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PRINCIPAL INVESTIGATOR: Dr. Ingrid H.C.H.M. Philippens

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Philippens, Ingrid, H.; Verhave, Peternella, S.; Jongsma, Marjan, J.; Blezer, Erwin; Mol, Marijke, A.

E-Mail: ingrid.philippens@tno.nl

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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. Patients suffer from motor dysfunction like tremors, bradykinesia and dyskineasia which results in serious functional impairment. The general course of the progression of PD is still largely unknown. Current standard drug treatment (like L-DOPA) tackles symptoms of PD however not the progression of the disease.

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 know: 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).

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. This strategy is used to induce parkinsonism in rats by the neurotoxin 6-hydroxydopamine. This compound induces oxidative stress associated with free radical production (Heikkila and Cohen, 1972).

It may be clear that, independently of the actual cause, neurodegeneration in PD is based on an endogenous excitotoxicity, i.e. a PD patient basically destroys, and has destroyed, its own *substantia nigra* neurons by endogenously generated activity, combined with a problem in the homeostatic control of the neurons. Since PD is a slow progressive disease, it is likely that the excitotoxic state does not always occur, and only occasionally induces neuronal death. This excitotoxic situation may be directly based on over-excitation, or suppression of neuronal maintenance processes, but most likely both.

To limit the impact of PD, early diagnosis and subsequent neuroprotective treatment is a valid and relatively easy strategy: progressive loss of neurons must be avoided.

Alternatively, factors related to neuronal maintenance processes may be of great importance for understanding slow gradual neurodegenerative processes. In neurodegenerative disorders, already weakened neurons may not survive glutamate concentrations that would normally not be lethal (Doble, 1999). These weakening factors may represent susceptibility processes, which are perhaps more easily influenced and less invasive to intervene with pharmacologically. The NMDA receptor antagonist riluzole is indeed able to prevent neuronal damage due to PD induction by MPTP in mice, marmoset monkeys and rhesus monkeys (Obinu *et al.*, 2002, Araki *et al.*, 2001, Benazzouz *et al*., 1995).

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 L-DOPA symptom treatment will be compared to untreated controls in the marmoset MPTP model. The integrative nature of this project and the link to human clinical testing (clinical scores and AIMS) will contribute to the understanding of the development and progression of PD symptoms, and offers an approach to limit the aggravation of symptoms in patients.

2. BODY

Statement of Work

Purpose of the study

The research 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.
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. Using enhanced dopaminergic action aims for symptom control. However, the progression of PD is not slowed down by this treatment. Therefore, the quality of life may be better served by alternative approaches serving neuroprotection, i.e. by treatments limiting excitotoxicity or oxidative stress. Critical changes indicating the (proteomic, physiological, and behavioral) 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 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.

Major goals and objectives

At the end of the first year, the dose range finding experiments of the neuroprotective and symptom control 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 effects of the progression of PD on the different read-out system levels can be established. This will give insight into the usefulness of the selected markers in this study. Furthermore, the correlation between the results will increase the insight of the mechanisms leading to PD induced symptomatology and brain damage.

At the end of the third year the protective effects on symptomatology and on brain damage by compounds preventing oxidative stress or excitotoxicity can be given. This will give information of 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 symptom control drug L-DOPA will give insight in the relative role of the different markers for neuroprotection and behavioral output.

Methods and materials

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 and hand-eye coordination (HEC),
- Continuous recording of movement activity, body weight, and body temperature,
- Clinical observations, AIMS (abnormal involuntary movements scale used for PD patients),
- Magnetic resonance brain spectroscopy,
- Proteomics of s*ubstantia nigra* and cerebellar control tissues,
- Tyrosine hydroxylase activity in the *substantia nigra*, Dopamine and DOPAC levels in the striatum

2.1 Material and methods

2.1.1 Animals

Two marmoset monkeys (*callithrix jacchus)* were used to test and evaluate the brain imaging and thereafter the brain activity, based on neurophysiological measurement, pilot (approved by the animal ethics committee #2053). Another 12 monkeys (of both sexes) of the same species were used for the dose response study (approved by the animal ethics committee #1985). 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), temperature was kept in between 23 and 25ºC and relative humidity was at least 60%. 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.

2.1.2 Drugs

Iron oxide nanoparticles (IRON; Sinerem; Utrecht University) and Amphetamine (RBI, Natick, USA) were used to activate dopamine neurons during the phMRI.
The anti-oxidant tested in the dose response study (-)-Epigallocatechin 3-O-gallate (EGCG; Teavigo®) was kindly provided bij DSM, Switserland. The anti-excitotoxic compound Riluzole (Rilutek) was obtained at Wippolder Pharmacy, Delft, Netherlands.

2.1.3 Test methods

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) and body weight are noted.

AIMS

Motor ability of the animals is scored using the Abnormal Involuntary Movements Scale (AIMS) adapted from the clinic (Guy, 1979). The effect of the compounds on abnormal involuntary movements is rated daily and also extensively after compound administering. In 9 different steps 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.

