VRIJE UNIVERSTEIT

Modelling early stage Parkinson's disease in mice and marmosets

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Aard- en Levenswetenschappen
op maandag 9 mei 2011 om 15.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Peternella Suzanne Verhave

geboren te Nijmegen

promotor:	prof.dr. A.B. Smit
copromotor:	dr. I.H.C.H.M. Philippens

Raken aan waarheid
Een zilveren spiegeling
Steeds onbereikbaar

Naar een gedicht van Hakuin Ekaku (1686-1768)
Omslag J.P. Verhave en C.J.W. Verhave-van Duijn

The cover is based on a Zen poem written by Hakuin Ekaku in Japanese. The poem is about a monkey reaching for the reflection of the moon in the water. The moon stands for enlightenment. The monkey is very persistent to reach the moon. However, if he would let go of the branch, it would only be water and not the moon that he finds.
The same paradox we find in experimental research, when using models for clinical situations. We look for answers to research questions about a disease (the moon), by using available models (the reflection). We need to keep reminding ourselves that we are studying the reflection.

Beoordelingscommissie:	prof.dr. P. Heutink dr. G. Torres prof.dr. B. 't Hart dr. W.D.J. van de Berg dr. J.B.F. van Erp prof.dr. E.J.W. van Someren
Paranimfen:	M.J. Jongsma R.M. van den Berg

Print
Gildeprint Drukkerijen
ISBN: 978-94-6108-156-8

The research decribed in this thesis was carried out at TNO Defence, Security and Safety, Rijswijk, The Netherlands and the department of Molecular and Cellular Neurobiology, VU University, Amsterdam, The Netherlands. The research described in chapters 2, 3 and 4 was supported by the U.S. Army Medical Research and Materiel Command, Award No. W81XH-05-1-0517.

The printing of this thesis was supported by Novartis Pharma B.V., UCB Pharma B.V., TNO Defence, Security and Safety and VU university.

Table of Contents

1

General Introduction

P.S. Verhave and I.H.C.H.M. Philippens

So slight and nearly imperceptible are the first inroads of this malady, and so extremely slow its progress, that it rarely happens, that the patient can form any recollection of the precise period of its commencement. (Parkinson, 1817)

1.1 Parkinson's disease

In the year 1817 the English surgeon James Parkinson first described the clinical features of Parkinson's disease (PD). In his 'An Essay of the Shaking palsy', he writes: *"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensy to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellect being uninjured"* (Parkinson, 1817). This was the basis for formulating three classical motor dysfunction symptoms for PD: are: tremor, rigidity and akinesia. The disease inherited Parkinson's name because the French neurologist Charcot recognized his work at the end of the 19th century (Goetz, 1986). Notably, it took another 100 years after Parkinson's first publication before the first PD brain pathology was discovered. The German neurologist Lewy described in 1913 the characteristic neuronal inclusions, later called Lewy bodies (Holdorff, 2006). Shortly thereafter, in 1919, Tetriakoff linked *substantia nigra* (SN) pathology to PD in his doctoral thesis (Lees et al., 2008).

The great breakthrough in PD started in the 1950's initiated by the Swedish scientist Carlsson (Fahn, 2008) who first recognized dopamine (DA) as a neurotransmitter. This subsequently led to research into Parkinsonian brains by the Austrian scientists Ehringer and Hornykiewicz. They demonstrated that the level of the neurotransmitter DA in Parkinsonian brains was dramatically reduced (Ehringer and Hornykiewicz, 1960). This reduction was caused by the degeneration of DA neurons in the SN. Only one year later the first patients were treated with the DA replacement drug L-dihydroxy-phenylalanine (L-DOPA) (Birkmayer and Hornykiewicz, 1961). The L-DOPA therapy for PD was designed and described by the English physician Cotzias (Fahn, 2008).

Parkinson had already described that the disease manifestation, in number of cases and severity, was more prominent with *advanced age*. However it is now well known that individuals are still in the prime of their lives when they start showing from PD symptoms. The progressive nature of PD gradually results in serious functional motor impairment. Nowadays, with improved conditions in nutrition and the increased quality of health care, life expectancy is increasing every year (Dorsey et al., 2007). This naturally makes age-related disorders like PD become more prevalent in the general population. Due to the progressive neurodegenerative properties, PD has a high impact on the patients and their social environment, and it also puts an economic burden on the health care system. Parkinson's disease is second to Alzheimer's disease (AD), one of the main chronic and progressive

neurodegenerative disorders. Currently, around 1.5% of Europeans over 65 years of age are diagnosed with PD (von Campenhausen et al., 2005).

Treatment today is still heavily dependent on the DA replacement therapy discovered in the 1960's, which is primarily aimed at symptom control and is not without severe side effects. Tackling PD, would be to focus on prevention and intervention of the cell death processes. This strategy has been well-recognized (Jankovic, 2005; Tolosa et al., 2009) and research efforts to develop ways to slow down or stop the progression of neurodegeneration is the current focus of many clinicians and basic scientists. Early identification of individuals at risk and an early start of neuroprotective treatment to prevent the progressive loss of neurons is then the priority. Parkinson already described these early stages of the disease. He writes: *So slight and nearly imperceptible are the first inroads of this malady, and so extremely slow its progress, that it rarely happens, that the patient can form any recollection of the precise period of its commencement.* This formulates well the obstacles in the investigation of early stages of PD and prompts us to investigate early stages of PD in humans and in animal models. In particular animal models offer an opportunity to link low level neurodegeneration to disease manifestation and a possibility to investigate possible routes for intervention therapy.

1.1.1 PD manifestation

The progressive neurodegeneration in PD results in a wide range of disabling motor and non-motor symptoms (fig. 1). Generally, patients enter the clinic with motor-related problems when they are in their fifties. At the time of diagnosis they are suffering from bradykinesia (slowness of movements) and one or more of the other classic motor-related problems: tremor at rest (typically in the hands), rigidity of movements, akinesia (impaired movements) and/or postural instability (balance problems). Besides motor problems, patients may suffer from disturbing non-motor problems like depression, constipation and dementia. Since there are no early diagnostic biological markers for PD, the clinical diagnosis is currently entirely based on the presence of the characteristic motor features. However, diagnostics based on live imaging of the DA transporter in brain scans (DaTSCAN) and non-motor symptoms like olfactory dysfunction are under current investigation (Deeb et al., 2010). Because before the now used motor symptoms-based diagnosis, many patients report to have been suffering for years from abnormal olfaction, mood disorders, autonomic dysfunction and sleep problems (Berg, 2008; Tolosa et al., 2009). Of these non-motor

symptoms, sleep problems are reported in around 80% of all PD patients (Tandberg et al., 1998; Oerlemans and de Weerd, 2002; Garcia-Borreguero et al., 2003). Sleep problems can range from reduced sleep efficiency, difficulty in turning in bed, to motor problems during rapid eye movement (REM) sleep, diagnosed as REM Sleep Behavior Disorder (RBD). The latter observation has been proposed to be a useful preclinical biomarker for PD (Iranzo et al., 2006; Postuma et al., 2006). Together with the classic motor symptoms these sleep problems are reported to be very disturbing for patients and (bed) partners. All together, with or without medication, patients are heavily disabled by a wide range of problems which invariably increase over time.

1.1.2 Neuropathology and mechanism of action

The control of movement is a result of the complex interplay of various groups of nerve cells in the central nervous system. Neurons in the *basal ganglia* (*striatum, pallidum, subthalamic nucleus* and SN) are the key players in motor function and are responsible for the fine-tuning of movements. They are regarded as components of several largely segregated *basal ganglia-thalamocortical* circuits serving cognitive, oculomotor and motor functions (Joshua et al., 2009). Most important to PD are a group of neurons located in the SN, situated in the *ventral midbrain*. Neurons of the SN communicate with other neurons in the *basal ganglia* through DA neurotransmission. At the time of PD diagnosis (when the diagnosis is based on typical motor symptoms), PD patients have already lost at least 50% of the DA neurons in the SN (Jellinger, 2008). Together with the loss of these neurons DA synthesis and DA release are drastically reduced. In- and output structures within the *basal ganglia* are connected through a direct monosynaptic pathway and an indirect polysynaptic pathway. DA regulates transmission of signals via the D1 receptors (direct pathway) and D2 receptors (indirect pathway). A reduction of DA neurons in the *substantia nigra pars compacta* (SNpc) results in a decrease of transmission via both D1 and D2 receptors localized within the *striatum* (Levey et al., 1993). The reduced D1 excitation leads to a reduced negative feedback to both the internal *pallidal segment* (GPi) and the s*ubstantia nigra reticularis* (SNr). The reduced D2 excitation leads to a reduced negative feedback signal to the external *globus pallidus* (GPe), which activates the *subthalamic nucleus* (STN). This in turn activates GPi and the SNr. So both, the indirect and the direct pathway ultimately lead to an overstimulation of the GPi/SNr. This causes an increase of negative feedback to *thalamocortical* projecting neurons,

reducing the usual reinforcing influence of the motor circuitry upon cortically initiated movements (Alexander and Crutcher, 1990; Wichmann and DeLong, 2003). Indeed in non-human primate models of PD the reduction of SNpc output leads to decreased facilitation of cortical motor areas and subsequent development of akinesia and bradykinesia in PD (Wichmann and DeLong, 2003). Together with this neurodegeneration in the SN, intraneural protein inclusions are found in the SN and other brain regions (Braak et al., 2003). The marked neurodegeneration in the SN together with the occurrence of protein inclusions called Lewy bodies form the post-mortem confirmation of the PD diagnosis.

1.1.3 Molecular and cellular mechanisms of PD

Although PD has been studied for almost 200 years, the precise mechanisms leading to progressive cell death still need to be resolved. PD is a multi-factorial disorder resulting from the combined effect of age, environmental factors, genetic susceptibility and complex genetic-environmental interactions (fig. 1) (Chan et al., 1998; Le Couteur et al., 2002; Migliore and Coppede, 2009; Schapira, 2009). Many epidemiological studies support the role of pesticide exposure in PD. For example, rural living (Chen et al., 2009), drinking well-water (Gatto et al., 2009) and occupation based exposure (Goldman et al., 2005) are potential risk factors that support the earlier stated role of pesticides. Recently, at least eight defined genetic loci have been associated with autosomal dominant or recessive familiar PD, wherein thus far five causative mutations have been identified (Nuytemans et al., 2010). Mutations in the following genes have been reported to cause familial PD: α-synuclein (SNCA), leucine-rich repeat kinase (LRRK2), parkin (PARK2), PINK1 (PARK6) and DJ-1 (PARK7). Although these familial forms of PD are rare compared with the frequency of sporadic cases, they are very important for understanding, the molecular basis or cause of the disease pathology.

The cellular processes involved, and thought to interact with age, environment, genes or a combination of these two are: mitochondrial dysfunction, glutamate excitotoxicity, oxidative stress, inflammatory responses and proteasome dysfunction (Alexi et al., 2000; Betarbet et al., 2000; Dauer and Przedborski, 2003; Jenner, 2003a). These mechanisms are described below. In this regard, neurons may die by necrosis, caused by changes in ion traffic, cellular swelling resulting in the disintegration of the cell and its organelles and their removal by

phagocytosis. Neurons can also go into apoptosis initiated by exogenous toxins which are mediated by e.g. oxidative stress and the release of cytochrome c by mitochondria.

