, aged 2-6 years with initial body weights between 350-550 g were obtained from the Biomedical Primate Research Centre (BPRC), The Netherlands and Harlan, United Kingdom. The ambient temperature in the housing room was regulated at 25 ± 2 °C and the relative humidity was always >60%. A 12-hour light-dark cycle was maintained, lights on from 7 am to 7 pm. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The independent 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 s.c. over 9 days (day 1: 2 mg/kg and days 2, 3, 6 and 9: 1 mg/kg). Six of these animals (4 males; 2 females) received a daily oral dose of 100 mg/kg modafinil from experimental day 1 until day 27. The remaining six animals (3 males; 3 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 (chapter 2), which was in accordance with a 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 contribution to research. The oral modafinil or vehicle treatment was given directly after the s.c. MPTP injections. Of the vehicle group one animal died during the anesthesia procedure before the MRI-scan, therefore the data of this animal is omitted.

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

The occurrence of parkinsonian symptoms were observed daily before and after administration of the treatment using two rating scales: clinical score and AIMS. On day 13, 17, 20, 24 and 27 the behavioral tests, namely the hand-eye coordination task, locomotor activity, startle response and small fast movements were tested in noninvasive test systems. The human threat test was executed on these days simultaneously with the behavioral observations. Before disease induction, the animals were trained on the hand-eye coordination task and baseline values of all test systems were obtained. The 'after administration' behavioral observations and tests were started two hours after administration. This time span reflects the peak activity of modafinil in marmoset monkeys based on the pharmacokinetic results of modafinil in our institute (Philippens et al. 2006) and the study of Jenner et al. (2000) and the t_{max} of modafinil in humans (Robertson and Hellriegel 2003).

As modafinil is a psychostimulant, these temporary symptomatic effects on motor function can be present besides the neuroprotective effects of modafinil. Therefore, a distinction is made between before and after administration: the behavioral observations were done twice a day and most tests were performed either before the daily administration on day 13, 20 and 27 or two hours after administration on days 17 and 24. Only the small fast movements test was tested in 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 surroundings; immobility; rigidity and presence of tremors. The degrees 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 to monkey research in our institute for more than 10 years. 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 due to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Movements that occurred due to stimulation by the observer were rated one step lower than those observed spontaneously.

Hand-eye coordination task: An automated robot-guided apparatus with positive reinforcement as a motivating stimulus (small pieces of marshmallow) has been used to asses the hand-eye coordination (Philippens et al. 2000). The marmoset is placed in front of a window in the test panel (8 x 5 cm). A robot arm presents a reward behind the window. With this system three types of trials were performed: one using a non-moving 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 one minute to grasp the non-moving reward. Each type of trial was presented 14 times per 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 performance of the animal. Before the start of the study, all animals were trained to successfully grasp a minimum of 80% of the presented rewards.

Spontaneous exploratory behavior (Bungalow test): The levels of spontaneous 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 variables and has been extensively described and validated (Wolthuis et al. 1994, Philippens et al. 2000). The apparatus consists of four horizontally placed non-transparent boxes (23 x 23 x 23 cm) all interconnected by 6 PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. There was one animal per session. The animals could freely move and change from one compartment to another during the 20-minute 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.

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 to 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. Since the animal, situated in the plastic tube between the plates, was a conducting medium, any change in posture of the animal lead to a change of plate capacitance. Both plates were, via a buffer amplifier, driven 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 human threat test is a non-human primate putative model of anxiety. 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). The 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 x 60 x 60 cm) with a hanging basket in the back of the cage, a wooden board (20 x 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-minute 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 pilo-erection), 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, 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 apparatus to measure startle response in marmoset monkeys has been described earlier and has been 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 (inter stimulus interval: 14 ± 4 seconds). For the duration of 200 ms, directly after the stimulus presentation, the force exerted by the animal was registered. The startle reflex was represented by the amplitude of the response.

