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14. ABSTRACT
It is still largely unknown what the general course is in the progression of Parkinson's disease (PD). Presumably more than one factor is responsible. There is evidence suggesting that metabolic compromise, excitotoxicity and oxidative stress are involved in the neurodegenerative process causing PD. To investigate the connection of excitotoxicity and oxidative stress with metabolic compromise in the development of the disease, anti-excitotoxic treatment with riluzole and anti-oxidant treatment with EGCG will be compared to untreated controls and to a standard treatment with L-DOPA in a MPTP induced Parkinson model. We hypothesize that critical changes indicating the nature of the gradual patho-physiological changes leading to PD will be revealed if anti-oxidants or anti-excitatory treatments are given in a situation where the brain is susceptible to develop PD. The comparison, of the results on the different levels of research, between the neuroprotective regimes and the symptom control drug L-DOPA will give insight in the relative role of the different markers for neuroprotection and behavioral output. In particular relatively new technologies such as differential proteomics and sleep research will yield novel insights. In this report period new test methods were developed, the use of brain imaging or neurophysiology was validated and the dose range finding of the test compounds was performed. The highest sign-free dose will be used in the neuroprotective experiments.

15. SUBJECT TERMS
Parkinsons' Disease, marmoset, MPTP, neuroprotection, oxidative stress, excitotoxicity, metabolic compromise, behavior

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1. INTRODUCTION

With the increasing average age of today's world population the prevalence of neurodegenerative diseases like Parkinson's disease (PD) increases. At this moment about 15% of the population over 65 years old suffers from this disease. However the cause, onset and general course of the disease are still not completely unraveled. PD patients have an extreme form of neurodegeneration especially in the *substantia nigra*. The decrease in dopaminergic neurons makes signaling to the *striatum* less effective and therefore patients suffer primarily from motor dysfunction like tremors, bradykinesia and dyskineasia which results in serious functional impairment. Saving motor function means saving dopamine neurons to make sure the inhibitory effect of the dopamine stays intact (reviewed by Lang and Lorenzo, 1998).

There is evidence suggesting that the 'lethal triplet', metabolic compromise, excitotoxicity and oxidative stress, are involved in the neurodegenerative process causing PD (Alexi *et al*., 2000; Jenner, 2003). Although extensive studies have been performed, the mechanism associated with the pathogenesis of PD is still not known: however several factors including oxidative stress, mitochondrial dysfunction, environmental toxins, proteasome dysfunction and genetic defects have been proposed to play a role (Betarbet *et al*., 2000).

The close releation between these factors suggest their possible interaction. Metabolic compromise in neurons results in a loss in mitochondrial function leading to a depletion of neuronal energy supply. This causes reduced membrane function and subsequent accumulation of intracellular Ca^{2+}, and to production of free oxygen and nitrogen radicals. These effects enter the neurons onto pathways leading to neuronal degeneration, i.e. apoptosis and/or necrosis. Ca^{2+} accumulation is similarly found after over-stimulation of glutamate receptors in excitotoxic conditions, which is also associated with enhanced production of toxic free radicals causing oxidative stress. These factors related to neuronal maintenance processes may be of great importance for the understanding of slow gradual neurodegenerative processes. In neurodegenerative disorders, already weakened neurons may not survive glutamate concentrations that would normally not be lethal (Doble, 1999). To limit the impact of PD, early diagnosis and subsequent neuroprotective treatment is a valid and relatively easy strategy: it is easier to save neurons than to replace lost neurons.

In this study PD will be mimicked in a non-human primate using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model. This specific neurotoxin induces degeneration through metabolic compromise of the dopaminergic neurons in the *substantia nigra* resulting in PD like symptoms (Colisimo et al., 1992; Fukuda, 2001). The effects of Riluzole (anti-excitotoxic) treatment, epigallocatechin-3-gallate (EGCG; anti-oxidative) treatment and (currently used) L-DOPA treatment will be compared to untreated controls in the marmoset MPTP model. Riluzole has shown to increase patient survival in the nerve degenerative disease ALS (Bensimon et al., 1994). Although the compound does induce apathy and sleepiness for a short time (Obinu et al., 2002; Philippens, 2006) it has generally accepted to have a well tolerated profile in human users (Bensimon et al., 1994). The 'drowsiness' does seem to have little effect on the motor and activity tests used in our lab nor on the clinical or movement scoring (Philippens, 2006). (-)-Epigallo-catechin 3-O-gallate (EGCG) is a compound derived from green tea and is beneficial for a number of conditions like obesity and cardiovascular failure (Chantre and Lairon, 2002, Morton et al., 2000). The positive effects of green tea or the effective anti-oxidant EGCG in brains have been generally accepted. Several studies have now been conducted to evaluate the effects of the compound on cell survival in PD models (Mandel and Youdim, 2004).

In the first year some test systems were evaluated and developed and the dose range finding studies have been performed.

- The use of the phMRI and neurophysiology measurements was evaluated. The impact of the scan procedure on the animals combined with the low yield of information especially from the phMRI made us decide not to go through with MRI in the actual experiment. MRI measurements limit the possibility of brain activity measurements, because of the metal electrodes used with EEG measurements. Because of the decision concerning the phMRI measurements, we can now precede with EEG brain activity measurement during sleep.
- Development of two new behavioral test methods: one for measuring the onset of movements, the so-called "Tower" and one to measure the dyskinesia, the so-called "Hourglass test".
- The dose response finding experiments were accomplished. Dose range effects of L-DOPA for chronic use were already performed in our institute. ECGC (5, 10 and 20 mg/kg) did not show any effect on the test systems used. Riluzole (1.25, 5, 10 and 20 mg/kg) showed a significant effect with the highest dose of 20 mg/kg in the observational tests. The activity was not affected.

Based on the dose range finding studies of the test compounds, doses for the efficacy experiments have been established. The highest sign-free dose is selected. For riluzole the highest dose without aversive effects is 10 mg/kg p.o. In the neuroprotection study animals will receive 10 mg/kg p.o. twice daily. This is also the dose which Obinu *et al*. used in their marmoset study (2004). For the EGCG no effects were found in all doses. Therefore, the dose of ECGC will be based on information from the literature. There is no information on EGCG dose in marmosets, in rodents daily oral dose varies from 0.5 to 50 mg/kg (Levites *et al.*, 2001; Goodin and Rosengren, 2003). Administration was done in several studies up to two weeks (Levites *et al.,* 2003). For this study 10 mg/kg twice a day p.o. will be used because large chronic i.p. dosages no longer protected again MPTP intoxication (Mandel and Youdim, 2004). The dose of L-DOPA is based on our experiences of this compound in counteracting the MPTP induced performance decline and symptoms (10 mg/kg twice a day p.o.). In this study we will test the full range of effects on protein expression, cell survival, neurophysiology, motor function and behavior. The impact of the MRI scan procedure on the animals combined with the low yield of information especially from the phMRI made us decide not to go through with MRI in the actual experiment. MRI measurements limit the possibility of brain activity measurements, because of the metal electrodes used with EEG measurements. Because of the decision concerning the phMRI measurements, we can now proceed with EEG brain activity measurement during sleep. PD patients suffer from problems during sleep especially related to the rapid eye movement (REM) sleep (Gagnon et al., 2002). Sleep and the diurnal rhythm are probably an important feature in the course of the disease because it is not only affected by the disease it can also be a part of progression of the disease (reviewed by Poewe and Högl., 2000). Cell regeneration as well as probably cell maintenance is strongly influenced by the amount and the content of sleep. Sleep is similarly affected in PD animal models: the amount of REM sleep affects the behavioral performance of Parkinsonian rats (Andrade et al., 1987) and sleep content is changed in MPTP treated marmosets (Almiral et al., 1999). To understand more about the interaction of sleep and Parkinsonism, this study will focus on the sleep architecture before, during and after disease introduction. This way a comparison can be made of the neurochallenged, neuroprotected and healthy sleep rhythm. The appearance and duration of different sleep stages can be analyzed using the EEG and EMG signal (Philippens *et al*., 2004) using the analysis program Polyman, according to Rechtschaffen and Kales (1968). The analysis program Polyman was developed at the Centre for sleep and wake disorders in The Hague (MCH), where it is used in the clinic. By using the same program the extrapolation towards the human situation is facilitated. Results will be presented in the next report period. Also two new behavioral test methods were developed: one for measuring the onset of movements, the so-called "Tower" and one to measure the dyskinesia, the so-called "Hourglass test". These tests will be used in the efficacy experiments. The link to human clinical testing will contribute to the understanding of the development and progression of PD and offers an approach to limit the aggravation of symptoms in patients.