Mitochondrial dysfunction: The involvement of mitochondria in neurodegeneration was reported at the end of the 1980's (Schapira et al., 1989), which entailed more specifically a deficiency in complex I of the inner membrane electron transport chain. The dysfunction of the mitochondria, specifically the electron transport chain leads to energy failure, increased reactive oxygen species (ROS) production and a release of apoptosis signaling molecules (Fulda et al., 2009). The Parkinson gene PINK1 and the experimental PD inducing toxins (MPTP and rotenone) also specifically affect complex I.

Excitotoxicity: Glutamate is the main excitatory neurotransmitter in the central nervous system and is essential for the communication between neurons. Alterations in its homeostasis can lead to lethal excitotoxic cascades. These cascades are primarily initiated following overactivation of the N-methyl-D-aspartate acid (NMDA) and α-amino-3-hyroxy-5-methylisoxazole propionic acid (AMPA) receptors, and voltage-gated calcium channels resulting in a lethal influx of extracellular calcium. This overload in calcium leads to cell death via the generation of free radicals, inhibition of protein synthesis, and ultimately mitochondrial damage (Beal, 1992).

Proteasome dysfunction: Proteasome dysfunction causes misfolding of proteins and protein aggregates. The Lewy bodies in PD expressing α-synuclein have been suggested to be the result of poor-performance of the proteasome (Cookson, 2009). Lewy bodies are also suggested to play an active role in pathogenesis (Chau et al., 2009; Cookson, 2009) affecting ER-Golgi transport, synaptic vesicles and mitochondria.

Oxidative stress: Oxidative stress is, besides PD, associated with several other neurodegenerative disorders such as Down syndrome and AD (Castellani et al., 1995; Iannello et al., 1999; Aksenov et al., 2001; Danielson and Andersen, 2008). Age is a main factor in the increase of oxidative stress in the brain (Stadtman and Berlett, 1998) and is directly associated with PD (Danielson and Andersen, 2008). In PD brains oxidative stress causes a decrease of the anti-oxidant glutathione (Pearce et al., 1997) and together with the increase of ROS as a result of α-synuclein (Junn and Mouradian, 2002) this points to an imbalance in the removal and production of ROS. Furthermore inherited mutations in the gene DJ-1 are directly linked to oxidative stress (Andres-Mateos et al., 2007) resulting in PD.

Inflammation: Together with DA neurodegeneration in the SN and the *striatum* of PD brains an increase in microglia is also found (Sawada et al., 2006). Microglia, inflammation-activated cells in the central nervous system, are associated with neuroprotection as well as neurotoxic activity in PD-associated brain areas, probably dependent of the disease stage (Sawada et al., 2006).

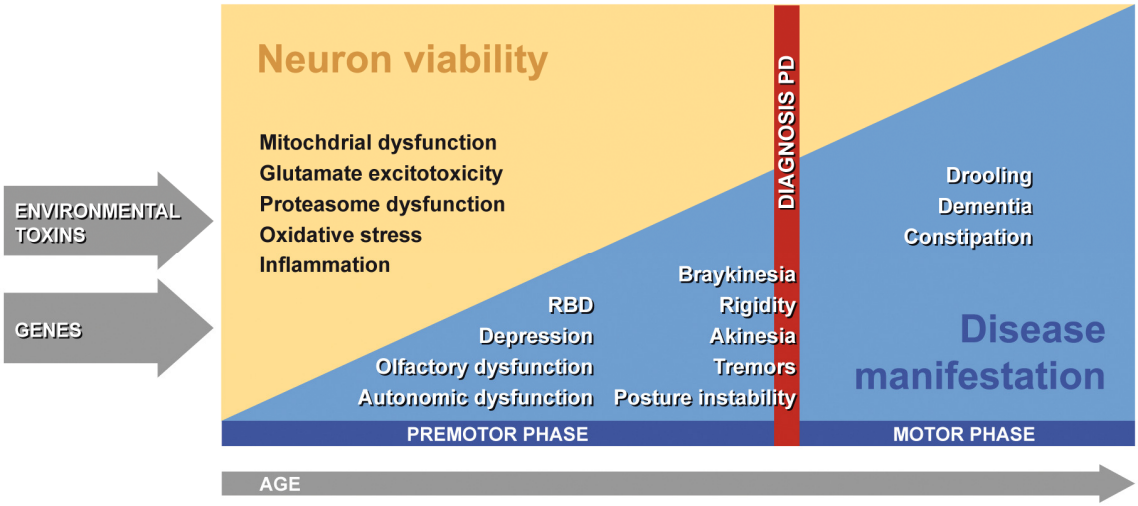

Figure 1. Schematic diagram depicting the pathology and symptomatology of PD. The disease manifestation is divided into the premotor phase, before PD diagnosis and the motor phase starting around diagnosis. The multi-factorial nature of the disease is depicted by the possible causes of PD: environmental toxins, genes and age are depicted by the grey arrows. The neuron viability is reduced over time as shown in the yellow triangle with the proposed features that cause the degeneration of neurons in random order. The occurrence of symptoms increases over time as shown in the blue triangle with the different symptoms that occur at each phase of the disease.

1.1.4 Treatment

Although the cause and progression of the disease cannot yet be prevented, there are several treatments available. Symptomatic treatment alleviates patients of some of the disturbing everyday symptoms of PD. It includes replacement or mimicking of DA in the *striatum* and functional (stereotactic) neurosurgery. The L-DOPA dopamine replacement treatment, which was initiated by Birkmayer and Hornykiewicz (Birkmayer and Hornykiewicz, 1961), is still the most successful treatment today, acting directly on postsynaptic DA receptors and substituting the absence of the endogenous neurotransmitter. Today physicians often

combine this treatment with COMT (Catechol-O-methyl transferase) inhibitors to maintain continuous levels of L-DOPA after oral dosing. Other therapies focused on DA availability or on replacement are, MAO-B (Monoamine oxidase type B) inhibitors (e.g. selegiline and rasagiline) and several DA agonists. Inhibition of MAO-B results in a decreased degradation of DA. DA receptor (DAR) agonists (e.g. ropinirole and pramipexole) result in symptomatic effects via the postsynaptic DARs. They were introduced for late-stage PD patients that were suffering from severe motor function related to side effects and fluctuations in the motor response due to treatment with the short-acting L-DOPA. Nowadays, they are considered as an alternative treatments for the early stages of the disease (Clarke, 2004). The problem is that the DAR drugs initially improve patient bodily function but over time they become less effective and start causing excessive, spasmodic movements (dyskinesia) and other major side-effects such as sedation and psychiatric complications (Rascol et al., 2003; Lewitt, 2008).

Anti-cholinergic treatment was used as a therapy for PD even before the DA breakthrough. It is, however, not as effective to manage the PD symptoms, and it has aversive cognitive side effects. Alternatively, apomorphine injections or infusions may act on the STN in late stage PD. Surgical procedures, e.g. lesioning the *pallidus, thalamus* or *subthalamic areas*, became less popular as treatment for PD after the introduction of L-DOPA because of the surgery risks and the irreversible nature. Recently, deep brain stimulation (DBS) has gained popularity to treat tremors in advanced PD (Sydow, 2008) by suppressing the neuronal firing pattern in the target area (STN) either directly or by inducing the release of inhibitory transmitters (Hilker et al., 2008). Thousands of patients worldwide have undergone DBS treatment. Although temporarily effective these PD therapies do not stop or reduce the neurodegenerative state and therefore do not actually cure the disease. Neurorestorative cell therapy is under investigation, however major ethical and practical issues need to be solved before it can advanced to clinical trails (Xi and Zhang, 2008). The current priority in PD research is, therefore, to move beyond symptom control to neuroprotective treatments.

1.1.5 Neuroprotective treatment

Neuroprotective therapies in PD can be defined as medical interventions that treat the underlying cause of PD neurodegeneration and thus delay the onset, or slow down the course of the disease. To find an effective neuroprotectant, the knowledge of the cause of

neurodegeneration is crucial. However, this is a major challenge because some of the identified pathological events can act separately or synergistically, and may act differently in individual patients and also during the various stages of the disease. Nevertheless, several neuroprotective strategies have been proposed to protect the brain from neurodegeneration. Most of these neuroprotective compounds are either acting as anti-oxidants or as anti-apoptotic agents. Some of the anti-oxidants have already been tested in the clinic such as tocopherol, the MAO-B inhibitor 1-deprenyl, the mitochondrial stabilizer coenzyme Q10 and the anti-apoptotic compound rasagiline (ParkinsonStudyGroup, 1993; Shults et al., 2002). Also anti-apoptotic compounds such as neuroimmunophilin, pramipexole and ropinirole have been tested for neuroprotective efficacy in PD patients (Sethi et al., 1998; Gold and Nutt, 2002; ParkinsonStudyGroup, 2002).

Besides these drugs, some treatments are directed against inflammation, glutamate release or excitotoxicity, or addressing the disturbed mitochondrial energy supply or neuronal maintenance, thereby ultimately aiming at reducing apoptosis and necrosis of the DA neuron. Examples are riluzole, a versatile anti-excitotoxic compound and a possible candidate for neuroprotection in PD (Bensimon et al., 2009), and the trophic factors like glial cell-derived neurotrophic factor (GDNF) (Nutt et al., 2003) and the DA replacement L-DOPA (Fahn, 2005). Some of the above mentioned compounds have shown promising neuroprotective effects in the clinic, whereas others did not. The neuroprotective efficacy is generally measured by the delay in time to start L-DOPA therapy, changes in PD symptoms, or imaging of DA markers. The following reasons may lead to difficulties in assessing the effectiveness of a neuroprotective compound (Ravina et al., 2003; Olanow et al., 2008): 1) none of the outcome measures used in clinical trials directly reflect neurodegeneration, 2) the outcome measures are confounded by the symptomatic or pharmacological effects of the intervention, 3) dosing to achieve neuroprotective action of a compound is often a guess based on possible non-relevant parameters from animal studies, 4) diagnosis can be mistaken with other related Parkinsonian disorders and 5) PD patients included in the trials were diagnosed for PD and are thus in a progressive state of neurodegeneration.

Although several neuroprotective compounds have been tested in both animal models and patients, none have led to an approved neuroprotective treatment for PD by the FDA. Neuroprotection in patients remains the ultimate goal. However it has to be combined with extensive preclinical screening, early diagnosis and exclusive neuroprotection markers.

In summary the history of PD begins with the first description by Parkinson. Despite almost 200 years of research the start and cause of the disease are not entirely unravelled. The disabling motor problems are a result of excessive cell loss in the SN and are preceded by a range of non-motor symptoms. Treatment is currently focused on replacing DA in the *striatum* thereby regaining motor function. Neuroprotective treatment for neurodegenerative diseases including PD is still scarcely effective but it could offer an opportunity to halt to the degeneration before motor symptoms occur.

1.2 Pre-clinical aspects of Parkinson's disease

1.2.1 Models for PD

Studying PD neurobiology in combination with disease manifestation in humans is limited to clinical trials and post-mortem material. Therefore, in order to find new targets for neuroprotective therapies, having adequate animal models available is an excellent asset in current PD research. Cell cultures or invertebrate models are useful (Botella et al., 2009; Schule et al., 2009) but they can only model PD to a certain extent.

Animal models should ideally mimic the main features of the disease pathology and additionally show the typical Parkinsonian syndrome. In this regard four scientific criteria have been designed (van der Staay et al., 2009) to test the validity of a model: face, predictive, construct and external validity. The DA deficiency observed in PD is the main event underlying the pathophysiology of the motor symptomatology.