Hand-eye coordination test

The hand-eye coordination was tested with an automated robot arm guided by a semi-randomized computer program according to Wolthuis *et al*., (1995) using positive enforcement (small pieces of marshmallow) as stimulus (see Figure 1). 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 marshmallows is presented by the robot arm. Marshmallows are presented at the tip of a 8.5 cm suction tube. Marshmallows are presented at three different speeds; still 0.00 m/s, slow moving at 0.04 m/s and fast moving at

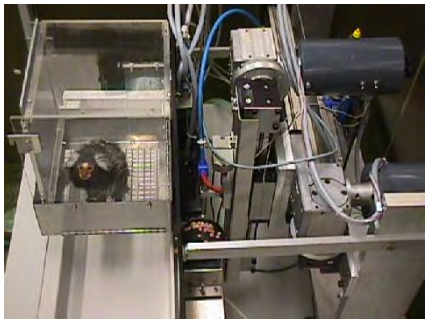

Figure 1: Picture of the Hand-Eye Coordination test

0.08 m/s. A sound signal alerts the animal before each new stimulus after which the door slides open and the marshmallow 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. Before the start of this test the animals are trained to grasp the reward from the suction tube. The animals are alerted before each trial by a sound signal. After the animals had reached a performance of 80% or more correct hits the animals are ready to start with their performance in this task. Training takes normally 6 to 8 weeks. In this specific group of animals it took twice as long.

Bungalow test

The bungalow test is an automated test to evaluate activity or exploration behavior 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 in the apparatus. The motor activity is expressed as the number of compartment changes in this period.

Hourglass test

The hourglass is a new non-automated test to evaluate the general motor ability. Animals are placed in front of a camera in a tube. These tubes are normally used to take the animals out of their cages. The tube is turned 180º in two seconds every 30 seconds. This way the incapacitated animals, which are no longer able to turn upright are never 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.

Tower test

Initiation of behavior is evoked in a new test the tower test. Animals appear to still be able to climb, however ability to jump seems to be damaged. The marmoset's ability to jump is measured using the "tower" test. This apparatus triggers the animals to jump different heights like there natural behavior in trees. The animals are released at the bottom of the tower via a small door operated from outside the room. On seven different levels the animals can find a sugar treat. Jumps 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 (small pieces of marshmallows) on every step in the tower. After inducing motor limitations the animal's ability to jump can be evaluated and compared to baseline levels.

Pharmacologic magnetic resonance imaging (phMRI)

All MRI scans were conducted at and in corporation with the Image Sciences Institute, University Medical Center Utrecht, Utrecht, The Netherlands (see Figure 2). A general diffusion scan (water disposition) measures dopaminergic cell loss and a T2* scan (iron disposition) measures cell functionality. Dr E.L. Blezer is our contact in Utrecht and conducted all our scans.

Figure 2: Picture of the NMR facility Utrecht. A marmoset is positioned in theNMR spectrometer.

The animals were transported in a climate controlled vehicle in special transport cages with openings on the sides of the cages and on the bottom a piece of cloth for a comfortable journey to Utrecht. Before the scans the animals were kept form eating and drinking to prevent them from nausea. The animals were anesthetized for scan preparation with Ketamine (Nimatek 15 mg/kg i.m. Eurovet, Bladel, The Netherlands). An oral tube was inserted for gas anesthesia and a canula was inserted in a tail vessel for administration of iron and amphetamine. Animals were kept under isoflurane/O_2 anesthesia during the scan period. The animals were immobilized in a specially designed stereotactic holder and placed in an animal

cradle, which was inserted into the NMR spectrometer. During the MRI-experiments animals were ventilated with isoflurane (1-2 %) in N_2O/O_2 (70/30). Heart rate, blood oxygen saturation and expiratory CO_2 were continuously monitored. Body temperature was maintained at $37^{O}C$ with a heated water pad. A homebuilt Helmholtz volume coil (∅ 85 mm) and with an inductively coupled surface coil (∅ 35 mm) were used for radio frequency transmission and signal reception, respectively. On a coronal scout image, 35 contiguous coronal slices of 1 mm were defined covering the complete brain. After the general diffusion scan and a T2* scan, iron oxide nanoparticles (IRON; Sinerem; Utrecht University) were injected to enhance the contrast of the amphetamine injection. Amphetamine (RBI, Natick, USA) was injected after the IRON particles. After the scan the animals were monitored closely and put back in a warmed transport cage. The warm water bottle was left with the animals in the cage. They also received some apple to prevent dehydration.

Neurophysiology

Under isoflurane/O_2 anesthesia combined with the local anesthetic lidocaine two stainless steel electrodes are placed into a small hole in the skull both 5 mm lateral to the *sutura sagitalis* and 5 mm cranial from intra-aural 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) (see Figure 3, left picture). Prophylactic antibiotic cover was provided by 0.02 ml/kg i.m. of 150mg/ml ampiciline before surgery and one day after surgery.
To measure sleep electroencephalogram (EEG) and electromyogram (EMG) animals were kept in special sleep cages (40*20*30 cm). The animals can move freely in the cage and have bedding material. The transmitter (Data science, USA) connected to the plug for telemetric registration of the EEG and EMG is mounted on the animals head (see Figure 3, right picture).