2. STATEMENT OF WORK

2.1 Purpose of the study

This study will test the hypothesis that neuroprotection in an early stage of PD limits the progression of PD symptoms, and thus prevents the long term functional and pathological outcome. Furthermore, a multilevel integrative approach will gather information on protein expression patterns, on *substantia nigra* and whole brain pathophysiology, on physiology, and on behavioral aspects of PD development, as well as the possible role of neuroprotective drugs in the prevention of PD development. Critical changes on protein expression, physiology, and behavior indicating the nature of the gradual patho-physiological changes leading to PD will be revealed if anti-oxidants or anti-excitatory treatments are given in a situation where the brain is susceptible to develop PD. Since usually the therapy against PD symptoms is based on enhancing dopaminergic activity by L-DOPA or dopamine receptor agonists, we will include a clinical control situation using this treatment. However, the test procedure will be focussed on a neuroprotective approach; tests will only be performed in the absence of the test compounds to role out the therapeutic effects.
The integrative nature of the approach and the open eye towards human clinical validity will contribute to understanding the development and detection of PD symptoms, and offers an approach to stop progression of PD symptoms.

2.2 Major goals and objectives

At the end of the first year, the dose range finding experiments of the neuroprotective drugs will be established. Knowledge of the side effects of these compounds on the read-out systems used will help to interpret the results of the neuroprotective effects gathered in part three.

At the end of the second year the behavioral (like the motor related behavioral tasks and tests) and physiological (like clinical scoring, brain activity, body weight and body temperature) effects of the progression of PD on different parameters and test systems can be established. This will give insight into the usefulness of the selected markers as a tool in this neuroprotective study. This will help set the priorities and the major factors of interest for the further course of the study.

At the end of the third year the protective effects on protein content and cell damage by the compounds preventing oxidative stress or excitotoxicity can be given. This will give information on the interaction between the different aspects (metabolic compromise, excitotoxicity and oxidative stress) of the lethal triplet leading to PD. If the hypothesized mechanisms are indeed the basis for PD related neurodegeneration, anti-excitotoxins or anti-oxidants should be effective against the neuronal loss and its functional impairments. The comparison, of the results on the different levels of research, between the neuroprotective regimes and the currently used control drug L-DOPA will give insight in the relative role of the different markers for neuroprotection and behavioral output.

2.3 Measurable output

In this study the MPTP treated marmoset monkey will be used. A multilevel integrative approach is taken to investigate the benefits of preventing the occurrence of related mechanisms to the metabolic compromise induced by MPTP. Measurements include:

- Spontaneous exploration, general coordination and hand-eye coordination (HEC),
- Recording of activity, body weight, and body temperature,
- Clinical observations and abnormal involuntary movement scale (AIMS),
- Neurophysiological measurements during sleep,
- Tyrosine hydroxylase activity in the *substantia nigra*,
- Dopamine levels in the *striatum*,
- Protein content of *substantia nigra* cytoplasm and *striatum* synaptosome.

2.4 Material and methods

2.4.3 Animals

Thirthy marmoset monkeys (*callithrix jacchus)* were used within this report period (approved by the animal ethics committee #1985). The experiments were executed in three batches of ten monkeys each. All animals used were bred and raised at the Biomedical Primate Centre (BPRC), Rijswijk, The Netherlands. The animals were housed in individual cages (61*61*41 cm) in a temperature (23-25ºC) and humidity (60%) controlled primate facilitation. Night and day was constant in a 12 hour cycle, with lights off at 19:00h. Animals were fed daily with pellet chow. Diet was enriched with peanuts, carrots, broad beans, green beans, apple compote, fruit, raisins, sunflower seeds and an occasional grasshopper. Water was available ad libitum. All animals were provided with a varying cage environment.

2.4.4 Drugs

The anti-oxidant, used as neuroprotective in this study, (-)-Epigallocatechin 3-O-gallate (EGCG; Teavigo®) was provided bij DSM, Switserland. The anti-excitotoxic compound Riluzole (Rilutek) was obtained at Wippolder Pharmacy, Delft, Netherlands. Methylcellulose, L-DOPA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were obtained from Sigma Aldrich, St. Louis, USA.
Vehicle consisted of 2:1 water:Karvan cevitam®. For oral administration EGCG and L-DOPA were dissolved in vehicle and rilozule was dissolved in vehicle and 0.5% methylcelluclose. MPTP was dissolved in Saline.

2.4.5 Test methods

2.4.5.1 Disability scoring

- Clinical score
  The animal's general well being is scored using the clinical score. The effect of the compounds on clinical components is rated daily using the clinical score. Six different components are rated from 0 (=normal/healthy) to 4 (=severely affected). This score alights specific reactions of parkinsonism, like apathy (no interest in their surrounding), immobility, rigidity (measured by the stiffness of the legs and tail) and tremors but also more general parameters of well being like appetite, grooming (by inspection of their fur) bodyweight and temperature (subcutaneous temperature chip) are noted. Observation and scoring of the signs is done by an experienced observer standing in front of the cage.
- AIMS
  Motor ability of the animals is scored using the Abnormal Involuntary Movements Scale adapted from the clinic (Guy, 1979). The effect of the compounds on abnormal involuntary movements is rated daily and also extensively after compound administering. On 9 different items the animals movements are rated from 0 (=normal) to 4 (=severely affected), rating different facial aspects like tongue and jaw movements and mask face, limb movements like repetitive movements with the feet and overall body posture. Also the severity of the global judgment is taken into account. Observation and scoring of the signs is done by an experienced observer standing in front of the cage.