Animal models can feature the typical –preferably progressive- loss of DA neurons in the SN in combination with the associated DA reduction in the *striatum*. This is called face validity or the degree of descriptive similarity between the symptoms in the animal model and in the human affected by PD. Often the presence of the typical PD behaviors or the PD specific Lewy body formation can be an important addition to answer the research question addressed in an animal model. In the pharmacological context, predictive validity refers to the ability of a model to correctly identify the efficacy of a therapeutic strategy. Therefore L-DOPA induced improvement of motor behavior is a key issue in animal models for PD.

Because of the multi-factorial nature of PD, construct validity is the most difficult scientific criterion in modeling idiopathic PD. Construct validity is the degree of similarity between the mechanisms underlying behavior in the model and that underlying the behavior in the condition, which is being modeled. Animal models can mimic the pathology and the

symptomatology of the disease but not easily the etiology. However, factors like genes, environment and age can be altered separately or in combination in animal models thereby insuring construct validity to a certain extent.

External validity, the way the results, which are obtained using a particular model, can be generalized or applied to and across models and populations. This is the information which is obtained in one situation or model and repeated in another lab or model.The ultimate PD model has not yet been described; however there are several experimental models that meet the above criteria. Some PD models rely on selective neurotoxins to chemically destroy DA neurons or on precise targeting of the specific brain regions using stereotactic surgery; others are focused on genetic deficits. In this thesis we focus on neurotoxicologically induced Parkinsonism.

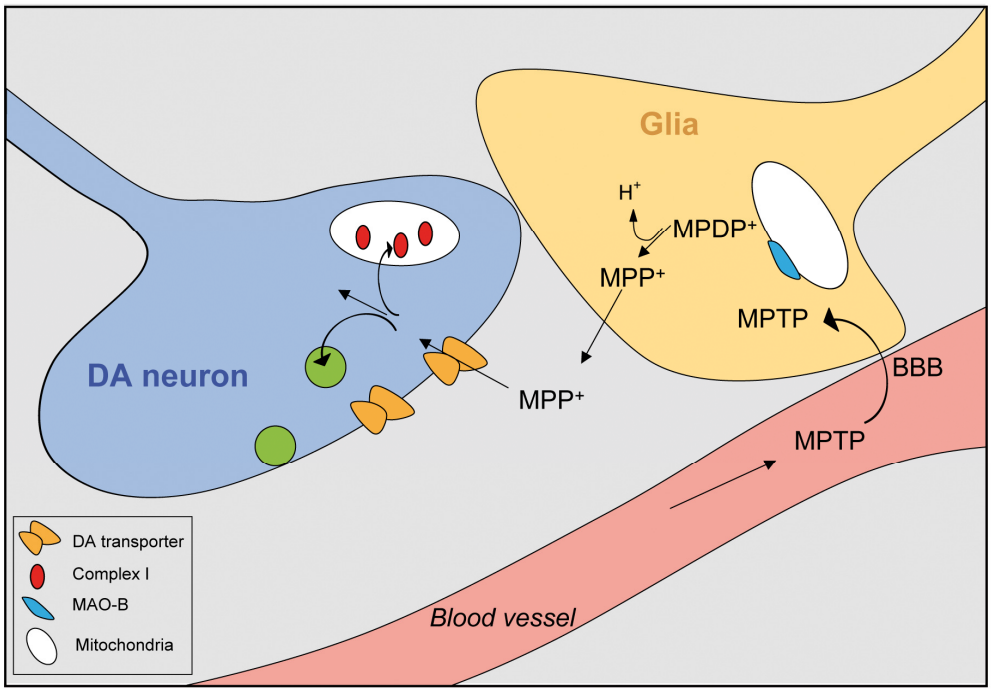

Figure 2. Schematic overview of MPTP-metabolism trafficking (Przedborski and Vila, 2003). After administration, MPTP, crosses the BBB and is metabolized into 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme MAO-B in glia cells. $MPDP^+$ is oxidized into 1-methyl-4-phenylpyridinium (MPP^+), the actual toxic compound. MPP^+ is released into the synaptic cleft and taken up by DA transporters. Inside DA neurons, MPP^+ concentrates in the synaptic vesicles, the cytosol and via an active process in the mitochondria. In the mitochondria MPP^+ impairs complex I of the electron respiration chain. The inhibition of complex I causes an increased production of free oxygen radicals.

1.2.2 MPTP

The most preferred toxin to induce Parkinson-like DA neurodegeneration is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a neurotoxin that easily crosses the blood brain barrier (BBB) and that is specific for DA neurons. The MPTP model induces face validity through a specific lesion in the SN and it shows predictive validity with the use of L-DOPA. The MPTP model is actually environmentally induced Parkinsonism; hence it has construct validity to a certain extent. And since MPTP induces reproducible PD in mice, monkeys and humans it offers appropriate great external validity.

Other useful toxins are the non-specific 6-hydroxidopamine (6-OHDA), rotenone and paraquat. 6-OHDA is recognized by SN neurons as DA and is taken up by the cell where it then exerts its toxic properties. As 6-OHDA does not cross the BBB it needs to be locally administered, which is not a trivial procedure. Additionally the severity of the lesion depends on the physical distance towards region of interest, which makes this model less suitable for studying the molecular mechanisms of neurodegeneration (Bove et al., 2005). An advantage of this model is that unilateral 6-OHDA lesions, generally used in rats, have proven to be very reproducible over time, in which the non-infused hemisphere can serve as inter-animal control (Blandini et al., 2007). Unlike paraquat which offers contradictory results in mice, repeated systemic administration of rotenone, which easily penetrates the BBB, is another potential alternative to induce Parkinson-like neurodegeneration in experimental animals (Sherer et al., 2003; Schmidt and Alam, 2006). Both paraquat and rotenone affect neurons by disturbing processes such as the glutamate balance, increasing ROS production, the mitochondrial respiration or misfolding of proteins as reviewed by Bove et al. (2005).

MPTP, the compound of choice for this thesis, was first discovered in the early 1980's when a group of young Californian drug users used the wrong temperature in one of the synthetic steps for the drug meperidine (Langston et al., 1983). MPTP was present in these preparations. The use of the preparations resulted in Parkinsonism in the users (Ballard et al., 1985). This observation led to the identification of MPTP as a drug derivative with specific neurotoxic properties. In animal experiments that followed this accidental Parkinsonism, it became clear that the MPTP caused selective cell death of DA neurons highly specific for the SN (Javitch et al., 1985). MPTP can cross the BBB in mice (Melamed et al., 1985), monkeys (Burns et al., 1983) and humans (Ballard et al., 1985) but not in rats due to the clearance of MPTP outside the brain (Kalaria et al., 1987). After entering the

brain, glia cells facilitate the conversion of MPTP into 1-methyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MOA-B). Subsequently, the actual toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) is formed by oxidation. MPP^+ leaves the glia cells and enters the DA cells via the dopamine transporter (DAT). Once inside the neuron MPP^+ is taken up in vesicles by the vesicular monoamine transporter (fig. 2) and taken up by mitochondria by means of an energy demanding process (Del Zompo et al., 1993). In mitochondria MPP^+ blocks the electron transport enzyme ubiquinone oxidoreductase (complex I) (Nicklas et al., 1985; Ramsay et al., 1991) and this leads to a reduction in cellular ATP. Additionally, MPP^+ has also been reported to directly inhibit other inner membrane complexes, namely complex III (ferrocytochrome c oxidoreductase) and IV (cytochrome c oxidase) of the electron transport chain (Mizuno et al., 1987; Smeyne and Jackson-Lewis, 2005). Corresponding to these mitochondrial effects of MPP^+, selective reduction of Complex I activity has also been found in the SN and *striatum* of PD patients. Additionally it where non-human primates, injected with MPTP to induce Parkinsonism, which where the main source of insight into the effect of DA reduction on the *basal ganglia* circuitry (DeLong, 1990).

1.2.3 Mouse models

Having a relatively short lifespan, genetic mouse models are of interest due to their quick course of progressing disease stages and their consistent neurological defect. This makes it possible to obtain biological, physiological and behavioral correlates of the restoration of lost functions by means of various treatments. There are several pharmacologically induced PD models available that can be used in mice with or without a genetic mutation. Currently, more than 140 spontaneous mutations are known to affect the nervous system of laboratory mice. These mutant mice are valuable models for investigating various pathological conditions that modify brain function either during development or in adulthood. For example in the Weaver mutant mouse, there is a selective decrease of neurons in the SN (Marti et al., 2007) due to a potassium channel mutation (Patil et al., 1995). Another example is the MitoPark mouse with a DA specific respiratory chain deletion resulting in a mouse with progressing DA neurodegeneration, motor problems and a time-dependent reaction to L-DOPA (Ekstrand and Galter, 2009).

Besides the genetic models, mice have also shown to be reproducible and sensitive animal models in acute MPTP intoxication studies (Schmidt and Ferger, 2001). However, unlike humans and monkeys, mice need a relatively high systemic dose of MPTP to induce DA neurodegeneration (Jackson-Lewis et al., 1995) and they show a relatively restricted PD-like symptomatology. Therefore, face validity is not optimal (Luchtman et al., 2009). Thus, mice are mainly of interest for neuropathology and molecular changes after neurodegeneration of DA neurons. To achieve a more progressive PD-like course of neurodegeneration more sub-acute administration protocols are under investigation (Petroske et al., 2001; Luchtman et al., 2009). The mouse MPTP infusion model (Fornai et al., 2005) aims to induce a more slowly progressive Parkinsonism with manifestation of protein inclusions analogous to Lewy bodies. The drawback of MPTP peak levels due to injections is prevented with this infusion model that accordingly generates a gradual intoxication of neurons. Consequently, this model has no immediate massive neurodegeneration due to high doses of MPTP, but provides a neuronal challenge model of the early phase of PD. This creates the opportunity for finding biomarkers expressed at the onset of the neurodegenerative process rather than at the final stage of neuron loss. Because of the interest in early Parkinsonism, the main focus in this thesis was not in cell death and the fatal disease endpoint in the animal model, but to investigate mechanisms involved at the start of the neuronal challenge.