Figure 3: Picture of a marmoset provided with a telemetric device for EEG registration

The appearance and duration of different sleep stages will be analyzed using the EEG and EMG signal (Philippens *et al*., 2004). The EEG and EMG signals are recorded using a system by Data Sciences International (DSI, a division of Transoma medical, Arden Hills, USA) and stored on the computer. After the recordings the data was transferred for analyses using Polyman (MCH, The Hague, The Netherlands) and Microsoft Excel (Microsoft corporation, USA). The raw EEG and EMG signals were used for manual scoring of the sleep phases using the analysis program Polyman, according to Rechtschaffen and Kales (1968). The analysis program Polyman was made 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. Also, the MCH had an advisory role regarding the overall setup and analysis techniques used in this study. Observations of video recordings of the animals during the night were used to verify the scored sleep stage in case of uncertainty.
The obtained scoring results were used to construct hypnograms. First, for the data of the entire night the hypnograms were constructed and the percentages of time per sleep phase were calculated. Because the first part of the sleep period is predominated by deep sleep and the second part by light sleep and (rapid eye movement) REM sleep. The presence of the different sleep stages will be subdivided into two halves. Furthermore, simple delta plots were made to achieve an indication about the impact of MPTP treatment on the sleep EEG. A Fast Fourier Transformation (FFT) was performed to obtain power spectra of the delta (0.2 – 4 Hz) band.

2.2 Study design

In the first year of this study the dose range finding experiments of the test compounds were 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.
After training in the hand-eye coordination task, a dose response study was conducted to measure effects of the test compounds EGCG and riluzole on different behavioral test systems. Several different dosages where used to test behavioral and clinical effects. Each week animals received a different dose of the compound and where tested and scored afterwards.
Twelve marmoset monkeys used in this study were subdivided into two groups based on their activity level measured in the bungalow test (naive values average of three sessions), gender, peer and weight. Animals were exposed to a weekly paradigm of tests and observations. The first week the animals did not receive any oral dosage, the second week they received an oral dosage of 2:1 water:Karvan cevitam® (vehicle). Thereafter, six animals received a weekly varying dose of EGCG (5, 10 and 20 mg/kg) and the other six animals a weekly varying dose of riluzole (1.25, 5, 10 and 20 mg/kg). For oral administration EGCG was dissolved in vehicle. For oral administration riluzole was suspended in vehicle and 0.5% methylcelluclose (Sigma; St. Louis, MO). Four hours after the EGCG administration (Rice-Evans, 1995) and one hour after the riluzole administration (Martinet *et al*., 1997) the animals were exposed to the different tests in a set paradigm (hand-eye coordination, loco-motor activity in the so-called bungalow test and hourglass test). The sensitivity of this multilevel approach is recently proved in our institute (Van Vliet *et al.*, 2006). Just before testing the animals were observed individually using the clinical score and the AIMS score.

Also extra experiments have been performed as pilot or preparation for the experiments planned in the second year of the study and two new test methods have been evaluated.

- A phMRI pilot was done to evaluate the possibilities of phMRI to measure dopaminergic cell survival in parkinsonian marmosets. phMRI is intended to analyze the deterioration of the dopaminergic cell function by evaluating the amount of neuron activation triggered by amphetamine. Using phMRI the cell function instead of the cells' presence will be evaluated with an amphetamine challenge. Amphetamine is a dopamine neurotransmitter (DA) releaser. When injected it leads a release of DA by the dopaminergic cells. The strong advantage is of this new type of MRI is that not only the degree of cell loss can be measured, the efficiency of the remaining dopaminergic cells can also be evaluated (Jenkins *et al.*, 2004). To increase the resolution of the regional activation iron oxide nanoparticles (IRON) are injected before amphetamine (Chen *et al*., 2001). Comparing images of dopaminergic and non-dopaminergic brain areas after amphetamine induction is intended to evaluate phMRI as a measurement tool. Region of interests (ROI) were defined by Stephan (1980) (see Figure 4). Naïve animals were taken to the scanner for imaging. After IRON particles for contrast enlargement, animals received two amphetamine injections to define the correct dose DA activation. ROIs of different brain areas were analyzed by the NMR in vivo department at Utrecht University.
- The effect of *substantia nigral* cell death on sleep is evaluated in a second pilot study. PD patients have altered EEG activity and increased muscle tension during certain sleep stages (Gagnon and Bedard, 2002). Changes in sleep rhythm have been found in MPTP treated monkeys (Almirall *et al.*, 1999). Using EEG combined with EMG sleep rhythm especially REM-sleep can be evaluated in parkinsonian marmosets. Using EEG measurements we can evaluate the very beginning of our cumulative MPTP paradigm, measuring sleep EEG at least once a week during the experiment period. The EMG measurements can outline the difference between REM sleep and non-REM sleep. To evaluate the changes detectable in EEG and EMG after MPTP induction two animals were used for a pilot. The animals received electrodes and EEGs were measured before, during and after MPTP induction. A similar paradigm will be used in the final experiments, two weeks of baseline measurements followed by a three week MPTP paradigm.
- Finally two new behavioral test systems are evaluated. The hourglass test and the tower test. In the hourglass test the rigidity caused by the *substantial nigral* damage can be evaluated by the animals' ability to turn upright. The hourglass test was already included in the dose response study. The tower test triggers the animals to jump as such measuring the marmoset's ability to initiate behavior.

2.3 Results

2.3.1 Pilot experiments

phMRI pilot

The animals recovered completely from the MRI test day; however the anesthesia, the scanning time and this in combination with the amphetamine challenge induced a visible level of discomfort in the animals. One of the two animals showed slowness in behavior for several days after the scan.