High performance liquid chromatography (HPLC) 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, ten 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 M perchloric acid containing 20 ng/ml 3,4-dihydroxybenzylamine hydrobromide (Sigma Chemical co., St. Louis, USA) and 20 ng/ml (±)-isoproterenol hemisulfate salt (Sigma Chemical co., St. Louis, USA) as internal standards. Homogenate was centrifuged at 22,000 g for 30 minutes at 4 °C and 1 ml of supernatant was adjusted to about pH 4.0 with 250 µl 2 M 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 x 4 mm i.d., 5 µm particle size; Merck, Darmstadt, Germany) connected to a Thermo Separations Products (San Jose, CA, USA) model P100 solvent delivery pump and AS300 autosampler and a Coulochem II Model 5011 electrochemical detector (ESA, Bedford, MA, USA). The mobile phase consisted of a 30 mM citrate/40 mM phosphate buffer, pH 4.0, containing 0.27 mM Na_2EDTA, 1.8 mM heptanesulphonic 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%.

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 (RM) analysis was applied on the results of day 13, 20 and 27 and of day 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 (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 day 13 and 20 vs. day 17, and pooled data of day 20 and 27 vs. day 24 with independent t-tests. Because 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 day 17 and 24 vs. day 20 with independent t-tests.

The difference between the monoamine levels of each treatment was tested with an 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 (Fig. 1). Hereafter, a discrepancy between the groups emerged. The vehicle treated parkinsonian animals showed a mild parkinsonian symptomatology, whereas the modafinil treated parkinsonian animals (from day 3) were not affected by the last MPTP injections and ended with rather weak parkinsonian symptoms. In the modafinil treated parkinsonian group all parameters of the clinical score were present until day 17. After day 17 decreased appetite, rigidity and tremors were most pronounced symptoms, whereas scores of the inadequacy of grooming, apathy and immobility were 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 on the hand-eye coordination task of the modafinil treated parkinsonian animals was clearly better than the performance of the vehicle treated parkinsonian animals (Fig. 2; RM, $p=0.002$ (before administration (BA)) and $p=0.004$ (after administration (AA)); t-test $p<0.05$). Although an improvement of the performance of the vehicle treated parkinsonian animals over time was observed (RM, $p=0.023$ (BA)).

The hand-eye coordination of the modafinil treated parkinsonian animals was comparable to the performance before the disease induction, only at day 13 and 20 the performance was slightly lower (paired t-test, $p<0.05$). The hand-eye coordination of the vehicle treated parkinsonian animals was worse on all test days than at baseline level (paired t-test, $p<0.05$).

Locomotor activity

The modafinil treated parkinsonian animals were more active in the Bungalow test than the vehicle treated parkinsonian animals (Fig. 3; RM, $p=0.012$ (BA), $p=0.004$ (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 also on experimental day 13 a difference between the two treatments was present (t-test, $p=0.09$).

The activity of the modafinil treated parkinsonian animals was comparable with the baseline activity before PD-induction. The activity of the vehicle treated parkinsonian animals was clearly reduced compared to 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. The small fast movements of the vehicle treated parkinsonian animals were less present than before the disease induction (Fig. 2). This was clear after experimental days 13, 17 and 20 (paired t-test, $p<0.05$). The small fast movements of the modafinil treated parkinsonian animals were at the same level as before the disease induction. Therefore, a difference between the experiment groups was also found (RM, $p=0.006$ (AA), $p=0.014$ (BA)).