1.2.4 Non-human primate models

Non-human primates are generally appreciated as model species because of their similarities to humans. Monkeys are, like humans, very sensitive to MPTP (Burns et al., 1983; Jenner et al., 1984) and after this treatment they express many features of clinical PD which might reflect their genetic, physiologic and behavioral proximity to humans. They have a similar *striatum* structure, similar hand-foot use and comparable responsiveness to all DA medications known to be effective in PD (Eslamboli, 2005); this makes them a valuable addition to the range of available PD models. Old world monkeys with high cognitive abilities, such as Macaque monkeys (Burns et al., 1983) and Baboons (Hantraye et al., 1993) are interesting because they can handle complex behavioral tasks enabling testing cognitive deficits in the late stage of Parkinsonism. New world monkeys, such as squirrel monkeys, capuchin monkeys and common marmosets are, although somewhat less sensitive to MPTP, especially useful because of the aforementioned abilities, but also because of their smaller

size and which makes them more suitable for a laboratory environment. The popular marmoset MPTP model has offered several advantages in studying disease therapies and neuroprotective methods. Unlike the rodent models (Jackson-Lewis et al., 1995; Mandel et al., 2002; Meredith et al., 2008) MPTP-treated marmosets show optimal face validity with a wide range of Parkinsonian behaviors (Jenner et al., 1984; van Vliet et al., 2006) including the L-DOPA-induced dyskinesia (Visanji et al., 2006) and hallucinations (Fox et al., 2006). The 'clinical' condition of the Parkinsonian state in this monkey model is generally focused on the motor symptoms. To measure motor symptoms in marmosets, extensive rating scales are available (Pearce et al., 1996; Iravani et al., 2003; Jenner, 2003b; van Vliet et al., 2006) and tests to quantify locomotor activity (Obinu et al., 2002; van Vliet et al., 2006) and hand-eye coordination (Annett et al., 1994; van Vliet et al., 2006) are also available. Non-motor symptoms in Parkinsonian marmosets are apathy (van Vliet et al., 2006) and bladder problems (Albanese et al., 1988). A more in-depth insight of the non-motor symptoms would be a valuable addition to this model in order to investigate the effect of DA drugs as reviewed by Jenner (2009). Because of the striking similarity in sleep macrostructure between marmosets and humans we evaluated the changes in sleep due to MPTP treatment in combination with the electromyogram (EMG) signal during the episodes scored as REM sleep. By doing so, early changes in muscle tension during sleep might be detectable in the marmosets such as those found in PD patients suffering from RBD.

1.3 Riluzole

The compound 2-amino-6-trifluoromethoxy benzothiazole (riluzole) was first described as an anticonvulsant compound (Benavides et al., 1985; Mizoule et al., 1985). Ten years after its introduction, the drug was subsequently approved by the Food and Drug Administration (FDA) as a life-prolonging treatment in amyotrophic lateral sclerosis (ALS) (Bensimon et al., 1994; Couratier et al., 1994). Riluzole is a prescription drug available since 1994 under the trade name Rilutek® (Sanofi-Aventis) and it is one of the few drugs that actually has been approved to be used as a neuroprotective compound in a neurodegenerative disorder. Ever since its FDA approval, riluzole has been studied for its versatile neuroprotective effects in experimental conditions. Central in all routes of action are its inhibitory effects on glutamate release and the availability of this compound in the synapse. Riluzole specifically acts on

active synapses (Lamanauskas and Nistri, 2008); it acts on the presynaptic glutamate release by affecting the calcium influx through a G-protein and protein kinase C (PKC) depended signaling pathway (Hubert et al., 1994; Doble, 1996; Martin, 2000; Wang et al., 2004). Persistent calcium influx leads to the repetitive firing of motor neurons. Riluzole indirectly inhibits the calcium channels by blocking sodium and potassium channels (Beltran-Parrazal and Charles, 2003). Although riluzole does not bind to the NMDA, AMPA, kainate or metabotropic glutamate receptors (Doble, 1996), it affects these receptors indirectly because:
1) Riluzole can bind directly to PKC (Noh et al., 2000). In this way riluzole reduces PKC activation, which in turn inhibits the NMDA trafficking, thereby inhibiting presynaptic NMDA receptors to favor glutamate release (Lamanauskas and Nistri, 2008).
2) Riluzole suppresses synaptic glycine release to presynaptic mechanisms. Glycine is a co-activator of the NMDA receptor, thereby riluzole indirectly inhibits NMDA receptor activity (Umemiya and Berger, 1995).
3) Riluzole can modulate AMPA-type receptor channels by a combination of open-channel blockade and binding site competitive-blockade mechanisms (Jin et al., 2010).
4) Riluzole acts directly on the astrocytic glutamate transporters (EAATs) (Debono et al., 1993). EAATs are regulators of glutamate homeostasis in the extracellular space, and prevent overstimulation of postsynaptic glutamate receptors and in this way avoid glutamate-mediated excitotoxicity.
Besides the direct glutamatergic effects of riluzole, also gamma-aminobutyric acid (GABA) effects have been reported (Kretschmer et al., 1998). It has been shown that riluzole blocks synaptosomal uptake of GABA in the *striatum* of rats. Subsequently, the increased endogenous GABA levels in the synaptic cleft enhance GABA activity. In a microdialysis study it was found (Wang et al., 2004) that GABA inhibited glutamate release in the cortex of rats. Hence riluzole can also indirectly reduce the available glutamate by blocking GABA uptake. In addition, riluzole's neuroprotective effect is also suggested to be caused by a riluzole mediated increase in neurotrophic factors (Fumagalli et al., 2006). Finally, it has been reported that riluzole has a direct anti-oxidative effect *in vitro* by blocking lipid peroxidation (Koh et al., 1999; Storch et al., 2000).

1.3.1 Neuroprotective riluzole treatment

Around the time that riluzole was introduced as an official treatment for ALS it also came under investigation as a treatment for PD (Boireau et al., 1994b; Boireau et al., 1994a). It became clear that riluzole treatment, when started around the initiation of neurodegeneration (Doble, 1996), was able to prevent damage to DA neurons in experimental PD models in mice, rat, marmoset and rhesus monkeys (Benazzouz et al., 1995; Araki et al., 2001; Douhou et al., 2002; Obinu et al., 2002; Scherfler et al., 2005). *In vivo* riluzole does neither interfere with the metabolism of the toxin MPTP, nor with DA uptake (Samuel et al., 1992; Boireau et al., 1994a) nor with MAO-B activity (Boireau et al., 1994b). Therefore, riluzole actually seems to be a good candidate for protective therapy in PD. A treatment to prevent cell loss can only be effective if started before or at the same time as the actual initiation of the apoptotic process. Clinical studies demonstrated that riluzole is less or not at all effective when started in the motor phase of the disease. Patients would therefore benefit from a premotor phase diagnosis of PD in order to start neuroprotective treatment at a stage when there are still enough DA neurons left to protect. Studying the survival of neurons combined with behavioral functionality is essential to establish the therapeutic value of a neuroprotective treatment. Insight in the multiple effects produced by riluzole on MPTP challenged neurons and their synaptic transmission can help to understand whether and why this drug might be clinically useful.

In summary animal models for PD are realized with either genetic modifications and or specific neurotoxins. MPTP is neurotoxin that induces Parkinsonism in humans, non-human primates and mice. MPTP-treated non-human primates offer a complete range of neuropathology and behavioral deficits analogous to those of PD and are therefore of specific interest for preclinical studies. Although mice offer less behavioral phenotypes they are specifically useful for extensive neuropathological and molecular research. Studying neuroprotective effects on early PD symptoms and early molecular changes, asks for a premotor phase PD model. The effects of the versatile compound riluzole on early PD models can offer insights into the timing of neuroprotection and molecular changes that underlie behavioral functionality.

1.4 Research aims

This research project was initiated to investigate early PD and the initiation of the neurodegenerative process. It builds on the notion that neuroprotective treatment at an early stage of PD will limit disease progression and that this might reduce the functional deterioration and pathology of the brain. This thesis aims to give insights into the behavioral and molecular markers of early PD, modeled in mice and marmosets.

In this introductory chapter 1, it is emphasized that there is no cure or satisfactory treatment for PD. A major factor for this is the multi-factorial nature of PD, a combination of endogenous and exogenous disease generating factors, which starts the neurodegenerative process many years before the disease can be diagnosed based on symptomatology. Therefore, research efforts should be focused on the identification of biomarkers for early diagnosis and neuroprotective treatment in relevant PD animal models.

In this study we evaluated motor and non-motor symptoms of PD in marmosets with an open eye towards human validity. Two new behavioral test systems have been developed for marmoset monkeys and described in chapter 2. These tests were developed to quantify jumping behavior as a measure for akinesia and the righting reflex as a measure for rigidity and axial turning. Furthermore sleep aspects are evaluated in chapter 3 as a potential indicator for moderate neurodegeneration in the marmoset MPTP model, in line with observed clinical manifestation of sleep problems in the premotor phase of PD. We describe changes during REM sleep analogous to RBD in patients, one of the suggested premotor symptoms of PD.

In chapter 4, the potential neuroprotective effect of (pre-) treatment with the anti-excitotoxic compound riluzole on the metabolically challenged DA neurons is examined in the MPTP-treated marmoset model for the early phase of PD. Not only the traditional observational scoring and histo-pathological evidence for neuroprotection is included in the analysis, but also a complete range of motor behavioral tests and sleep aspects is used in order to find the most efficient way of predicting the therapeutic value of a compound in a preclinical trial. Additional post-mortem research on DA neurons and DA levels was performed to give insight in the neuroprotective efficacy of riluzole.

In chapter 5, riluzole is evaluated for its effect on the early molecular changes in neurodegeneration. Because the actual start of the neurodegenerative process cannot be diagnosed or predicted in humans, we investigated changes in protein expression in a

chronic low level MPTP mouse model. The compound MPTP was slowly infused into mice to generate a chronic progressive cell challenge. By sampling brain tissue at different time points, neurodegenerative as well as neuroprotective protein expression changes will be evaluated directly after the initiation of DA neuron specific neurodegeneration.
In chapter 6 all measurements varying from clinically relevant behavioral parameters to biochemical and pathological parameters are integrated and discussed.

Acknowledgements
The authors would like to thank H. van Westbroek for his assistance with the design of fig. 1 and fig. 2.

2

Two new test methods to quantify motor deficits in a marmoset model for Parkinson's disease

P.S. Verhave, R.A.P. Vanwersch, H.P.M. van Helden, A.B. Smit and I.H.C.H.M. Philippens

Behavioural Brain Research (2009) 200(1):214-219

The submission of the limbs to the directions of the will can hardly ever be obtained in the performance of the most ordinary offices of life. (Parkinson, 1817)

2 Abstract

The validity of the common marmoset as a model for human disease depends on the development of parameters with clinical relevance. We tested the effect of treatment with MPTP in two newly developed non-invasive motor behavioral paradigms in the context of Parkinson's disease. The "Tower" was designed to quantify the marmoset's natural jumping behavior as a measure for akinesia, the "Hourglass" to test the marmoset's natural righting reflex as measure for rigidity, analogous to axial motor behavior in humans.

MPTP treatment affected marmoset behavior in both testing paradigms. The marmoset's righting reflex in the Hourglass remained significantly impaired during the full 3-week period after the MPTP intoxication. In the Tower, the marmosets were not able to jump the largest distances one week after MPTP and showed a persistent reduction in activity during the 3-week period after the MPTP intoxication.

Because not all aspects of motor behavior are similarly affected by MPTP, a complete behavioral sketch of Parkinsonian marmosets should preferably include a range of motor behavior functions to create an overview of the full range of motor impairments. Both the Hourglass and Tower test provide important behavioral parameters in a clinically relevant multiple testing approach for motor disorder models.

2.1 Introduction

The occurrence of neurodegenerative disease increases with age (Mattay et al., 2002; Ward and Frackowiak, 2003). Thus, with an aging world population more people tend to suffer from movement disabilities as manifested in Parkinson's disease (PD). The slow onset of these clinical impairments in relation to the progression of neurodegeneration makes studies correlating neuropathology to symptom manifestation in human subjects difficult. Animal models may fill this gap and provide insight into the course of behavioral effects in relation to the underlying neurodegeneration of the brain.

Results from animal studies for motor disorders have an increased value when translation to human motor behavior is feasible at all levels of investigation. Physiological and pharmacological questions can often be studied well in rodent models; however issues concerning complex behavior can be addressed more accurately in primates. Therefore, non-human primate models are preferred in clinically focused behavioral studies because their behavioral repertoire can be translated directly to human behavior (Annett et al., 1994; Di Monte et al., 2000; Eslamboli, 2005).