The optimal increased relaxation that should have been induced by the iron oxide nanoparticles was not obtained in either one of the pilot animals (respectively 12.5 and 30 mg/kg). This resulted in no extra contrast after amphetamine injection.

Pixels were fitted along a proposed curve to find contrasts in the scan images. No contrasts were found in the *substantia nigra* or striatum however contrast was found in the cortex with the amphetamine injections of 2.5 and 5 mg/kg. This implies that the scans can show a contrast however not specifically contrasts of the dompaminergic cells in the *substantia nigra* which was aimed for.

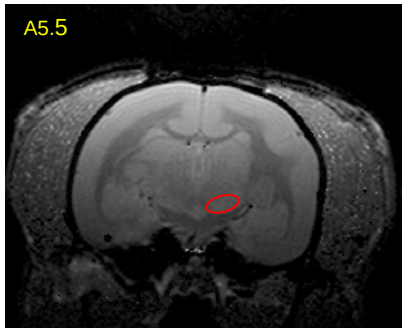

Figure 4: Example of a slice of the marmoset brain by MRI. The ROI of substantia nigra is shown in red.

EEG pilot

The present results showed that the marmoset monkey model is a valid model for the research of sleep. Therefore, the effects on the quality of sleep after MPTP treatment can be extrapolated towards the human situation.

The hypnograms showed a clear cyclic pattern (see Figure 5). A cycle often started with light sleep, after which the animal falls into deep sleep, usually followed by a REM-phase. After this REM phase the animal shortly regained wakefulness, after which a new cycle started. Such a cycle lasted approximately 45 minutes in the marmoset monkeys.

It was clearly observed that the first phase of the night is predominated by deep sleep and the second phase of the night by light sleep and REM sleep (see Figure 6).

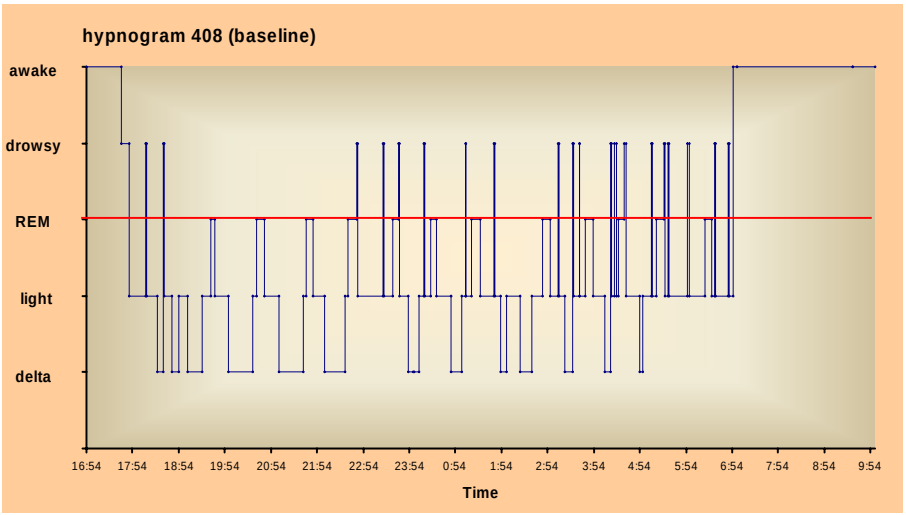

Figure 5: Example of a hypnogram of a marmoset

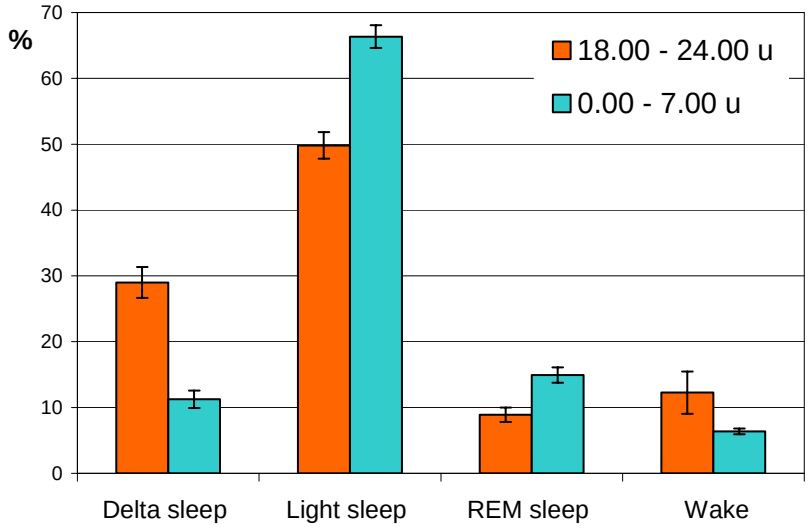

Figure 6: The percentage of observed sleep phases during the two parts of the night (first: before 0.00 h; second: after 0.00 h) ± standard error of the mean.

In the power plots of the sleep EEG it was clearly shown that PD induction by subchronic MPTP injections affected the sleep pattern. These plots are added in Appendix A.

2.3.2 Dose range experiments

AIMS

In figure 7 the cumulative scores of the different items average per treatment per dosage are shown. No significant differences were found on the abnormal involuntary behavior the AIMS (see Figure 7). Animals could score a maximum of 36 in the AIMS scoring system (scores higher than '1' per item means a deviation from normal). All animals scored an average no larger than 1, meaning that the animals showed no involuntary abnormal behaviors. Neither the compounds nor the different dosages had an effect on abnormal involuntary behaviors.