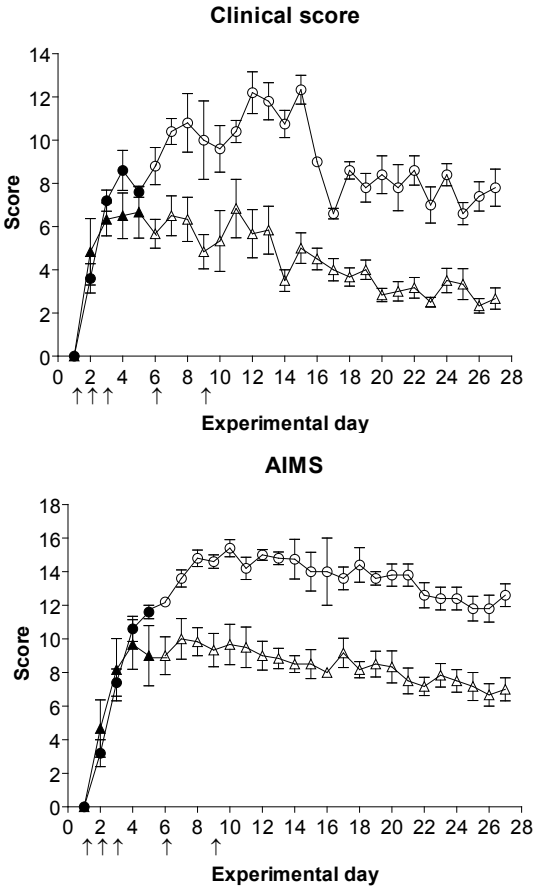
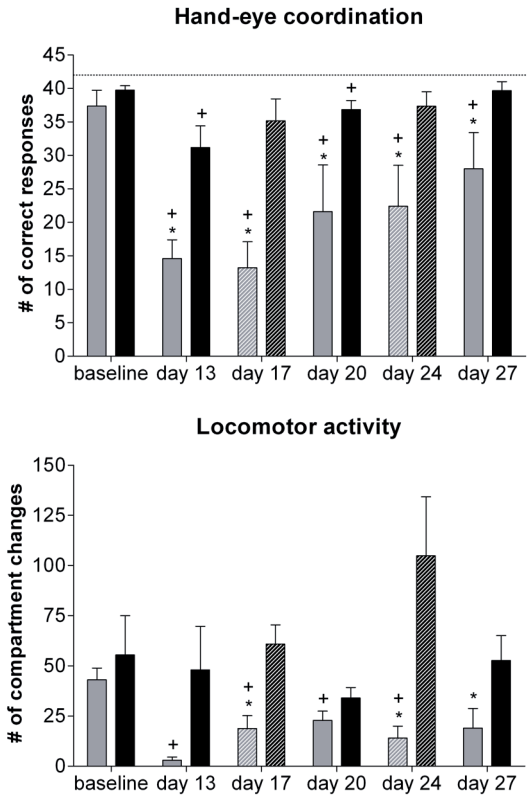


Fig. 1 Behavioral observation scales. Mean (± SEM) of the clinical (a) and AIMS (b) score before daily administration. Circles indicate the vehicle treated parkinsonia animals and triangles modafinil treated parkinsonian animals. On experimental days 14-16 only n=2-4 were scored due to MRI and MRS-scans of part of the animals (chapter 7). Arrows indicate day of MPTP injection. Modafinil or vehicle was given from day 1-27. Open data points indicate significant difference between vehicle and modafinil treated parkinsonian animals (t-test, $p<0.05$).

Human threat test

The two anxiety-related parameters of the human threat test, namely the 'number of body postures' and 'time spent in front', did not differ between the two treatment groups (data not shown; RM, body postures and front, $p>0.05$). No changes were found in both treatment groups compared to baseline.

The activity parameter of the human threat test: 'the number of position changes' showed a significant difference between the vehicle and modafinil treated parkinsonian animals (Fig. 2; RM, $p=0.002$ (BA), $p=0.001$ (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 parkinsonian animals was comparable to baseline values (except day 24 and 27). The activity of the vehicle treated parkinsonian animals was clearly reduced compared to baseline values (paired t-test, $p<0.05$, except day 27).



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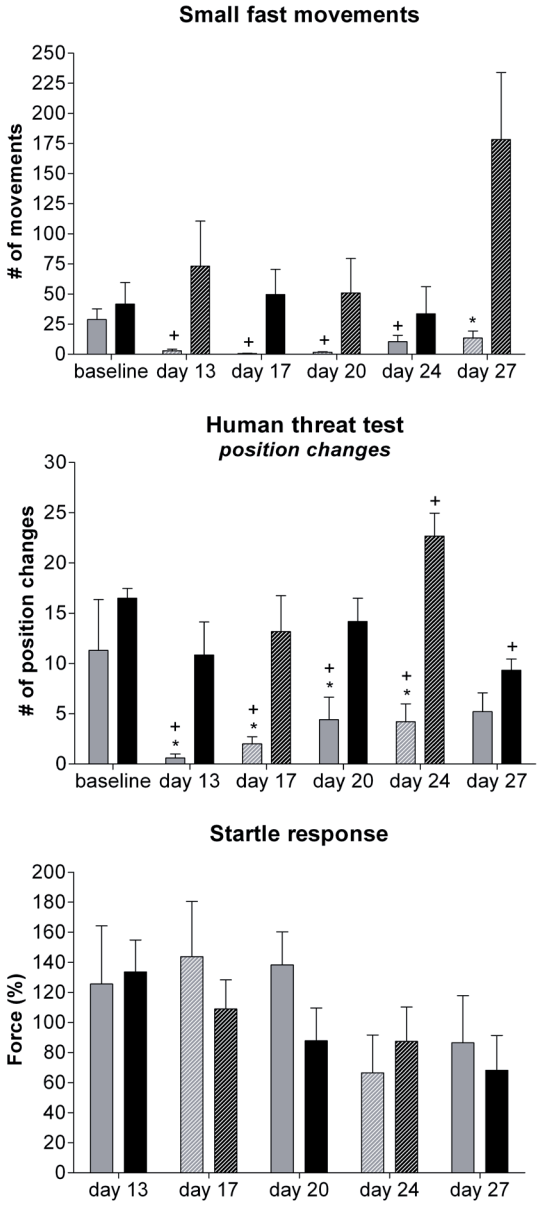