2.4.5.2 Behavioral test

- Hand-eye coordination test (HEC)
  The HEC was tested with an automated robot arm guided test system with a semi-randomized computer program according to Wolthuis *et al*., (1995) using positive enforcement (small pieces of marshmallow rewards) as stimulus. Animals are placed in a special cage (32.5*24*24 cm) with stainless steel bars at one side, spaced specifically to allow the animal to reach its arm at full length through a window (8*5 cm) with a sliding door. The door is opened and closed pneumatically and opens when a reward is presented at the tip of a 8.5 cm suction tube by the robot arm. Marshmallows are presented at three different speeds; still 0.00 m/s, slow moving at 0.04 m/s and fast moving at 0.08 m/s. A sound signal alerts the animal before each new stimulus after which the door slides open and the reward is presented at one of the speeds. A hit is registered

when the animal successfully retrieves the reward, the number of attempts and failures are also registered. The animals are alerted before each trial by a sound signal. Before the start of this test the animals are trained to grasp the reward from the suction tube. After the monkeys had reached a performance of 75% or more correct hits they are ready to start with this task. The total number of correct hits is used to measure the HEC performance.

- Bungalow test
  The bungalow test is an automated test to measure the exploratory loco-motor activity of the marmoset (Wolthuis et al., 1994; Philippens et al., 2000). The bungalow consists of four equal compartments (23*23*23 cm) connected with each other by six PVC tubes. The compartments are closed off at all sides except for the roof (small wiring). The tubes are also closed off. The animals are always placed in the same compartment before the start of a session after which they can move freely from one compartment to the other during a 20 min. period. A video tracking system and infra red beams in the tubes registers the movement pattern and the position of the animal in the apparatus. The loco-motor activity is expressed as the number of compartment changes during a session.
- Tower test
  PD animals appear still be able to climb, however ability to jump seems to be disturbed. The marmoset's ability to jump is measured using the "Tower" test (Figure 1). In this test the initiation of movements is measured. This apparatus triggers the animals to jump increasing heights like their natural behavior in trees reinforced by positively reinforced by small sugar treat (small pieces of marshmallows) as an extra stimulator on each level. The animals are released at the bottom of the tower via a small door operated from outside the room. There are 7 different levels which vary increasingly from 10 cm to 50 cm. Time to reach every level, jump attempts and general activity can be monitored by following the animals using led infrared beams and video analysis. Animals are habituated to jump to every step of the tower to find the treats on every step in the tower. The animal's ability to jump to the increasing levels can be evaluated and compared to baseline levels.

Figure 1: Picture of a monkey performing in the Tower test

- Hourglass test
  The hourglass is a new non-automated test to evaluate the dyskinesia. Animals are placed in a tube in front of a camera. These tubes are normally used to handle the animals and translocate them to the test rooms. The tube is turned 180º in two seconds every 30 seconds. This way the incapacitated animals, which are no longer able to turn upright will never be longer than 30 seconds up side down. Three tubes (consecutively 11, 13 and 15 cm ∅) are used each session, each tube is turned 5 times. The time(s) it takes the animal to turn back upright is noted after video analysis. All tubes are used regularly to take the animals out of their cage.

2.4.5.3 Neurophysiological measurements on EEG

- Sleep evaluation
  Monkeys are equipped with electrodes to measure the electroencephalogram (EEG) and the electromyogram (EMG). Under isoflurane/O_2 anesthesia combined with the local anesthetic lidocaine two stainless steel electrodes were placed into a small hole in the skull leaving the dura mater intact. To measure muscle activity a flexible electrode is attached with a single stitch to the chin muscle. Another flexible electrode is attached to the neck muscle and both lines are tunneled to the head of the animal. All electrodes are connected by a plug and fixed to the skull with dental cement (Fuji plus capsule; GC corporation, Tokyo, Japan). Prophylactic antibiotic cover was provided by 0.02 ml/kg i.m. of 150mg/ml ampiciline before surgery and one day after surgery. Direct after surgery animals were provided with systematic analgesia (Carprofen). To measure telemetric sleep EEG and EMG monkeys were kept in special sleep cages provide

with bedding material. The animals can move freely in the cage. The transmitter (Data science, USA) connected to the plug for telemetric registration of the EEG and EMG is mounted on the animals head. The sleep EEG and EMG signals are recorded on weekly basis using a system by Data Sciences International (DSI, a division of Transoma medical, Arden Hills, USA) and stored on the computer. The data is transferred for analyses using Polyman (MCH, The Hague, The Netherlands). The raw EEG and EMG signals will be used for manual scoring of the sleep phases using the analysis program Polyman, according to Rechtschaffen and Kales (1968). Observations of video recordings, simultaneously with the EEG and EMG recordings, of the monkeys during the night will be used to verify the scored sleep stage in case of uncertainty. The obtained scoring results will be used to construct hypnograms to calculate the percentages of time per sleep phase. Analysis of the data will take place in the comming year.

2.4.5.4 Preparation of brain material

At the beginning of week 8 of an experimental period all animals were deeply anaesthetized and subsequently decapitated. At this point material was prepared for neurophysical analysis. Brains were isolated within 15 minutes and different brain areas were dissected. Parts of the areas were fixated in paraformaldehyde for histological analyses and were subsequently imbedded in paraffin for sectioning. The areas for protein and dopamine expression areas were snap frozen in liquid nitrogen, these areas were stored for analysis at -70 ºC. In Table 1 an overview of the used fixation methods of the different brain areas is given. Furthermore, the planned analyses are mentioned. TH staining is an immunocytochemical staining technique of thyrosine hydroxylase (TH) containing cells. This will be done to measure the active dopaminergic neurons expressing TH in the *substantia nigra*. The presence of these neurons will be quantified using light microscopy. Protein expression of the soluble fractions of the *subtantia nigra* cytoplasm will be analysed with differential gel electroforesis (DIGE) technique. Differences in protein expression will be further analysed using Mass-Spectrometry (MS). Protein expression of the non-soluble fractions of the synaptosome will be analysed using isobaric tags for relative and absolute quantitation (iTRAQ) followed by fragmentation using MSMS. The monoamine assay will be used to measure the level of dopamine and its metabolites and related monoamines in the *striatum*, the main projection area of the *substantia nigra* neurons. This will give insight in the level of neuronal damage. Monoamines will be quantified using ion-pair reversed-phase liquid chromatography.

Table 1: Overview of preparation of the different marmoset monkey brain areas.

Area	L/R	Fixation method	Analyses
Substantia Nigra	R	4% paraformaldehyde in PBS	TH staining for dopaminergic cells
Substantia Nigra	L	Snap freezing	Cytoplasmic protein expression
Striatum	L	Snap freezing	Synaptosomic protein expression
Striatum	R	Snap freezing	Monoamine assays (dopamine level)

L: left hemisphere; R: right hemisphere.