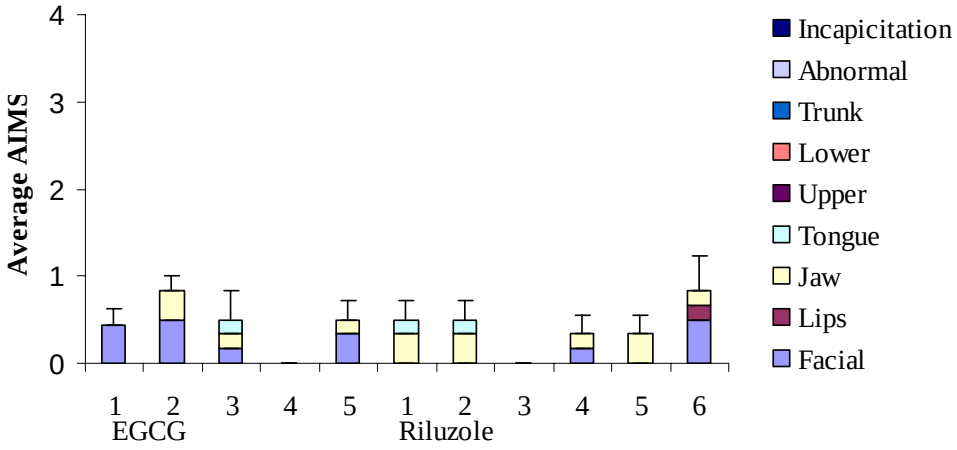

Figure 7. Average (+SEM) AIMS per test compound (n=6/group). The different items are shown in colour. 1: baseline value, 2: vehicle, 3 low dose of the test compound, 4 middle dose of the test compound, 5: high dose of the test compound, 6: one dose higher than the high dose of the test compound.

Clinical Score

For the treatment of EGCG no effect was found on any of the scored items. At all doses of EGCG the scores were '0' (not shown in Figure 8). In Figure 8 the averaged cumulative scores of the different items of each dose of riluzole are shown. Animals could score a maximum of 16 in the clinical scoring system (score 1 or lower per item showing no clinical abnormalities).

Significant effect was found in the clinical score data (ANOVA, P> 0.05). The animals which received a dose of 20 mg/kg (High2) riluzole showed a significant increase in their clinical score in comparison to the other Riluzole dosages (Bonferroni, P<0.001). However, the individual items did not reach a score higher than '1'.

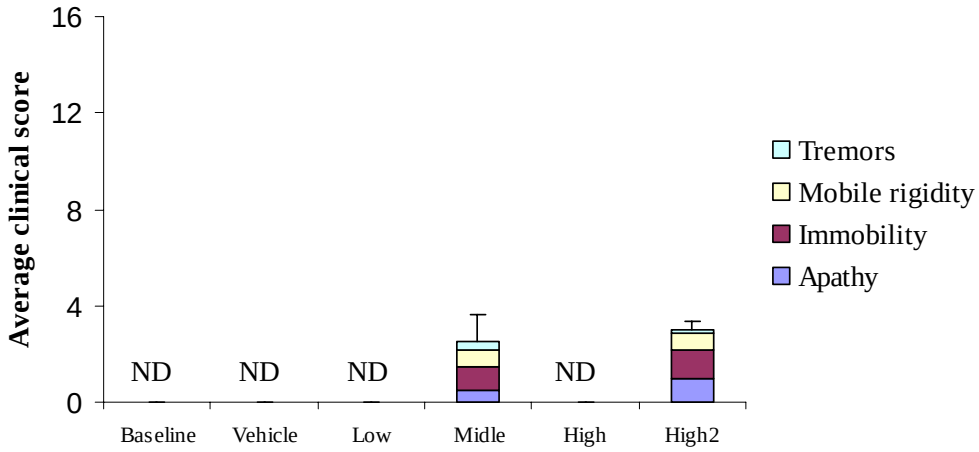

Figure 8: Average (+SEM) clinical score for Riluzole group(n=6) (ND = Not Detected).

Hand-eye coordination

No effects were found of EGCG dosage on the Hand-eye coordination test (see Figure 9). The High2 20 mg/kg riluzole dosage did significantly reduce the performance in this task in comparison to the vehicle, low and middle dosage (P < 0.05). This effect was mainly due to the increased failures in the fast moving trials. The performance on the non-moving and slow moving trials was not affected.