Especially the common marmoset is an appreciated model in neuroscience. Compared to humans these monkeys have a similar *striatum* structure, hand-foot use and motor behavioral reaction (construct validity) and for example a similar response to dopamine (DA) replacement therapy (predictive validity) (Hardman et al., 2002; Jenner, 2003b; Blanchet et al., 2004; Eslamboli, 2005). Additionally non-human primates are genetically closer to humans than rodents and react similar to pharmacological interventions (face validity) (Smith et al., 2001). The small and easy to handle marmoset is thus of major importance as a model in laboratory behavioral studies (Willner, 1986).

The translation of animal models for movement disorders depends on a correctly designed motor behavior experiment with objective and quantitative parameters. Additionally the outcome gains interest when the parameters are directly related to clinical measures (Emborg, 2004). Methods to evaluate patient's motor function in the clinic are based on the ability of subjects to perform movements (Guy, 1979; Mattay et al., 2002; Ward and Frackowiak, 2003; Nicoletti et al., 2005; Ruiz et al., 2007). Parkinsonian behavior has several distinct motor features in both humans and marmosets. Like human patients, Parkinsonian marmosets display, dependent on the disease state and impact, slowness of movements (bradykinesia), rigidity, and disability to initiate movement (akinesia), here defined as the

absence of movement (Jenner et al., 1984; Przedborski et al., 2001). These behaviors can be scored using a clinical or behavioral assessment scale (Di Monte et al., 2000; van Vliet et al., 2006). This scale is a useful tool for indicating the condition and the Parkinsonian state of the marmoset, however, the scale is dependent on investigator-dependent subjective criteria with low quantitative value despite blind assessment.

Several test systems and quantification methods have been described for marmoset motor behavior. There are methods to evaluate the 'full body' motor behavior, for example the general activity tests (Philippens et al., 2000; Przybyszewski et al., 2006). Some other methods are designed to evaluate unilateral performance, such as the Sticky label test or the Rotation task (Annett et al., 1994). And there are methods that focus on more specific motor performance like 'arm reaching' motor behavior such as the Staircase test (Marshall and Ridley, 1996) and the Hand-eye coordination task (Wolthuis et al., 1995). In some test systems it is necessary to motivate the animals to move by using positive or negative reinforcement, which may influence the outcome of the performance. To minimize the adverse effects of reinforcement on motor behavior, the animals' motivation should be as close to its natural behavioral paradigm as possible.

In this study we investigated the potency of two newly developed non-invasive motor behavioral testing paradigms to evaluate Parkinsonian motor behaviors such as akinesia and rigidity in the marmoset, using treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to model aspects of PD. The neurotoxic compound MPTP induces neurodegeneration selectively to the dopaminergic neurons and has been shown to cause signs and symptoms of PD in humans and primates (Jenner et al., 1984; Ballard et al., 1985). In behavioral research the marmoset is a widely used species for the MPTP model (van Vliet et al., 2006) and to increase the available behavioral measures, the "Tower" was designed to quantify the animals jumping behavior as a measure for akinesia. The "Hourglass" was designed to test quantitatively the animals' righting reflex as measure for rigidity, analogous to axial motor behavior as assessed in the clinic. Both tests made use of the natural motivation of the marmosets, such as body rotation and their natural jumping behavior.

2.2 Material and Methods

Animals

Experiments were conducted on twelve marmoset monkeys of both sexes (*Callithrix jacchus,* between 2 and 3 years of age, purchased from BPRC, the Netherlands). They were housed in individual primate cages (61x61x41 cm) under controlled conditions of humidity (60%), temperature (23-25ºC) and lighting (12 hour light/dark cycles). Marmosets were fed daily with pellet chow. Diet was enriched with peanuts, fruit and vegetables, raisins, sunflower seeds and an occasional grasshopper. Water was available *ad libitum*. All marmosets were provided with a varying cage environment. Experiments were conducted after approval by the Ethical Review Committee (TNO).

Study design

The marmosets were randomly divided into two groups. One group (n=6) received 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP, purchased from Sigma Aldrich, St. Louis, USA) by subcutaneous injections in the abdominal area. MPTP (7 mg/kg) was administered in four injections on Mondays and Thursdays (subsequently 2, 2, 1.5, and 1.5 mg/kg) within two weeks till moderate Parkinsonian symptoms were established. The other group (n=6), received time and volume-matched sc 0.9% saline injections. The marmosets were tested weekly on Wednesdays in both test paradigms. Tests were performed one week before, during, and one, two and three weeks after the MPTP treatment. To rule out the direct effects of MPTP the last three weeks after the MPTP treatment was completed, were used for evaluation of test setups.

Tower

The trespa tower (35x35x250 cm) has a transparent Plexiglas front (figure 1). It contains 7 levels of horizontal mounted trespa crossbars with an increasing distance varying from 10 to 50 cm. A camera is placed in front of the Tower for video analysis. The marmoset was transported to the Tower setup in a Plexiglas cylinder (13x24 cm) and placed in front of a sliding door at the bottom of the Tower. At time point '0' the marmoset could enter the Tower after opening the sliding door from outside the room. During each test the marmoset could freely move around on the 7 levels for 5 min. To motivate the marmoset to visit each

Figure 1. The Tower test setup: three successive snapshots of a marmoset performing jumping behavior in the Tower. The animal can move freely in the Tower setup and the time is noted when the marmoset changes level.

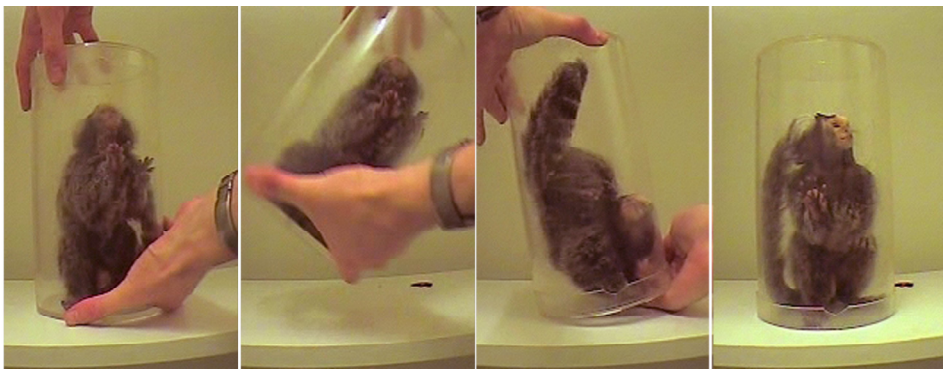

Figure 2. The Hourglass test setup: four successive snapshots of a marmoset in the Hourglass. The cylinder is turned in one continuous movement and the time it takes for the marmoset to turn upright is noted during video analyzes.

level there was a small piece of marshmallow (2x2x2 mm) available on each level. All marmosets were habituated to the Tower before testing.
The marmoset's location (level) was noted by non-automated video analysis, done by an observer blinded to the treatment. A marmoset had reached a certain level, when it was observed that the centre of gravity of the marmoset was on the crossbar representing that level.

Hourglass

This method is based on the earlier mentioned transportation cylinders. Marmosets were transported to the Hourglass setup in such a Plexiglas cylinder (13x24 cm) and were then transferred into a smaller Plexiglas cylinder (11x27 cm) and placed in front of a camera (fig. 2). One trial consisted of one manual 180º turn of the cylinder (like turning an Hourglass). After 30 sec a new trial started by turning the cylinder again. A test consisted of five subsequent trials. The cylinder was always turned after 30 sec, keeping the marmoset (when they do not right themselves) no longer than 30 sec upside down. After five trials the marmoset was transferred back into the wider cylinder (13x24 cm) in which it was tested again, also consisting of 5 subsequent trials.
The time it took for the marmoset to turn back in the upright position was noted with non-automated video analysis, done by an observer blinded to the treatment. Intervals between cylinder turn and the marmoset's ability to recover its position (head above legs) was noted, maximum time noted was 30 sec (also for marmosets which did not turn upright at all). To analyze the same number of turns for each animal (the ones that turn upright and the ones that do not) only the three fastest turns were taken into account for the analysis.

Immobility

All marmosets are scored for immobility as a measure for akinesia in their home cage twice a day on test days. A '0' is noted for normal mobile marmosets, a '1' is noted for marmosets which are slightly less able to move but still within a normal range, a '2' is noted for marmosets which are slightly less able to move than normal, a '3' is noted for marmosets which show little ability to move and a '4' is noted for marmosets that do not move at all.

Statistics

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 15.0. Data are described per test paradigm. All data tested were ordinal, therefore tested in a non-parametric fashion. All overall data were tested for overall effects with a Friedman test for repeated measures followed by Mann-Whitney t-test for differences within test days. Statistical differences were tested two-sided and considered significant when $p < 0.05$.

2.3 Results

Tower

First we measured the jumping ability. Vehicle treated marmosets reached the top level 6 or 7. Marmosets treated with MPTP were severely impaired in their jumping ability, and as a result they could not reach the higher levels in the Tower (fig. 3). The average top level reached measured at 6 days after the last MPTP injection was level 2 (Mann-Whitney $p < 0.01$). The marmosets recovered over time and at two weeks after the MPTP treatment their average top level was at level 5 and remained at this level in the period thereafter.

To evaluate the activity of the marmosets the average number of level changes was used (fig. 4). Vehicle treated marmosets had an average of about 20 level changes per test session. Directly after MPTP intoxication the marmosets show a significant reduction in their activity in comparison to their own baseline measurements (Mann-Whitney $p < 0.05$) and also in comparison to the vehicle-treated marmosets (Mann-Whitney $p < 0.01$). The activity of the MPTP-treated marmosets recovered with time but was still lower compared to the activity of vehicle-treated animals.