2.4.6 Study design

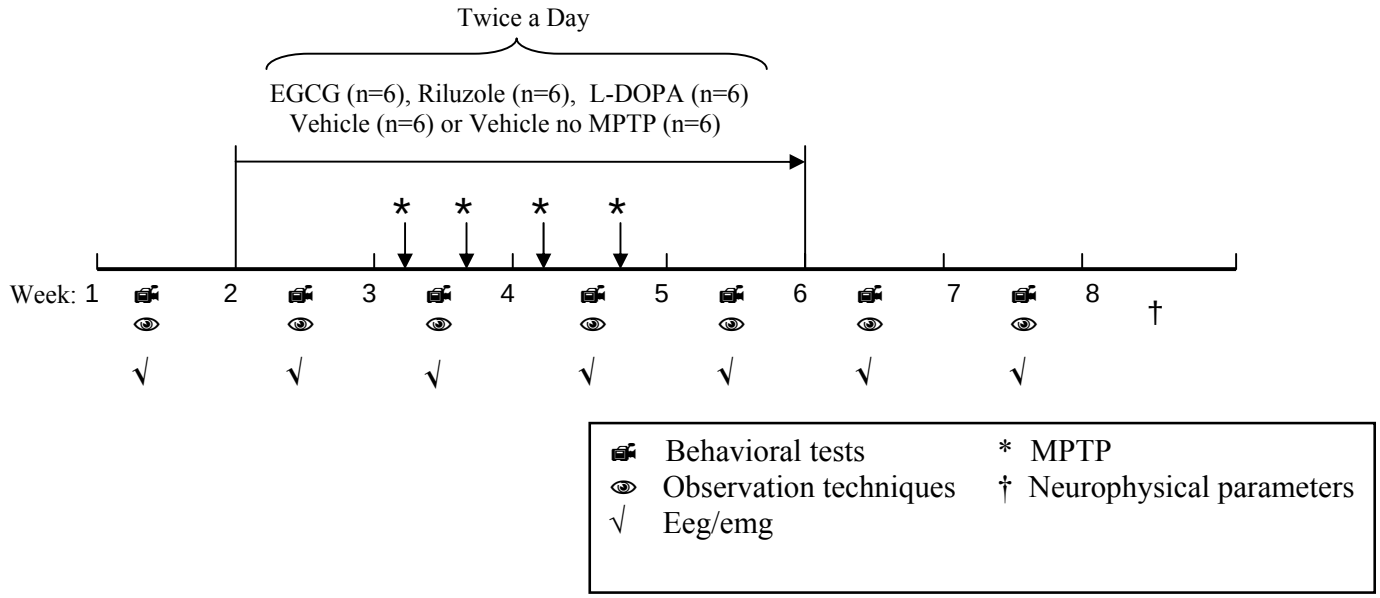

Figure 2: Testing paradigm

The neuroprotective properties of three different compounds with different targets were tested in a MPTP primate model. The behavior of six marmosets per compound treatment was compared to the behavior of a group of untreated parkinsonian and a group of 'healthy' marmosets. Animals were semi-randomly divided over the five groups, making sure that both sexes and siblings were equally distributed over the groups. All animals were submitted to an eight week long experiment with a weekly returning behavioral and neurophysiological testing paradigm and daily observations (Figure 2).
MPTP was given by four subcutaneous injections. Two injections of 2 mg/kg each in the third week on Tuesday and Friday and two injections of 1.5 mg/kg each in the fourth week on Tuesday and Friday of the experiment. The cumulative dose was 7 mg/kg.
The test compounds were given twice daily (8:00 am and 6:00 pm) during four weeks, started one week before the PD induction. Compound treatment is ceased at the end of the fifth week. Fortuitously all test compounds were administered in a dose of 10 mg/kg p.o. based on literature data and the previous reported dose range finding studies (annual report, 2006).

The first week in the test schedule is included to acquire basic performance levels, the second week to acquire insight in the effects of the test compounds in healthy animals. The testing paradigm is continued for another two weeks after drug administration and brain isolation took place at the beginning of week eight.

Each week starting at Monday (day 1) an identical scheme of behavioral and physiological parameters was obtained. Every day at 17:45 animals were clinically rated (including bodyweight and temperature) and observed using the abnormal involuntary movements scale. Body temperature was measured every two hours (7:00h - 19:00h) at day 1. Day 2 and 3 were used for behavioral testing; animals were submitted to the Hand-eye coordination test and the Bungalow test (activity) on day 2 and tested for motor ability in the Tower and the Hourglass on day 3. Sleep EEG/EMG was measured starting at 19:00h day 3 finishing at 7:45h day 4. Days 6 and 7 were used for home cage activity measurements.

2.5 Results

In the first week of a test period baseline values were obtained from the monkeys on all parameters. All MPTP treated monkeys were significantly affected on all parameters. Both observation scores, the physiological parameters and the different test paradigms were affected after the MPTP injections. In this report all measurements are compared to the vehicle treated control monkeys.

2.5.1 Observations: Clinical and Abnormal involuntary movements

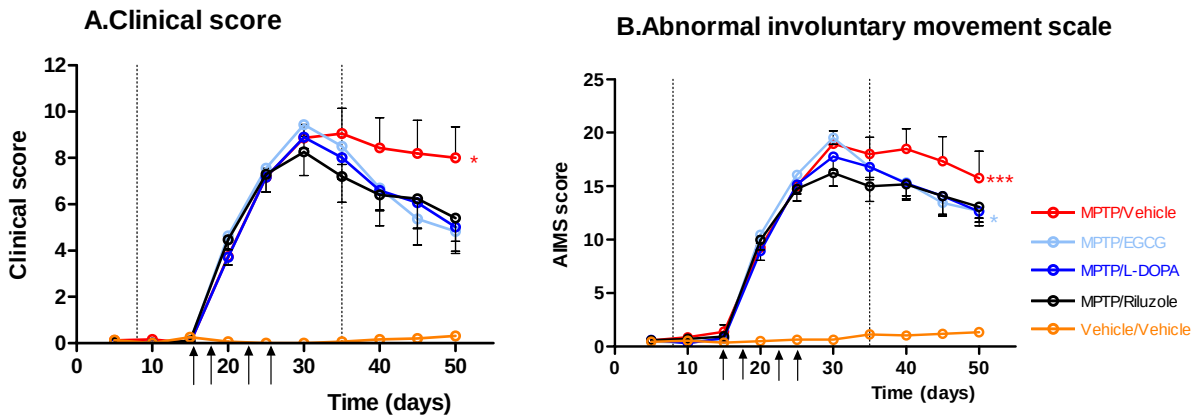

Figure 3: Daily observed signs and symptoms of MPTP intoxicated marmoset monkeys treated with vehicle, EGCG, L-DOPA or riluzole and of control marmoset monkeys only treated with vehicle (n=6/group). Observations took place before any treatment (first data point), during neuroprotective treatment (second data point), during neuroprotective treatment and PD induction by MPTP (until the second vertical dotted line), and after ending of the treatment (beginning at the second vertical dotted line). Each data point represents mean ±SEM of five days observation. (A) Clinical score and (B) AIMS. Red curve: MPTP/vehicle, orange curve: vehicle/vehicle; light blue curve: MPTP/EGCG, dark blue curve: MPTP/L-DOPA, black curve: MPTP/riluzole. Arrows indicate MPTP injections and time period between the two vertical dotted lines indicates period of treatment. Stars indicate significant differences in comparison to vehicle/vehicle group; *** P<0.001, * P<0.05.

Clinical score

Apathy, immobility, appetite, grooming, muscle rigidity and tremors were scored daily before treatment starting at 17:45h (Fig. 3A). There was no difference in the basal clinical score between groups and the different treatments (before MPTP intoxication) did not change the scoring results. After MPTP induction, all treated animals scored significantly higher than the control animals from day 20 until day 30. The animals treated with only MPTP stayed in a significant worse clinical condition than the control animals (Friedman, Dunn multiple comparisons, P<0,05).

AIMS

Abnormal involuntary movements were scored daily before treatment starting at 17:45h (Fig. 3B). The scale included facial and full body movements as well as an overall ability assessment. Abnormal involuntary movements were significantly affected by the MPTP intoxication. The non treated animals show the highest overall scoring until the end of the experiment (Friedman, Dunn multiple comparisons, P<0.001). The Riluzole and the L-DOPA treated animals show an increase in abnormal involuntary movements however not to this significant level. The EGCG treated animals show more abnormal involuntary movements just after the end of MPTP intoxication, however they recover to the same level as the Riluzole and the L-DOPA treated animals (Friedman, Dunn multiple comparisons, P<0.05).