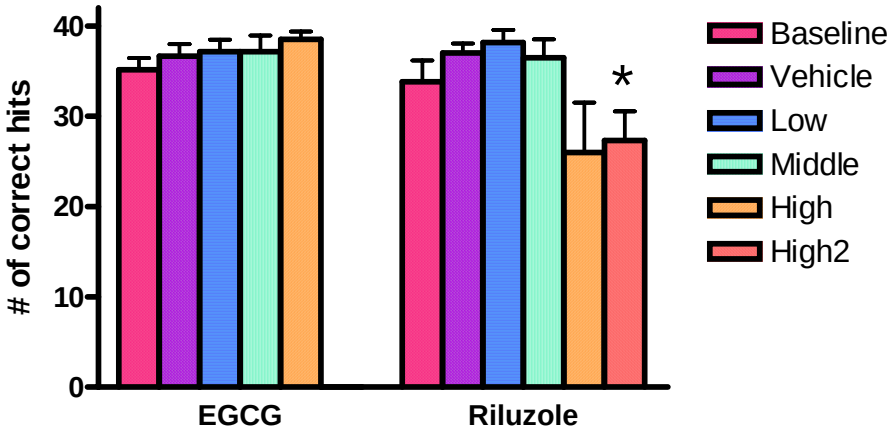

*Figure 9: Mean (+SEM) number of correct hits after EGCG and riluzole in different doses (n=6/group). * Significantly different compared to Vehicle, Low, and Middle dose (p<0.05).*

Bungalow

In Figure 10 the effects on loco-motor activity is shown. No effects of either compound were found on the number of compartment changes in the Bungalow test (log (# compartment changes)) transition to induce normality, ANOVA). The normal activity of naive monkeys in this test is 40 to 60 compartment changes. These groups of animals show a normal activity in this test.

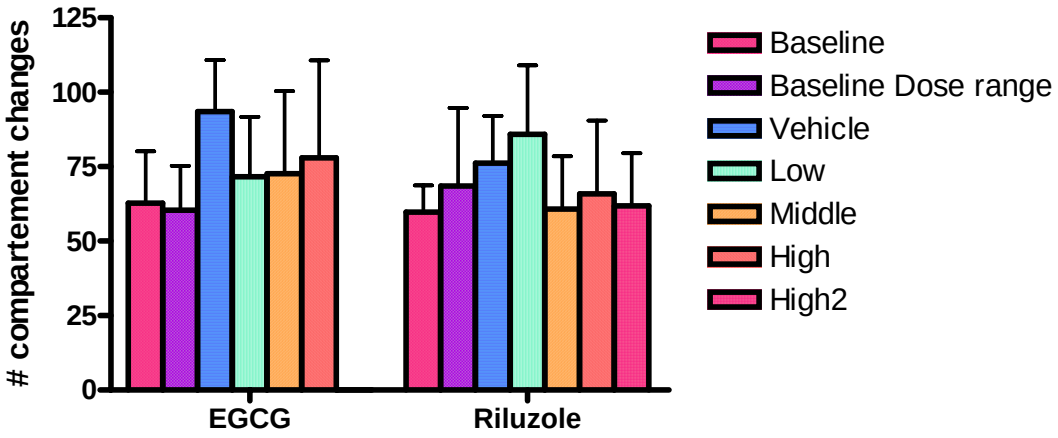

Figure 10: Mean (+SEM) number of compartment changes in 20 minutes after EGCG and riluzole in different doses (n=6/group).

Hourglass

Differences were found between the average of three fastest turns and the tube size. Healthy animals tested in the hourglass test with different sized tubes turned faster in a broader tube (see Figure 11, left figure). It took the healthy untrained animals respectively less than a second, about one second and about three seconds to turn upright. Animals turn significantly faster in a wider tube then in a smaller tube (Kruskal-Wallis $P<0.001$). Differences were found between the smallest tube compared to the two larger tubes (Post Hoc Bonferroni $P<0.001$) (see Figure 11, right figure). With both compounds an effect was found over the different test weeks (Kruskal-Wallis EGCG $P<0.01$, riluzole $P<0.001$). In the EGCG group a trend was found between the baseline values and the middle dosage (Tamhane $P<0.10$). In the riluzole group significant effects (Tamhane $P<0.01$) were found with the middle dosage and the second high (High2) dosage. No overall treatment effects were found between treatments.

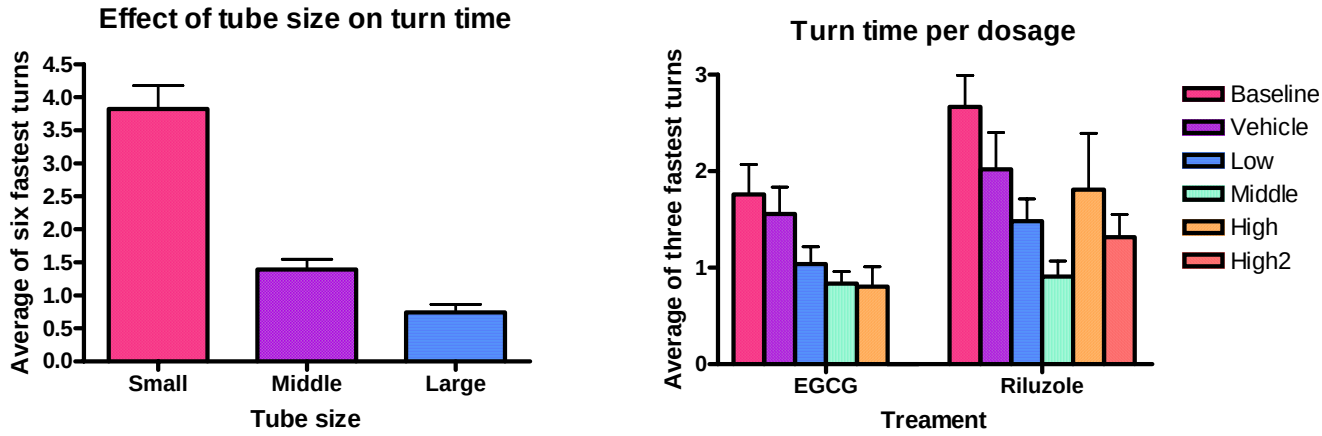

Figure 11: Left figure: Average (+SEM) of six fastest turns per tube in two hourglass sessions. Right figure: Average (of all tubes) (+SEM) number of three fastest turns per test compound.