Fig. 2 Behavioral tests. Grey bars show mean (+ SEM) of vehicle treated parkinsonian animals. Black bars show mean (+ SEM) of modafinil treated parkinsonian animals. Solid bars indicate the results before administration. Striped bars indicate results two hours after administration. Dotted line indicates maximum correct responses. *p<0.05 vehicle vs. modafinil treated parkinsonian animals. +p<0.05 before vs. after disease induction.

Startle response

Neither the shape of the curve nor the timing of the startle response of both groups was affected after the induction of parkinsonian symptoms by MPTP (Fig. 2). 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. There was a decrease of the amplitude over time (day 13, 20 and 27, RM, vehicle: p=0.06, modafinil: p=0.03), but this habituation of the startle response was also seen in naïve marmosets.

Difference symptomatic and neuroprotective effects of modafinil

In the protocol a shift in time of observation was included to rule out the temporary symptomatic effects of modafinil on motor function. The animals were observed daily before and after administration and the tests were executed on day 13, 20 and 27 before administration and on days 17 and 24 two hours after administration (the small fast movements test in opposite order). No difference was found between the clinical and AIMS score obtained before and two hours after treatment. Also 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 human threat test), the activity on day 24 (AA) was higher than the activity of days 20 and 27 (BA, Fig. 2, t-test, $p<0.05$). More small fast movements were present on day 27 (AA) than on day 24 (BA; Fig. 2; t-test, $p<0.05$).

Monoamine levels

DA level in the striatum of modafinil treated parkinsonian animals was reduced to 41% of control DA level, whereas vehicle treated parkinsonian animals showed a reduction of 95% (see Table 1). The DA metabolites, HVA and DOPAC, were reduced in the vehicle treated parkinsonian animals compared to control values. In the modafinil treated parkinsonian animals HVA content was lower than control

Table 1 Monoamine and metabolite levels. Mean ± SEM in μg/g tissue of the striatum of untreated controls or vehicle and modafinil treated parkinsonian animals.

	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 ± 0.40	4.42 ± 1.37	8.34 ± 0.27 ***/+

Dopamine turnover: ((DOPAC +HVA)/ DA); serotonin turnover: (5-HIAA/5-HT); ***p<0.001 vs. control levels, *p<0.05 vs. control levels, ++p<0.01 vs. vehicle treated parkinsonian animals, +p<0.05 vs. vehicle treated parkinsonian animals (ANOVA followed by t-test).

values, but higher than the level of vehicle treated parkinsonian animals. Therefore, a change in the DA turnover, the ratio between degradation and synthesis of DA ((DOPAC + HVA)/DA) is found. DA turnover of the modafinil treated parkinsonian animals was comparable to the turnover of control animals. The vehicle treated parkinsonian animals had a higher DA turnover ratio than the modafinil treated parkinsonian animals and naïve animals. The 5-HT level was reduced in both MPTP treated groups. The 5-HT metabolite, 5-HIAA, was slightly reduced in the vehicle treated group, however the modafinil treated parkinsonian animals were not different from controls. Therefore, an increase in the turnover ratio between 5-HIAA and 5-HT was found in modafinil treated parkinsonian animals and not in vehicle treated parkinsonian animals. The NA levels of the three groups were comparable since MPTP had no effect on these levels.