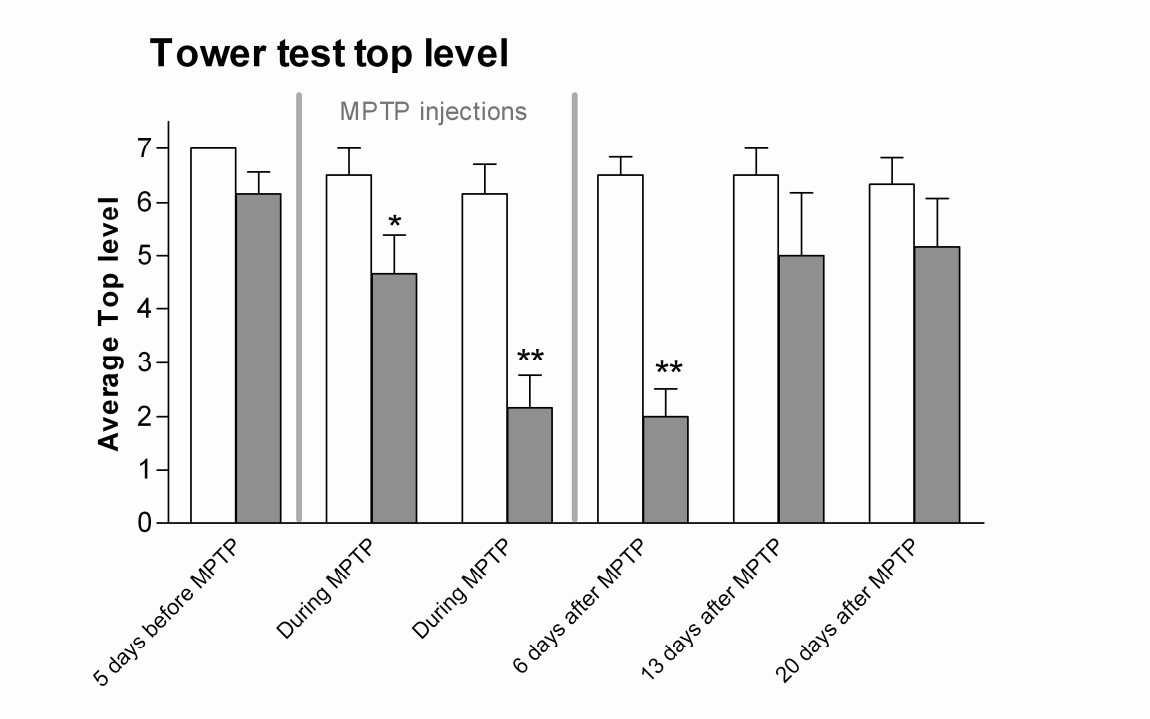

Figure 3. Tower test top: Average top level in relation to the MPTP treatment (Mean of 6 + SEM). The performance of vehicle (white bars) and MPTP (grey bars) treated marmosets is shown before, during and 6, 13 and 20 days after MPTP treatment. The maximum level achievable in the Tower is level 7. Grey vertical lines outline the Parkinsonism induction period of four injections (total 7 mg/kg MPTP) in two weeks. Asterisks indicate significant difference between the MPTP-treated group and the vehicle-treated group, * $p < 0.05$ and ** $p < 0.01$ (Friedman followed by Mann-Whitney tests).

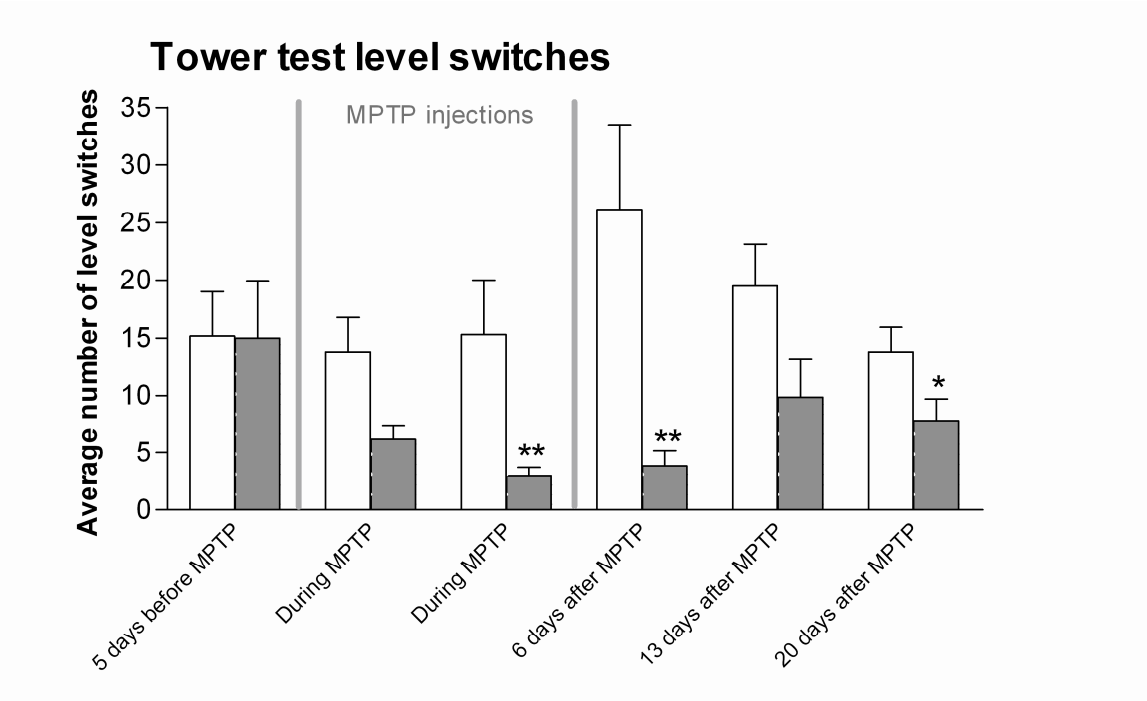

Figure 4. Tower test level switches: Average activity of the marmosets in relation to the MPTP treatment (Mean of 6 + SEM). The number of level switches of vehicle (white bars) and MPTP (grey bars) treated marmosets is shown before, during and 6, 13 and 20 days after treatment. Grey vertical lines outline the Parkinsonism induction period of four injections (total 7 mg/kg MPTP) in two weeks. Asterisks indicate significant difference between the MPTP-treated group and the vehicle-treated group ** $p < 0.01$; * $p < 0.05$ (Friedman followed by Mann-Whitney tests).

Hourglass

It took a healthy marmoset about two sec to turn upright. Marmosets treated with MPTP were severely impaired in their full body turning ability (fig. 5). For the Parkinsonian marmoset it took 24 sec on average, including the marmosets that did not turn upright within the 30-sec period. The marmosets hardly recovered during the experimental period. After 13 and 20 days the MPTP-treated marmosets still turned significantly slower than the vehicle-treated marmosets and needed an average of 19 ± 5 sec to turn to the upright position (Mann-Whitney, $p < 0.05$).

Also in the wider cylinder marmosets treated with MPTP were severely impaired in their turning ability. In this wider cylinder it took a healthy marmoset less than a second to turn upright, while it took the MPTP-treated marmosets measured 6 days after the last MPTP injection, again about 26 ± 4 sec to turn upright. In contrast to the response in the narrow

cylinder, the righting reflex in the wider cylinder improved to an average of about 14 ± 3 sec and 8 ± 3 sec measured respectively 13 and 20 days after the last MPTP treatment.

Most marmosets remained to have problems with the turning behavior after the MPTP treatment was completed (fig. 6). Marmosets were given five trials; the three fastest turns were used for analysis. Respectively 6, 13 and 20 days after the last MPTP treatment the marmosets turned themselves in the narrow cylinder on average 0.7, 1.3 and 1.7 times out of the three analyzed turns. A similar moderate improvement is observed in the wide cylinder where they turned themselves respectively 0.5, 2.3 and 2.2 times out of the three analyzed turns. The same marmosets (before MPTP treatment) as well as the vehicle-treated marmosets (data not shown) never missed a turn.

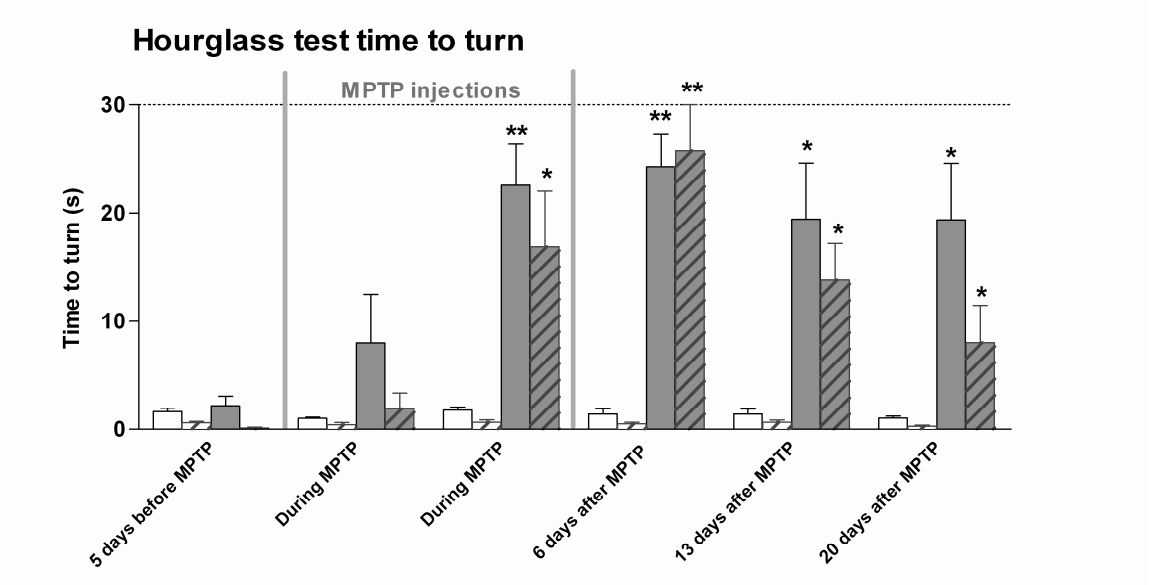

Figure 5. Hourglass test: Time to turn in the Hourglass test in relation to the MPTP treatment (Mean of 6 + SEM). The performance of vehicle (white bars) and MPTP (grey bars) treated marmosets is shown before, during and 6, 13 and 20 days after treatment. Solid bars represent the narrow cylinder, striped bars the wide cylinder. Dotted line indicates the maximum time (30 s) set for one turn. Grey vertical lines outline the Parkinsonism induction period of four injections (total 7 mg/kg MPTP) in two weeks. Asterisks indicate significant difference between the MPTP and the vehicle-treated group ** $p < 0.01$ and * $p < 0.05$ (Friedman followed by Mann-Whitney tests).

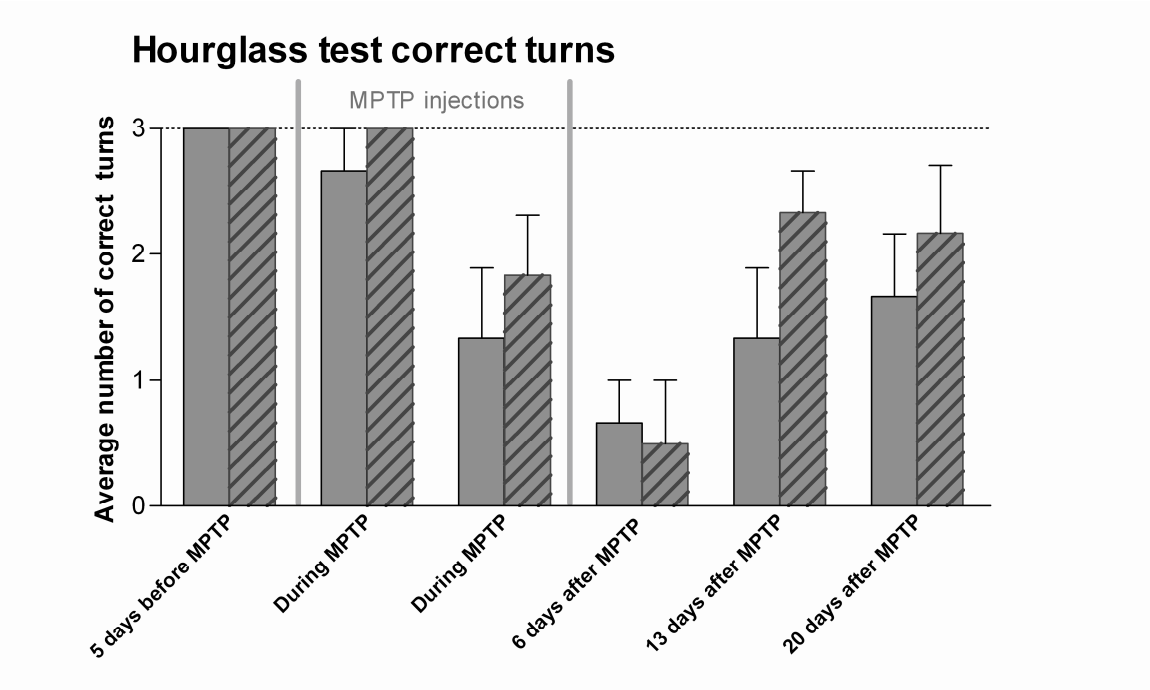

Figure 6. Hourglass test correct turns: Average number of correct turns of the MPTP-treated marmosets (Mean of 6 + SEM). The performance before, during and 6, 13 and 20 days after treatment MPTP treatment is shown. Solid bars represent the narrow cylinder, striped bars the wide cylinder. Dotted line indicates the maximum number of turns. Grey vertical lines outline the Parkinsonism induction period of four injections (total 7 mg/kg MPTP) in two weeks.