2.5.2 Physiological parameters

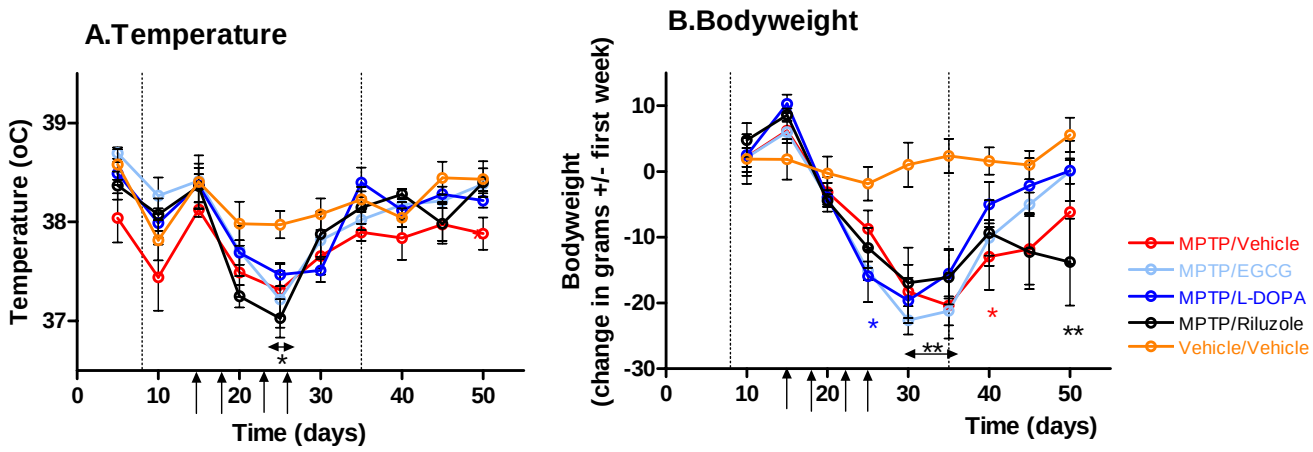

Figure 4: Daily measured body temperature (A) and delta bodyweight (B) of MPTP intoxicated marmoset monkeys treated with vehicle, EGCG, L-DOPA or riluzole and of control marmoset monkeys (only treated with vehicle). Each data point represents mean ±SEM of five days of registration for each animal group (n=6). Arrows indicate MPTP injections and time period between the two vertical dotted lines indicates period of treatment. Stars indicate significant differences in comparison to vehicle/vehicle group; ** P<0.01, * P<0.05. ↔ indicates that all groups are significant for those data points.

Body temperature

Every morning the animals' temperature was taken with the use of a subcutaneous temperature chip (Fig. 4A). The animals had an average temperature of around 38,5 ºC. After the start the treatments (day 8), the handling seems to effect the temperature of all animals also the vehicle treated animals. There is a trend for effects on body temperature by the MPTP intoxication. Around day 25 we see all MPTP treated groups decreasing in temperature (Kruskal-Wallis, Bonferonni P<0.05). After this reduction in temperature at the end of the MPTP treatment we see all animals recovering to there normal body temperature.

Relative body weight

All monkeys were weighed every day (Fig. 4B). The change in body weight relative to the average weight in the first week of the experiment was used. There was a trend of body weight decline in all treated groups. The L-DOPA treated animals showed a siginificant weight reduction from of the end of the MPTP induction. The MPTP injections induced a weight loss in all groups. This weight loss was significant until ten days after the end of MPTP intoxication (day 30 and 35, Friedman, Bonferonni P<0.01). The non treated MPTP animals took longer to start regaining the weight (day 40 Friedman, Bonferonni P<0.05). The Riluzole treated animals recovered at day 35, however they lost more weight in comparison to the control animals during the final days of the experiment (Friedman, Bonferonni P<0.01). The weight loss in all animals stayed within the safe range.

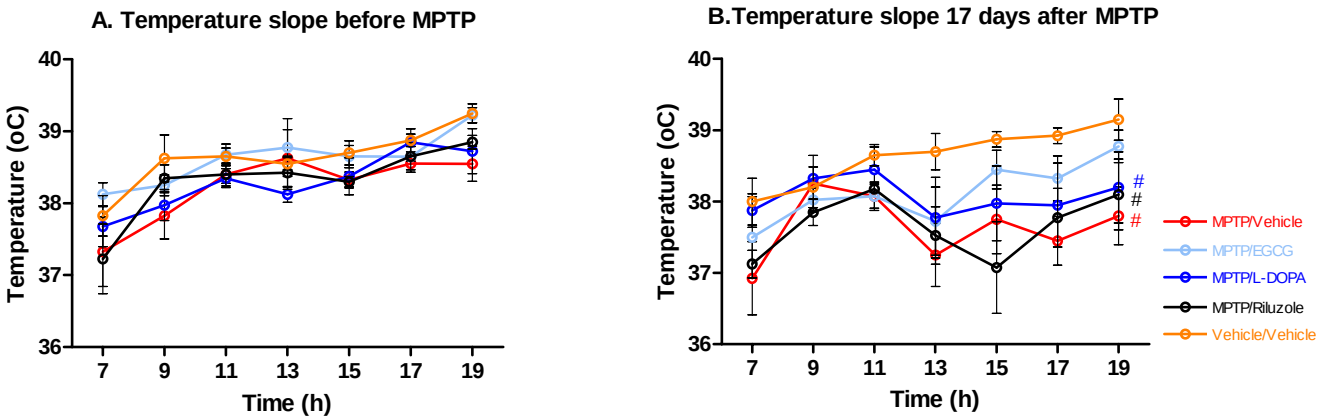

Figure 5: Temperature curves of marmoset monkeys during one day before (baseline values) and 17 days after MPTP. (A) Temperature curve before MPTP, (B) Temperature curve 17 days after MPTP. The temperature was measured by a subcutaneous placed chip every two hours started at 7:00 pm. Each data point represents mean ±SEM for each test group (n=4). Number signs (#) indicate significant differences in temperature curve (Pearson r).

Temperature curve

Once a week the temperature was measured every two hours (Fig. 5). All animals have a similar day time temperature slope at the start of the experiment. Awakening temperature is around 37.5 ºC and during the day the temperature increases to about 39 ºC. After MPTP treatment only the EGCG treated animals regain their diurnal rhythm (Pearson r). The temperature slope after MPTP treatment indicates that especially the afternoon body temperature is affected.

2.5.3 Behavioral performance

Bungalow

Animals were tested weekly in the bungalow for their spontaneous loco-motor activity (Fig. 6A). Activity was severely affected by the MPTP treatment. All MPTP treated animals showed a dramatic reduction in activity as compared to the control animals just after the MPTP intoxication (two way ANOVA, Bonferonni $P<0.001$). EGCG and L-DOPA treated animals recovered to almost their baseline activity. The significant difference with the vehicle/vehicle group is caused by a very active control group.