3. AMENDMENTS TO THE PROJECT

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.

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”.

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. For the biosketches of Peternella Verhave see Appendix B.

4. KEY RESEARCH ACCOMPLISHMENTS

- 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”.

- Marmoset monkeys were trained in the hand-eye coordination task; a learned behavior for coordinated motor skills. These monkeys were used for the dose range finding study and will be re-used for the first PD experiments.

- The dose response finding experiments were accomplished. Dose range effects of L-DOPA for chronic use were already performed in our institute.

5. REPORTABLE OUTCOMS

Dose response

All animals were in good health at the end of the dose range experiments. The animals did not show aversive behavior to the oral EGCG; however they did not seem to enjoy the largest dose (50 mg/kg). Most animals reacted strongly to oral Riluzole (reacting stronger to the higher dosages). It is probably not so much the taste of the compound but the local anesthetic-like effect in the mouth of the animals and the strong sedative reaction shortly after dosage. After the high dose (20 mg/kg) some animals showed behavior which was probably due to this sedative effect. Their coordination was affected and they seemed mildly sedated (eyes closed, affected movements). The clinical score was significantly affected after the 20 mg/kg Riluzole dosage.

However the reaction to oral treatment was very different between the two compounds, the performance in the different test was not strongly affected. No effects were found on activity (Bungalow) or abnormal involuntary movements (AIMS). An effect was found on the hand-eye coordination task after the 20 mg/kg (High2) dosage of Riluzole in comparison to the Vehicle, Low and Middle dosages. This effect was probably due to the sedative affect of the drug because it was specifically the trails with the fast moving rewards which the animals missed.

It is hard to rule out the learning effects when discussing the hourglass. The graphs suggest a strong learning, underlined by the effects found on the middle dosage. However a compound effect or difference is suggested especially comparing the two high dosages. The significant difference found in the EGCG group, not found in the Riluzole group could suggest a compound effect. This is underlined by the behavior of the animals treated with Riluzole observed during the hourglass test. Some animals continuously dropped their head after 10 and 20 mg/kg dosage. More thorough conclusions could have been drawn with either a control group or a training period before hourglass testing. We have added a control group to exclude the learning effects (data not yet available).

Information about L-DOPA for long term use is already present in our institute. L-DOPA in a dose of 8 mg/kg p.o. was not able to prevent the PD symptoms. A dose of 10 mg/kg p.o. was able to reduce the clinical signs and the symptoms in the AIMS test by 30%. Also the hand-eye coordination performance and the loco-motor activity was improved. 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.).

The highest sign-free dose will be used will be used. 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 a day. 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).

Publications and presentations

- Poster session held at the 10th Endo-Neuro meeting, 07-06-06, Doorwerth, The Netherlands.
  Title: Oxidative stress and excitotoxicity in the marmoset MPTP model.
- Internal presentation Department of Diagnosis and Therapy 30-05-06, Rijswijk, The Netherlands
  Title: Neuroprotection in the marmoset MPTP model
- External presentation Molecular and Cellular Neurobiology, 09-11-06, Amsterdam, The Netherlands
  Title: Neuroprotection in the marmoset MPTP model

6. CONCLUSIONS

Based on the effects of the dose range finding study and information from the literature it was decided to use riluzole 10 mg/kg p.o. twice daily, EGCG 10 mg/kg p.o. twice daily, and L-DOPA 10 mg/kg p.o. twice daily. The analogy between the dosages is fortuitously.
Two motor functional tests will be added to the study to gather more information about the functional outcome of the progress of the disease.
The MRI scan will be replaced by telemetric neurophysiology measurements on sleep aspects.