Immobility

From the onset of MPTP treatment home cage mobility was affected (fig. 7). The MPTP-treated marmosets were hardly able to move just after treatment (Mann-Whitney $p < 0.01$) and were moderately disabled to move at the end the study (Mann-Whitney $p < 0.05$). The vehicle-treated animals were scored normally mobile for a healthy animal during the complete course of the study.

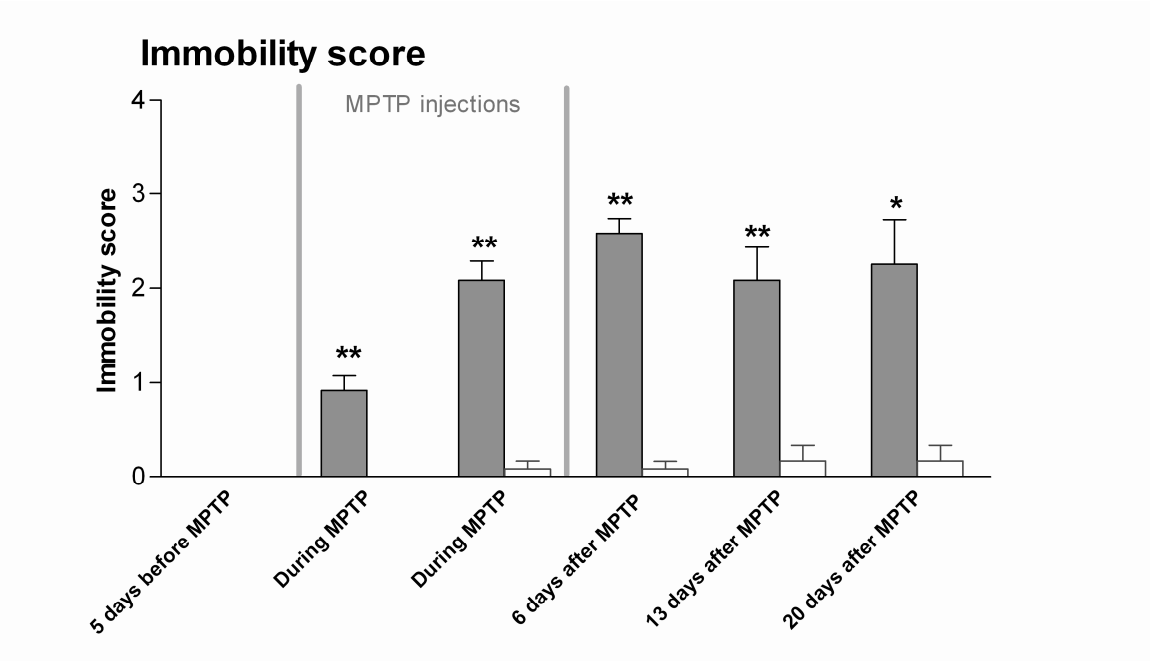

Figure 7. Immobility score: Average immobility score in relation to the MPTP treatment on the same day as Hourglass and Tower tests. The home cage immobility of vehicle (white bars) and MPTP (grey bars) treated marmosets is shown before, during and 6, 13 and 20 days after MPTP treatment is shown. Grey vertical lines outline the Parkinsonism induction period of four injections (total 7 mg/kg MPTP) in two weeks. Asterisks indicate significant difference between the MPTP and the vehicle-treated group ** $p < 0.01$ and * $p < 0.05$ (Friedman followed by Mann-Whitney tests).

2.4 Discussion

To measure the efficacy of a treatment on symptom control in a marmoset model the test methods used are crucial for the outcome of the study. In this study two new non-invasive motor-related behavioral tests, the Tower and the Hourglass, for quantification of the motor behavioral deficits in marmosets, were evaluated. Both tests are sensitive methods to measure MPTP induced motor impairments in marmosets: In the Hourglass the turning behavior and in the Tower test the amount of spontaneous jumping behavior was affected by MPTP treatment.

The Tower test is directed to the marmoset's preference to be on a high level in trees for foraging or, when in captivity, in the top part of their enclosure (Buchanan-Smith et al., 2002). The Hourglass is directed to the marmoset's specialization in various vertical postures for foraging purposes (Garber, 1992) which include upside-down clinging. These tests are

closely linked to the natural behavior (jumping and vertical rotating) of the marmosets to keep stress levels to a minimum. It is of importance to prevent stress as this might interfere with the motor performance of Parkinsonian marmosets. It is known that on occasion, PD patients can react with normal movements to a stressful event, a phenomenon called kinesia paradoxia. This phenomenon is suggested to be mediated through non-dopaminergic pathways and reflects a psychological response (de la Fuente-Fernandez and Stoessl, 2002).
In both tests we found a slight recovery that normally occurs in marmosets treated with MPTP (Jenner et al., 1984; Obinu et al., 2002; van Vliet et al., 2006), however the marmosets behavioral reaction did recover differently in both tests. The inhibiting effect on the righting reflex in the Hourglass test lasted for the whole test period; this was two weeks longer than the effects observed in the Tower (fig. 3).
The first explanation of the difference in persistence of the MPTP effects can be found in the marmosets' motivation to move. Motivation in mentally sane human patients will be of no or little effect. In animal research however motor behavioral studies rely on the motivation of the animal to perform. This has to be taken into account when assessing evoked behavior in animals. Evoked motor behavior is the outcome of the animals' motivational status and the ability to perform. Positive reinforcement is often induced with a motivational stimulus such as a food reward, like in most 'arm reaching' tasks, a preferred environment like an illuminated area or social interaction. Negative reinforcement is often induced by physical constraint to some level. In this approach the effect of stress cannot be neglected as it may affect the behavior. The Tower test is a reward related test. The anticipation to the occasional (on each level one) reward might trigger the DA release in the *striatum* like it does in humans (de la Fuente-Fernandez et al., 2002; de la Fuente-Fernandez and Stoessl, 2002). This increased DA level then might facilitate jumping to the upper levels in the Tower. Once the expectation of reward is reduced (all rewards are eaten) the dopamine trigger is reduced and jumping is no longer activated. The persisting reduction in activity in the Tower subscribes this. Instead, the Hourglass test is centered around a motor response to a geotaxically induced sensory stimulus. This stimulus will not trigger the DA release like the expectation of a reward would do. Therefore, the marmosets will not be triggered by an increase of dopamine to perform the righting reflex.
The second explanation might be found in the complexity level of the movements required in these tests. In the Tower test we see that the Parkinsonian marmosets show a significant

recovery over two weeks after MPTP intoxication, in contrast to the behavior in the Hourglass test where the motor deficits were more persistend over time. The jumping behavior is thus not similarly affected by the Parkinsonism as the complex axial turning behavior. This is in line with data on to the motor performance of PD patients. In patients, it has been reported that difficulty with turning behavior is apparent whilst walking is unaffected (Nieuwboer et al., 1998; Crenna et al., 2007). The suggestion has been made that the turning relies on more complex neural mechanisms and are more susceptible to impairments associated with PD. The main area affected in PD, the *substantia nigra*, plays a role in head orientation and initiation of the executive pathways in axial motor behavior (Crenna et al., 2007) However, it is the supplementary motor area (SMA) and its eye fields, which is the initiator of an axial motor behavior (Wichmann et al., 2001; Chan et al., 2005). The SMA is affected in PD as well as in the MPTP model (DeLong and Wichmann, 2001; Wichmann et al., 2001; Brownell et al., 2003) and might therefore also cause problems in the complex axial motor behavior.

The righting reflex in marmosets was designed as to parallel axial motor impairments or turning behavior in humans. In 6-OHDA-treated rats the righting reflex is also used as a model for axial motor impairments in humans (Martens et al., 1996). Rats are placed on their side, fully restrained (front and hindquarters) and only the hindquarters are released. In both, marmosets and rats with a disturbed dopaminergic system, the righting reflex is affected, suggesting that the dopaminergic system is the main cause of axial motor impairments. However, in Parkinson patients axial motor impairments are not completely ameliorated by dopamine replacement, e.g., by L-DOPA therapy (Lakke, 1985; Steiger et al., 1996; Stack et al., 2005). Moreover, patients can still perform well in axial challenges under stressful conditions (Lakke, 1985). Therefore, it is suggested that the axial motor impairments make use of different neurotransmitter systems. For example the serotonergic system affects turning behavior (Belforte and Pazo, 2004) and is also influenced by the reduction of dopaminergic cells after MPTP or 6-OHDA intoxication (Russ et al., 1991; van Vliet et al., 2006), while the DA replacement compound L-DOPA does not influence this behavior. Thus, the righting reflex is not a simple motor reaction, but a series of actions mediated by the concerted action of serotonergic and dopaminergic systems.

Locomotor activity of marmosets has been a parameter in several test setups in the marmoset literature (Wolthuis et al., 1994; Moussaoui et al., 2000; van Vliet et al., 2006). An

interesting subdivision has been made in the research group of Imperato (Moussaoui et al., 2000; Obinu et al., 2002). They divided the locomotor behavior in small, medium and large movements, in which large movements are jumps from one perch to another. MPTP-treated marmosets showed less large movements than before treatment. Coherent with this data the current study shows that the marmosets perform less level switches (jumps) after MPTP treatment and they were akinetic in the home cage environment. In combination with our top data in the Tower the conclusion can be drawn that although the MPTP-treated marmosets are able to perform the jumps, they perform less of them in the same amount of time. This difference in the ability to perform and the amount of performance can also be seen in the clinic. Patients rarely show akinesia in the clinic while 75% reports suffering from the phenomenon in the home environment (Nieuwboer et al., 1998). Therefore, evaluating the ability of a marmoset to perform versus the prevalence of the movement is valuable for translation to the clinic.

In conclusion, both the Hourglass and Tower test can be used to measure different aspects of motor related behavior in the marmoset, in which probably not only the DA depletion, resulting from DA neuron cell death, plays a crucial role. This holds especially for the Hourglass test, in which other pathways indirectly connected to the main *basal ganglia-thalamocortical* circuits are responsible for the effects on the motor output system. Like Parkinsonian behavior in the clinic, not all types of motor behavior are affected in the same manner by the MPTP intoxication. Therefore, a complete behavioral sketch of a Parkinsonian marmoset should preferably include a range of motor behavior test systems to create an overview of the full range of motor impairments. The Hourglass as well as the Tower may contribute to preclinical research on the *basal ganglia-thalamocortical* signaling. The Hourglass test for righting reflex and the Tower test for jumping behavior provide important behavioral parameters strengthening the translational aspects of non-human primate locomotor behavior to the clinic.