Hand-eye coordination

Hand-eye coordination was a weekly returning task in the test paradigm (Fig. 6B). The MPTP treatment significantly reduced the amount of correct hits (Friedman, Bonferroni). However, the riluzole treated monkeys were not affected in their performance. Just after the treatment until the end of the experiment the monkeys remained at their original level. Just after MPTP treatment (5 days post MPTP) they show a significant better performance than the MPTP treated animals ($P<0.05$). Both L-DOPA and EGCG treated animals were similarly affected as the untreated MPTP animals, however they seemed to recover to their baseline performance much better than the MPTP treated animals. Especially the EGCG treated animals, they return almost to their baseline performance.

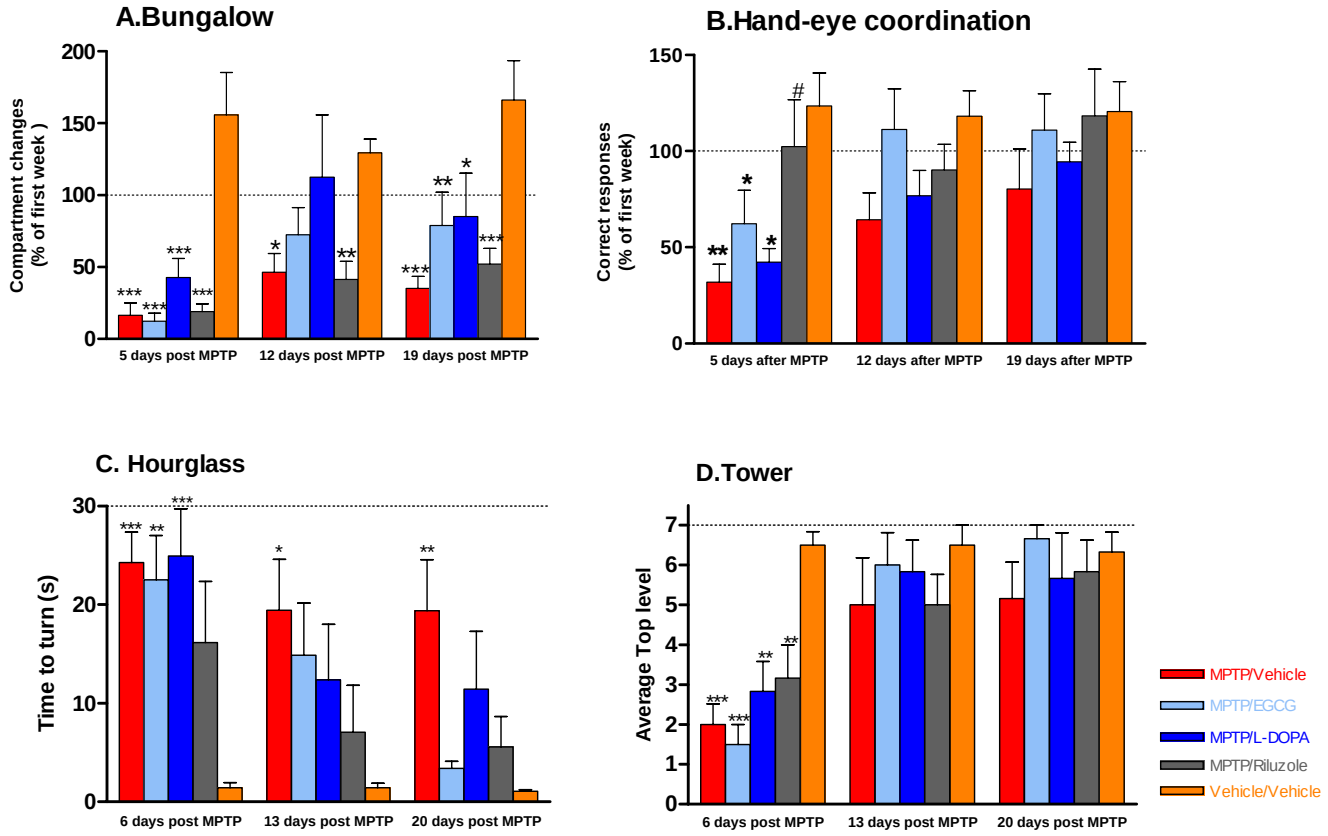

Figure 6: Mean ±SEM of weekly performance on behavioral tests of MPTP intoxicated marmoset monkeys treated with vehicle, EGCG, L-DOPA or riluzole and of control marmoset monkeys treated with vehicle (n=6/group). A. Bungalow activity measurement measured by the number of compartment changes expressed as % of baseline activity, B. Hand-eye coordination measured by the total number of correct hits expressed as % of baseline activity, C. Hourglass a measure for akinesia measured by the time needed to correct the body position and D. Tower jumping ability a measure for movement onset measured by the average top level the animals were able to reach.
Stars indicate significant differences in comparison to vehicle/vehicle group; *** P<0.001, ** P<0.01, * P<0.05. Number sign (#) indicates significant difference in comparison to MPTP/vehicle group P<0.05.

Hourglass

Monkeys treated with MPTP took more time to turn upright in a confined space (Fig. 6C). The MPTP treated animals did not recover to a normal level in the three weeks after MPTP treatment (Friedman, Bonferonni P<0.01). However, there was a large difference between not treated MPTP animals and treated MPTP animals. Riluzole treated monkeys turned relative fast and did not reach a significant different time in comparison to the vehicle/vehicle treated animals. From two weeks after MPTP treatment EGCG and L-DOPA treated animals show less akinesia. Three weeks after MPTP treatment the Riluzole and EGCG treated animals recovered to almost a normal level.

Tower

The animals were placed in the Tower every week and were free to visit the different levels (Fig. 6D). The top level of the Tower test is branch 7. All treatment groups had a significant lower top level after MPTP treatment when moving around freely for 5 minutes in the Tower (Friedman, Bonferonni). All animals recovered almost to baseline level from of 13 days after MPTP treatment. The control animals show a very steady top level.

2.6 Discussion

Three identical PD neuroprotection experiments with in total 30 marmoset monkeys were conducted in the past year. Our behavioral results demonstrate that the compounds used for neuroprotective treatment against PD progression are all effective on one or more parameters compared with the untreated MPTP marmoset monkeys. These results are consistent with previous studies on riluzole in rhesus monkeys, marmosets and mice (Benazzouz et al., 1995, Obinu et al., 2002 and Boireau et al., 1994) and on EGCG in mice (Choi et al., 2002). Interestingly, the positive effect of L-DOPA suggests the neuroprotective prospect of this compound (Ogawana et al., 2005). In Table 2 the effectiveness on different parameters of the three different test compounds in comparison to the untreated MPTP marmoset monkeys is summarized.

Table 2: Overall performance of treated MPTP monkeys in comparison to non treated MPTP monkeys.

Type of parameter	Parameter	EGCG	Riluzole	L-DOPA
Observations	Clinical score	*	*	*
	AIMS	-	*	*
Physiology	Bodyweight	*	-	*
	Body temperature	-	-	-
	Diurnal rhythm	*	-	-
Test paradigms	Bungalow activity	*	-	*
	Hand-eye coordination	-	**	-
	Tower	-	-	-
	Hourglass	**	*	*

Minus symbol (-) = significantly affected like MPTP in comparison to control, star symbol (*) =significantly not effected unlike MPTP treated animals, Two stars (**) =significally better performance than untreated MPTP monkeys.