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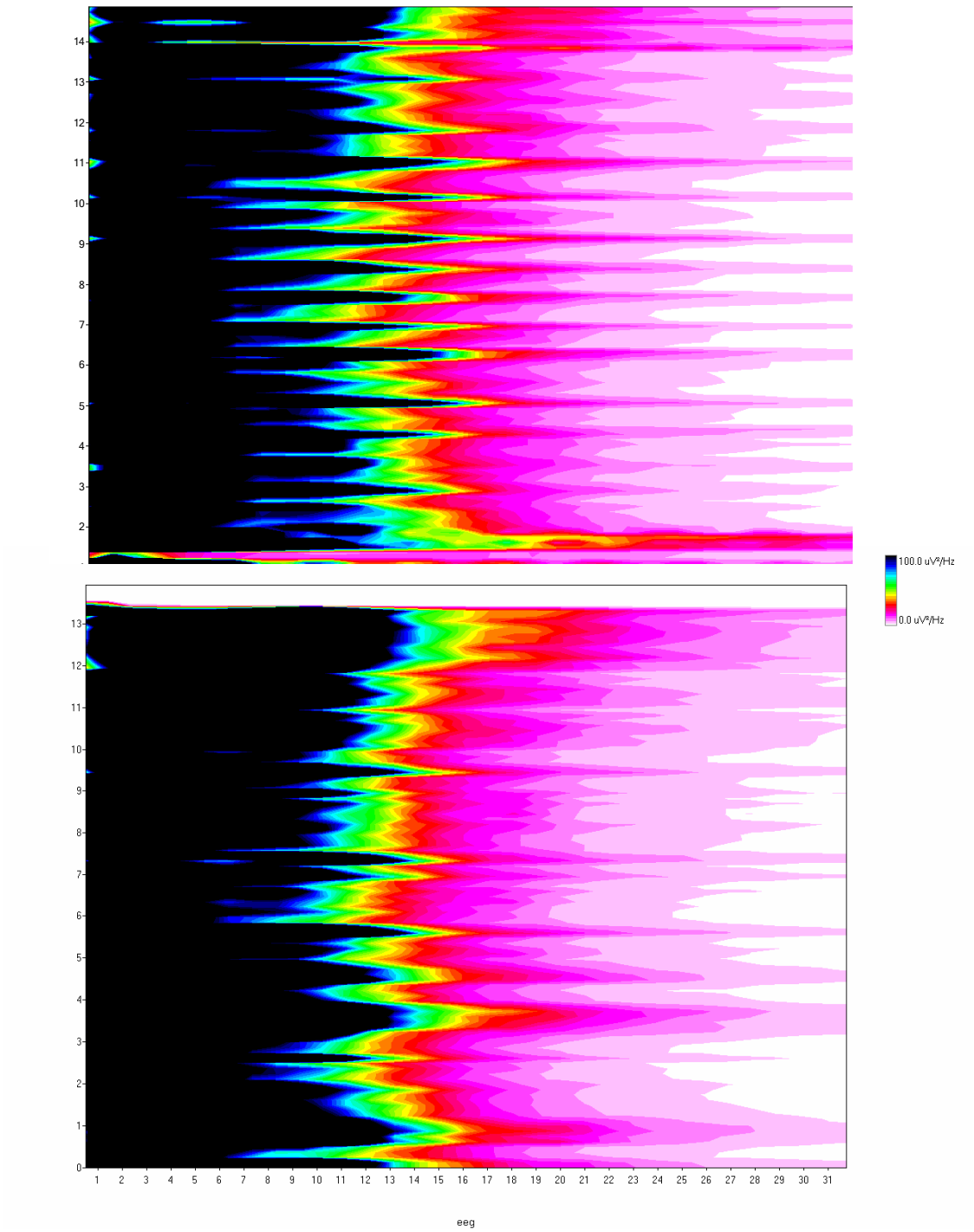
8. APPENDIX A: POWER PLOT OF SLEEP EEG



Power plots from sleep EEG registration. On the x-axis the frequency is plotted (0-32 Hz) and on the y-axis the time of the night in hours is plotted. In the plot at the top the sleep pattern of normal sleep is shown. On the second plot the sleep pattern after PD induction is shown.

9. APPENDIX B: BIOGRAPHICAL SKETCHES

NAME	POSITION TITLE
P. S. (NELLEKE) VERHAVE	NEUROBIOLOGICAL SCIENTIST

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training).

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
• Animal science, Wageningen University, The Netherlands • Environmental genetics and parisitology, Edinburgh University, Great Britain • Stress sensitisation and the glutamate receptor, RMI institute, UU, The Netherlands • Internship animal science, Massey University, New Zealand	Bachelor and Masters degree	1999-2004 2001 2003 2004 2004	Ethology and neurobiology
Employer	TNO Defence, Security and Safety, Rijswijk, The Netherlands		

RESEARCH AND PROFESSIONAL EXPERIENCE.

Peternella S. Verhave MSc, Nelleke is a behavioral neurobiologist and was born in 1981. She graduated highschool in 1999 in Nijmegen, where she was born and raised. She then started her studies of Animal Science at Wageningen University. She combined her studies as a committee member of the animal ethics committee of Wageningen University. This committee decided on all animal experiments done at Wageningen University and Research Centre. During her studies Animal Science she worked on anticipation behaviour in catfish at the Ethology department (Prof. Dr. BM Spruijt). During her studies she studied at several other universities. She studied three months at Edinburgh University with an Erasmus Scholarship. She worked on the effect of stress sensitisation on glutamate receptors at the Pharmacology and Anatomy department of the Rudolf Magnus Institute in Utrecht (Prof. Dr. V.M. Wiegant). She worked five months at Massey University in New Zealand, during this internship she was involved in several behavioural studies and she wrote a report on Animal Ethics legislation in New Zealand (Prof. Dr. K.J. Stafford). After her studies she worked at Wageningen University as a technical assistant at the Ethology department and as research assistant at the Adaptation Physiology department. As PhD student she is currently involved in Parkinson research.

Research experience:

- Research on different animal models for e.g. activity, stress, anticipation behavior, animal welfare, social behavior, reproduction and motor behavior.
- Development and working with different behavioral test systems for rats, fish, sheep, cattle, pigs, chickens, rabbits, and monkeys.
- Laboratory experience on cortisol measurements, in situ hybridization and histology on brain tissue.
- Brain activity by electro-encephalogram (EEG).
- Evaluation of animal experiments on completeness and ethical grounds (Animal Ethics Committee).