Discussion

This study shows 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, as this is not extensively studied in the marmoset. Clinical and abnormal involuntary movement scores show a clear difference between the modafinil treated parkinsonian animals and vehicle treated parkinsonian animals. Locomotor activity, hand-eye coordination and small fast movements of the modafinil treated parkinsonian animals were comparable to values before disease induction and were clearly better than in vehicle treated parkinsonian animals. DA levels in the striatum showed the same 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. The locomotor activity is an often used and well validated parameter in MPTP-marmoset studies (Jenner et al. 2000, Kupsch et al. 2001). In this study two other tests, namely hand-eye coordination task and small fast movements, were 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 shape of the curve nor 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 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). 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-hydroxydopamine lesions in the locus coerulus (LC) (Adams and Geyer 1981) and in the Parkin null mouse, which has a clear loss of LC neurons, but

not of the nigrostriatal DA system (von Coelln 2004). Anxiety-related behavior as measured with the human threat test was not changed after the devastating effects of MPTP. It can be concluded that despite deprived movements of vehicle treated animals, anxiety-related behavior as compared to before disease induction was not changed.
In this study vehicle treated parkinsonian animals show an improvement of symptoms over time on clinical score and three tests reflecting movement. This is a general outcome in marmoset monkeys. This is due to compensatory mechanisms to improve DA function like higher DA turnover, reflecting neuronal activity or 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, eventually resulting in residual parkinsonian symptoms (Rose et al. 1993).

Modafinil may act as a symptom controlling drug by temporary short-living 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 is included in the behavioral tests (see materials and methods section). Measurement two hours after administration showed increased activity (Bungalow test and the number of position changes of human threat test) which was not present when measured before administration. This psychostimulating property of modafinil is also apparent in naïve animals (chapter 2). The small fast movements test showed a clear increase after modafinil treatment on the last test day, when tested after administration. When modafinil is given to naïve animals the small fast movements are not changed (chapter 2). As the small fast movements of the modafinil treated parkinsonian 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 were already shown at neuronal level in both mice and marmoset MPTP models, whereas tyrosine hydroxylase immunoreactivity, a marker of viable dopaminergic neurons, and DA uptake was higher in protected animals than in vehicle treated parkinsonian animals (Fuxe et al 1992, Jenner et al. 2000). The mechanism of protection of modafinil is not clarified yet. Clear is that modafinil does not act as a MAO-B inhibitor, to obstruct conversion of MPTP into 1-methyl-4-phenylpyridinium (MPP^+), the actual damaging compound, as these are ineffective if administered 5 minutes after MPTP (Sundstrom et al. 1986). Fuxe et al. (1992) showed that the neuroprotective abilities of modafinil against MPTP are independent of the time of administration (15 minutes before until 3 hours after administration). Therefore, modafinil is not a DA uptake blocker, because a blocker cannot prevent damage when administrated 2 hours after MPTP (Sundstrom et al. 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 liquidation 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 (Wichman 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 (Wichman and DeLong 1998). The improved function of the dysfunctional motor loop is reflected in this study, whereas the motor behavior of the modafinil treated parkinsonian animals is 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 parkinsonian animals stayed at the same level, whereas scores of vehicle treated parkinsonian animals still worsened. Second, the last two MPTP injections on days 6 and 9 did not affect the modafinil treated parkinsonian animals. In the marmoset study of Jenner et al. (2000) a comparable picture was shown: The MPTP injections were given on five subsequent days and after this period the difference between the modafinil and vehicle treatments became apparent due 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 covering 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 DAergic demise (Teisman et al. 2003). The more glial cells are present due to MPTP, the more 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 parkinsonian animals against the last two MPTP injections can on one hand be a result of the above described processes, but on the other hand the result of a change in neurotransmitter balance in the basal ganglia after the 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 parkinsonian animals were lower than control values, although behavior of these animals was nearly normal. The reason of this deviation in the parameters might be that due 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 parkinsonian 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 parkinsonian animals are comparable with chronic and more severe MPTP studies (Perez-Otano et al. 1991, Russ et al. 1991). Remarkable is the changed 5-HT turnover in the modafinil treated parkinsonian animals due to reduced 5-HT levels, but normal metabolite production. As the animals are 10 days off-treatment before decapitation, 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 shows 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 about neuroprotective effects of modafinil in PD-models. It is as yet unclear what the actual protective mechanism of modafinil is, although it is likely 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 reduction of the sensitivity of the neurons and restoration of the basal ganglia-thalamo-cortical loop.