In general different scoring systems are used when the effects of MPTP treatment in marmosets are evaluated (Emborg et al 2004; Obinu et al., 2002; Costa et al., 2001 and Nomoto et al., 1998). All scoring systems have the emphasis on motor behavior. However, precise observations vary. Ratings can generally be divided in two parts: clinical observations (grooming, apathy and appetite) and abnormal movements (posture, rigidity and akinesia). To have a complete view on the observed performance two separate scoring paradigms are used in this study; the clinical score and, because MPTP will mainly affect motor behavior in the marmosets, the abnormal involuntary movements scale (AIMS) (van Vliet et al., 2006a, van Vliet et al., 2006b). On the scoring scales the EGCG and the L-DOPA treated marmoset monkeys show a similar pattern. The '–' in Table 2 for the EGCG animals on AIMS score is probably caused by the increase in scoring during the MPTP treatment rather than the overall recovery of the animals. As seen in Figure 3B the EGCG treated animals recover similar to the other treated animals. The L-DOPA treated animals do not show this increase during the MPTP treatment which can be caused by the symptom treatment of the compound. However this cannot completely explain the recovery because of the delay of observation after drug administration.
Ten hours after administration of L-DOPA, the level of L-DOPA is to low to influence the scoring. The similar pattern of these two compounds however can be explained by a similar effect of both compounds, the effect of anti-oxidation. EGCG, the green tea component (Mandel and Youdim, 2004) and L-DOPA the dopamine precursor (Ogawana et al., 2005) are both reactive oxygen species scavenger.
The positive effects of EGCG on diurnal rhythm (Figure 5B) could be caused by the effects the compound has on energie expendure as suggested by Berube-Parent et al., (2005). The question is however if this would still be the case two weeks after the last EGCG administration. Therefore, we expect that this recovery on temperature is be caused by the neuroprotective effects of the compound rather that the direct effects of the compound on body temperature.

Besides observational scoring scales and physiological measurements, behavioral tasks can be used to measure motor performance in the marmoset monkey. It is important in motor disability models to role out the effects of general activity. For that reason differences will be found when activity is measured in the home cage compared to

an experimental test condition. Activity can be measured in several ways depending on measurement interest and animal housing; examples are video analysis (Obinu et al., 2002; Van Vliet et al., 2006), infrared cells and photocells (Pearce et al., 1996; Philippens et al., 2000) or accelerometer (Mann et al., 2005). Tests or tasks can generally be divided in two types: 1) 'full body' motor behavior for example the Sticky label test or the Rotation task (Annet et al., 1994) or 2) 'arm reaching' motor behavior for example the Staircase test (Marshall and Ridley., 1996) and the Hand-eye coordination task (Wolthuis et al., 1995). This latter test system has proven to be very sensitive for functional performance decrements after MPTP treatment in marmoset monkeys (Philippens et al., 2000) and usefull to measure neuroprotective effects (van Vliet et al., 2006). In this task positive reinforcement by a food reward (like in most 'arm reaching' tasks) is used. In this way we prevent the development of stress by negative reinforcers. On the other hand, Macht and his group (2007) stated that stress will not influence PD impairments under cognitive control.

In this study behavioral tests are included containing the four different test types; the Tower test and the Hourglass test evaluating 'full body' motor behavior, the Hand-eye coordination task for 'arm reaching', the Bungalow test for measuring the loco-motor activity (Philippens et al., 2000; Van Vliet et al., 2006), and home cage activity - measured by using seismographic changes in the home cage- to supplement the motor behavioral measurements. Results from the home cage activity will be presented in the third annual (final) report.

Despite the absence of positive effects on the physiological measurements, riluzole showed to have a strong effect on two functional motor tasks, the Hand-eye coordination and the Hourglass test. On both parameters the MPTP treated monkeys without any therapeutic treatment were severely affected. Besides the correct coordination the timing of the performance is crucial in the hand-eye coordination task. The performance in the Hourglass test is evoked by the motivation of the monkey to correct his or her posture (the upside down position of the monkey). In both tasks the akinesia plays an important role. The fact that this symptom recovery is long lasting (up to the end of the experiment) suggests that this compound has a neuroprotective effect. This compound helps effectively against the akinesia, although the monkeys remain slower than untreated control monkeys. This decline in activity was also found in the so-called bungalow task for loco-motor activity. This is in line with the findings from the dose range finding study in the first year of this study and from literature (Philippens, 2006; Obinu et al., 2002). The sedating effects of riluzole may affect the loco-motor activity.

Based on the present results of the functional parameters and behavioral aspects, all three test compounds riluzole, EGCG, and L-DOPA seems to have neuroprotective effects on Parkinsonian symptoms. Riluzole has also a positive effect on functional motor effects. The neuroprotective effects of L-DOPA are presumably based on anti-oxidative effects. Taken into account that metabolic compromise induced by MPTP results in loss of mitochondrial function leading to a depletion of ATP and subsequently to accumulation of intracellular Ca^{2+}, and to production of free oxygen and nitrogen radicals, we may conclude that both oxidative stress and excitotoxicity interact with this process in a synergistic way. Because NMDA antagonists, like riluzole, play a role in preventing accumulation of intracellular Ca^{2+} and subsequent production of free oxygen and nitrogen radicals, the functional motor effects are presumably related to the accumulation of intracellular Ca^{2+}. The Parkinsonian symptoms, on the other hand, are related to the effects of oxidative stress. This explanation should be verified by the proteomics data. Furthermore, the relation of these factors to brain damage has to be confirmed by the histological and biochemical data in the forthcoming period. Insight in these factors related to neuronal maintenance processes may be of great importance for the understanding of slow gradual neurodegenerative processes.

With this study on the behavioral performance and physiological parameters of common marmosets treated for MPTP intoxication we underline the interaction of the lethal triplet, metabolic compromise, excitotoxicity and oxidative stress, and the neuroprotective prospects of the three compounds tested, Riluzole, EGCG and L-DOPA. All three compounds show significant lasting performance improvement on several parameters.

3 AMENDMENTS TO THE PROJECT

3.1 Drugs

In the original proposal the NMDA antagonist riluzole and the anti-oxidant dextromethorphan were selected. However, dextromethorphan shows besides its anti-oxidative action also activity against excitotoxicity. This would interfere with the outcome of the study. Therefore, we replaced dextromethorphan by EGCG with a strong anti-oxidative profile without affecting the excitotoxicity.

3.2 Methods

Because of the low sensitivity of the phMRI for the *substantia nigra* and the expected small changes in the brain we decided not to use this method in the neuroprotection experiments, but to replace this technique by the telemetric EEG measurement for following the effects on sleep during the PD progress. This MRI method is still very new and at this moment we do not possess over the experience level required to go through with the pharmacological MRI scans. This decision was made in close deliberation with Dr E.L. Blezer (Utrecht University).
We added two test systems to the repertoire of behavioral tests: 1) One for measuring the onset of movements, the so-called "Tower" and 2) one to measure the dyskinesia the so-called "Hourglass test".

3.3 Personnel

The work load deviation for this project for the different staff members is altered. P.S. (Nelleke) Verhave is appointed to work on this project. She has a master's degree in Animal Science with emphasis on brain and behavior. Her work will exclusively consist of this project. Practical work will be done together with and closely monitored by Ingrid Philippens, Marjan Jongsma and Raymond Vanwersch. The biographical sketch of Peternella Verhave was already added in the annual report of 2006 (Philippens, 2006).
Marjan Jongsma has left our institute. Roland van den Berg with a similar level of education took over her work. Roland was already working within TNO in the same research group.

4 KEY RESEARCH ACCOMPLISHMENTS

- Evaluation of two new behavioral test methods with parkinsonian monkeys: one for measuring the onset of movements, the so-called "Tower" and one to measure the dyskinesia, the so-called "Hourglass test".
  - Two new behavioral test methods were evaluated with parkinsonian marmoset monkeys: one for measuring the onset of movements, the so-called "Tower" and one to measure the dyskinesia, the so-called "Hourglass test". With the Tower test the level of disturbance of the movement initiation, which is expressed by the disability to jump, can be measured. In this study only a mild PD induction is used leading to a maximum effect only a short period of a week after the MPTP injections. Therefore, only during this short period of time the effects of MPTP treatment can be measured with this system. The Hourglass test is much more sensitive. During the complete test period the effects of MPTP treatment on akinesia can be quantified with the hourglass test. Furthermore, this system is also sensitive to measure neuroprotective effects of the different treatments.

- Training of sufficient monkeys to perform the hand-eye coordination task; a learned behavior for coordinated motor skills. These monkeys were used for the PD neuroprotection experiments.
  - For the neuroprotective efficacy experiments 30 marmoset monkeys were trained on a learned task for coordinated motor skills in the hand-eye coordination test system. These animals were trained to a level of 75% or more correct hits.

- Providing all monkeys with telemetric EEG electrodes and successfully measuring sleep EEG/EMG over a period of 7 weeks.
  - The marmoset monkeys were provided under inhalation anesthesia with telemetric EEG and EMG electrodes to measure the sleep pattern during several nights over a period of 7 weeks. Telemetric data acquisition is necessary to enable the monkeys to move freely without any restrictions of wires.

- Performance of the large neuroprotection experiments, including the data acquisition of observational, behavioral and physiological parameters.
  - These experiments were carried out in three separated experiments of 10 animals each (n=2/group). From all animals data acquisition of observational, behavioral and physiological parameters has taken place.

- Evaluation of the behavioral performance of the MPTP treated monkeys in comparison to the control monkeys and the treated MPTP monkeys.
  - Evaluation of the behavioral performance and observational measurements of the MPTP treated monkeys in comparison to the control monkeys and the treated MPTP monkeys was carried out, which is presented in this report.

- Preparation of brain material for histology, proteomics and biochemistry analysis.
  - The brains of these animal has been prepared for different ex-vivo tests like the evaluation of brain damage by histology, changes in cytoplasmic and synaptosomic protein expression by electroforesis or isobaric tags and determination of the level of monoamines for measuring the functionality of the dopaminergic system.

5 REPORTABLE OUTCOMES

The behavioral results demonstrate that the test compounds used for treatment of the MPTP disabled marmoset monkeys are all effective on one or more parameters.
Clinical score: After MPTP induction, all treated animals scored higher than the control animals. The animals treated with only MPTP stayed in a significant worse clinical condition than the control animals.

AIMS: The non treated MPTP animals show the highest overall scoring on the abnormal involuntary movements until the end of the experiment. An increase was also found in the riluzole and the L-DOPA treated MPTP animals, however not to this significant level. The EGCG treated animals show a higher score just after the MPTP injections, however they recover to the same level as the riluzole and the L-DOPA treated animals.

Body temperature: One week after MPTP injections all MPTP treated groups decreased in body temperature, which was recovered in time to normal body temperature. Concerning the day time temperature curve we found that MPTP treatment affects the afternoon body temperature from ca 39 ºC to 37.5 ºC. Only the EGCG treated animals regain their normal diurnal rhythm.

Body weight: There was a trend of body weight decline in all MPTP treated groups. This weight loss was significant until ten days after the end of MPTP intoxication. Thereafter the animals gain weight again which took longer in the non treated MPTP animals. The weight loss in all animals stayed within the safe range.

Bungalow: Loco-motor activity was severely affected by the MPTP injections in all MPTP treated animals. EGCG and L-DOPA treated animals recovered to almost their baseline activity.

Hand-eye coordination: The MPTP treatment significantly reduced the amount of correct hits in the Hand-eye coordination task. Strikingly, the riluzole treated monkeys were not affected in their performance at all. Both L-DOPA and EGCG treated animals were similarly affected as the untreated MPTP animals, however they seemed to recover to their baseline performance much better than the MPTP treated animals. Especially the EGCG treated animals, they return almost to their baseline performance.

Hourglass: Monkeys treated with MPTP without additional treatment took more time to turn upright in a confined space. These animals did not recover to a normal level during the experimental period. Riluzole treated monkeys turned relative fast and did not reach a significant different time in comparison to the control animals on akinesia. Two weeks after MPTP treatment EGCG and L-DOPA treated animals improved their akinesia. Three weeks after MPTP treatment the Riluzole and EGCG treated animals recovered to almost a normal level.

Tower: All treatment groups reach a lower top level in the Tower test after MPTP treatment and recovered almost to baseline level. The control animals show a very steady top level.

The EGCG and the L-DOPA treated animals show a similar pattern. This similarity can be explained by a similar effect of both compounds, the effect of anti-oxidation. With the riluzole treatment a strong effect was found on two functional motor tasks, the Hand-eye coordination and the Hourglass test, related to akinesia. The fact that this effect is long lasting (up to the end of the experiment) suggests a neuroprotective effect of this compound. With these results on the behavioral performance and physiological parameters we underline the neuroprotective prospects of the three compounds tested, riluzole, EGCG and L-DOPA. All three compounds show significant lasting performance improvement on several parameters.

6 PUBLICATIONS

- Annual progress report: 'Report Monitoring and counteracting functional deterioration in Parkinson's disease: A multilevel integrative approach in a primate model system.' October 2006.

- External presentation Molecular and Cellular Neurobiology, 09-11-06, Amsterdam, The Netherlands
  Title: Neuroprotection in the marmoset MPTP model

- Postersession at 11th Endo-Neuro meeting, 05-06-07, Doorwerth, The Netherlands.
  Title: Tower and Hourglass, two motor behavior tests for marmosets

7 CONCLUSIONS

The conclusions are based on the present results of the functional behavioral parameters and physiological aspects and is valid for the MPTP marmoset model used in this study. Some of the conclusions should be verified by the proteomics data. Furthermore, the relation of the neuroprotective effects to actual prevention of brain damage has to be confirmed by the histological and biochemical data in the coming period. Insight in these factors related to neuronal maintenance processes may be of great importance for the understanding of slow gradual neurodegenerative processes.

- The selected read-out systems are very sensitive for MPTP induced PD effects. There is a slight recovery seen in time due to the mild PD induction.
- Based on the present results of the behavioral and clinical parameters all three test compounds riluzole, EGCG, and L-DOPA seem to have neuroprotective effects reducing Parkinsonian symptoms.
- The neuroprotective effect of L-DOPA is presumably based on anti-oxidative effects. The therapeutic effects of L-DOPA are ruled out by the long delay after administration of L-DOPA .
- The NMDA antagonist riluzole has also a positive effect in preventing the functional motor disturbances based on akinesia.
- Anti-oxidative effects seem to reduce the Parkinsonian symptoms.
- Anti-excitotoxicity effects, which prevent accumulation of intracellular Ca^{2+}, seem to prevent the decline in functional motor performance.

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