Acknowledgements

The authors would like to thank R.M. van den Berg and M.J. Jongsma for their excellent technical support. This study was supported by the U.S. Army Medical Research and Materiel Command, Award No. W81XH-05-1-0517.

3

REM Sleep Behavior Disorder in the marmoset MPTP model for early Parkinson's disease

P.S. Verhave M.J. Jongsma, R.M. van den Berg, R.A.P. Vanwersch, J.C. Vis, A.B. Smit E.J.W. van Someren and I.H.C.H.M. Philippens

Sleep (2011) Manuscript in press

It now seldom leaves him for a moment; but even when exhausted nature seizes a small portion of sleep, the motion becomes so violent as not only to shake the bed-hangings, but even the floor and sashes of the room. (Parkinson, 1817)

3 Abstract

Sleep problems are common phenomena in most neurological and psychiatric diseases. In Parkinson's disease (PD), for instance, sleep problems may be the most common and burdensome non-motor symptoms in addition to the well-described classical motor symptoms of the disease. Since sleep disturbances generally become apparent in PD before motor symptoms emerge, they may represent early diagnostic tools and a means to investigate mechanisms of PD onset. The sleep disturbance REM sleep behavior disorder (RBD), precedes PD in one-third of patients. We therefore investigated sleep changes in marmoset monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP), the non-human primate model for idiopathic PD.

Mild Parkinsonism was induced in five marmoset monkeys (3M/2F) over a two-week period of sub-chronic MPTP treatment. Electroencephalograms (EEG) and electromyograms (EMG) were recorded weekly. Motor activity and hand-eye coordination were also measured weekly and any signs of Parkinsonism were noted each day. Sleep parameters, motor activity and performance data, before and after MPTP treatment, were compared between MPTP-treated marmosets and four control marmosets (1M/3F).

MPTP increased the number of sleep epochs with high-amplitude EMG bouts during REM sleep relative to control animals (mean ± SEM percentage of REM 58.2 ± 9.3 vs. 29.6 ± 7.7, respectively; $p < 0.05$). Of all sleep parameters measured, RBD-like measures discriminated best between MPTP-treated and control animals. On the other hand, functional motor behavior, as measured by hand-eye coordination, was not affected by MPTP treatment (MPTP: 23.40 ± 3.56 vs. control: 36.13 ± 5.88 correct trials; $p = 0.32$).

This REM sleep-specific change, in the absence of profound changes in wake motor behaviors, suggests that the MPTP marmoset model of PD could be used for further studies into the mechanisms and treatment of RBD and other sleep disorders in pre-motor symptom PD.

3.1 Introduction

Rapid eye movement sleep behavior disorder (RBD) is characterized by increased muscle activity during rapid eye movement (REM) sleep, which can lead to injury either to oneself or to a bed partner. The core symptom of RBD, namely the lack of normal muscle atonia during REM sleep (Comella et al., 1993; Ondo et al., 2001), can emerge in two ways: (1) tonic muscle activity characterized by at least 50% of muscle activity in a 30-sec REM sleep epoch or (2) phasic muscle activity and twitches within 3-sec epochs (Lapierre and Montplaisir, 1992). RBD is a disorder of considerable interest for understanding early pathological processes in Parkinson's disease (PD). At least one-third of PD patients have increased and irregular chin muscle tone during REM sleep (Comella et al., 1993; Gagnon et al., 2002) and many meet the criteria for RBD. While the clinical diagnosis of PD is primarily based on overt motor symptoms, these symptoms emerge relatively late in the course of the underlying neurodegenerative process. It has been estimated that at least 60% of the dopamine (DA) neurons are lost by the time motor symptoms emerge. In order to investigate early pathological processes of the disease and to develop early treatment approaches, the stages that precede the motor phase of PD need to be identified. Reported symptoms preceding the motor phase of PD include: reduced ability to smell and taste; alterations in mood and autonomic function; and most notably disturbed sleep (Berg, 2008; Tolosa et al., 2009). For instance, complaints of insomnia are reported in approximately 80% of all PD patients (Tandberg et al., 1998; Oerlemans and de Weerd, 2002), and excessive movement during sleep frequently occur in PD patients (van Hilten et al., 1994). More importantly, disturbances in sleep usually begin years before PD is diagnosed (Iranzo et al., 2005; Postuma et al., 2006; Tolosa et al., 2009). RBD is a key symptom during the early phases of PD, since a third of all patients initially diagnosed with RBD are later diagnosed with PD within 3 to 13 years after the initial RBD diagnosis (Schenck et al., 1996; Gagnon et al., 2002; Iranzo et al., 2006). Moreover, 40% of all RBD cases reported eventually go on to develop a neurological disorder, most notably PD (Ferini-Strambi and Zucconi, 2000).

Because the etiology of idiopathic PD still remains largely unknown, research into the immediate pathogenic mechanisms of the disease relies heavily on appropriate animal models that mimic certain manifestations of PD. The neurotoxic compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) specifically targets DA neurons of

the *substantia nigra* via uptake by the DA transporter where it causes cell death by compromising the mitochondrial energy supply system of these DA neurons. The marmoset MPTP model, in this regard, has been the most valuable preclinical model for mimicking core symptoms of PD. The 'clinical' condition of the Parkinsonian state in this model is generally based on assessments and observations while the monkey is awake, for which a large number of quantitative measures have been developed and validated (Pearce et al., 1996; Iravani et al., 2003; Jenner, 2003b; van Vliet et al., 2006; Verhave et al., 2009). However, it is not known whether abnormalities during sleep are present *prior* to the pronounced wake motor disturbances in animal models of the disease. Because of the striking similarity in sleep macrostructure between marmoset monkeys and humans, a demonstration of early abnormalities during sleep in the marmoset MPTP model would be of significant value as potential biomarkers for the early stage of idiopathic PD. Therefore, we investigated the effects of MPTP treatment on sleep architecture in marmoset monkeys, with special attention to RBD-like changes in muscle tone during REM sleep. In order to evaluate these changes relative to the emergence of wake and sleep symptoms, the development of wake motor symptoms was quantified as well.

3.2 Material and Methods

Animals

Common marmoset monkeys (*Callithrix jacchus*) (5M/4F) between two and three years of age were obtained from the Biomedical Primate Research Centre (BPRC), Rijswijk, the Netherlands. They were housed in individual primate cages (61x61x41 cm) under controlled humidity (60%), temperature (23-25ºC) and lighting (12 hour light/dark cycles). Marmosets were fed daily with pellet chow. Diet was enriched with peanuts, fruit and vegetables, raisins, sunflower seeds and an occasional grasshopper. Water was available *ad libitum*. All marmosets were provided with enriched-varying cage environments. Protocols were reviewed by the Ethical Review Committee on Experimental Animals at TNO prior to the start of experiments. All animals were observed closely throughout the course of the experiments. General welfare and changes in body weight were monitored twice a day.

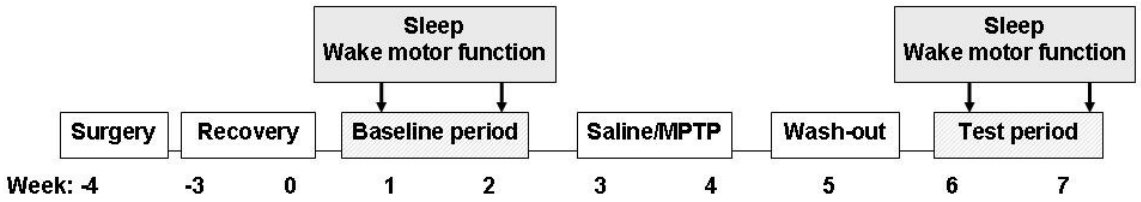

Figure 1. Schematic diagram depicting the experimental design. Surgery: placing EEG and EMG electrodes, Recovery: four weeks recovery from surgery, Baseline period: baseline measurements (sleep and motor function) before treatment, Saline/MPTP: Saline (n=4) or MPTP (n=5) treatment, Wash-out: period to allow recovery from direct MPTP effects and Follow-up period: all measurements (sleep and motor function) after treatment.

Surgical procedures

Two stainless steel electroencephalogram (EEG) electrodes were placed into the skull, one above and the other 5 mm anterior to the intra-aural and both were placed on the right hemisphere, 2 mm from the *sutura sagittalis*, leaving the *dura mater* intact. Surgery was performed under isoflurane/O2 anesthesia combined with the local anesthetic lidocaine. To measure muscle activity, one flexible electromyogram (EMG) electrode was attached with a single stitch to the chin muscle (*trigonum submandibularis*) and a second flexible EMG electrode was attached to the neck muscle (*trapezius*). Both EEG and EMG electrodes were fixed to the skull with dental cement. For sleep measurements, the electrodes were connected to a two-channel telemetric transmitter (F20-EET, Data Sciences International, a division of Transoma Medical, Arden Hills, USA) for wireless recording of endogenous brain signals.

Drug treatment

The MPTP group (n=5) received subcutaneous (sc) MPTP (Sigma Aldrich, St. Louis, USA) injections into the abdominal area. The neurotoxin was administered on Mondays and Thursdays (in the first week 2 mg/kg and in the second week 1.5 mg/kg). The vehicle group (n=4), received time and volume-matched sc 0.9% saline injections. After a one-week wash-out period, the two-week experimental phase, followed under otherwise identical conditions,

the baseline period (see follow-up period, fig. 1). Behavior was observed twice daily. Sleep recording and wake motor function tests were recorded weekly during both the baseline and follow-up period. Data from each animal were combined to give an average value for either the baseline or the follow-up period.

Sleep analysis

Animals were placed in special sleep cages (40x20x30 cm) and provided with bedding material, food and *ad libitum* water access. The marmosets could move freely around the sleep cages while having olfactory, visual and auditory contact with other marmosets. Climate and light/dark cycles were kept constant throughout the experiment. Sleep recordings started one hour before lights were switched off and lasted until one hour after they came back on again. The endogenous brain signals, recorded with a sample frequency of 100 Hz, were transferred to a receiver (RPC-1, Data Sciences International) and then to an acquisition program (Dataquest A.R.T., Data Sciences International, a division of Transoma Medical, Arden Hills, USA) and stored in European Data Format (EDF). Software Somnologica (Embla Inc, Broomfield, U.S.A.) was used for sleep staging to obtain hypnograms. Without knowing the treatment received by an individual animal, an experienced sleep technologist classified each 30-sec EEG epoch into sleep stages according to the criteria of Rechtschaffen and Kales (Rechtschaffen and Kales, 1968) and adapted for marmosets (Edgar et al., 1993; Almirall et al., 1999). Sleep macrostructure was quantified into sleep efficiency, total sleep time, number of wake bouts, wake time, number of sleep stage transitions and the duration of three different sleep stages. Duration of sleep stages 1 and 2 were combined into the variable "light sleep" and duration of sleep stages 3 and 4 were combined into a variable "deep sleep" (fig. 2A-C). Four categories of muscle tone were distinguished for the 30-sec REM epochs: (1) complete atonia (fig. 2D), (2) up to three twitches, (3) increased tone for 10% to 50% of the epoch (fig. 2E), and (4) increased tone for more than 50% of the epoch. Outcome variables were calculated as the percentage occurrence of the category relative to the total number of REM epochs.

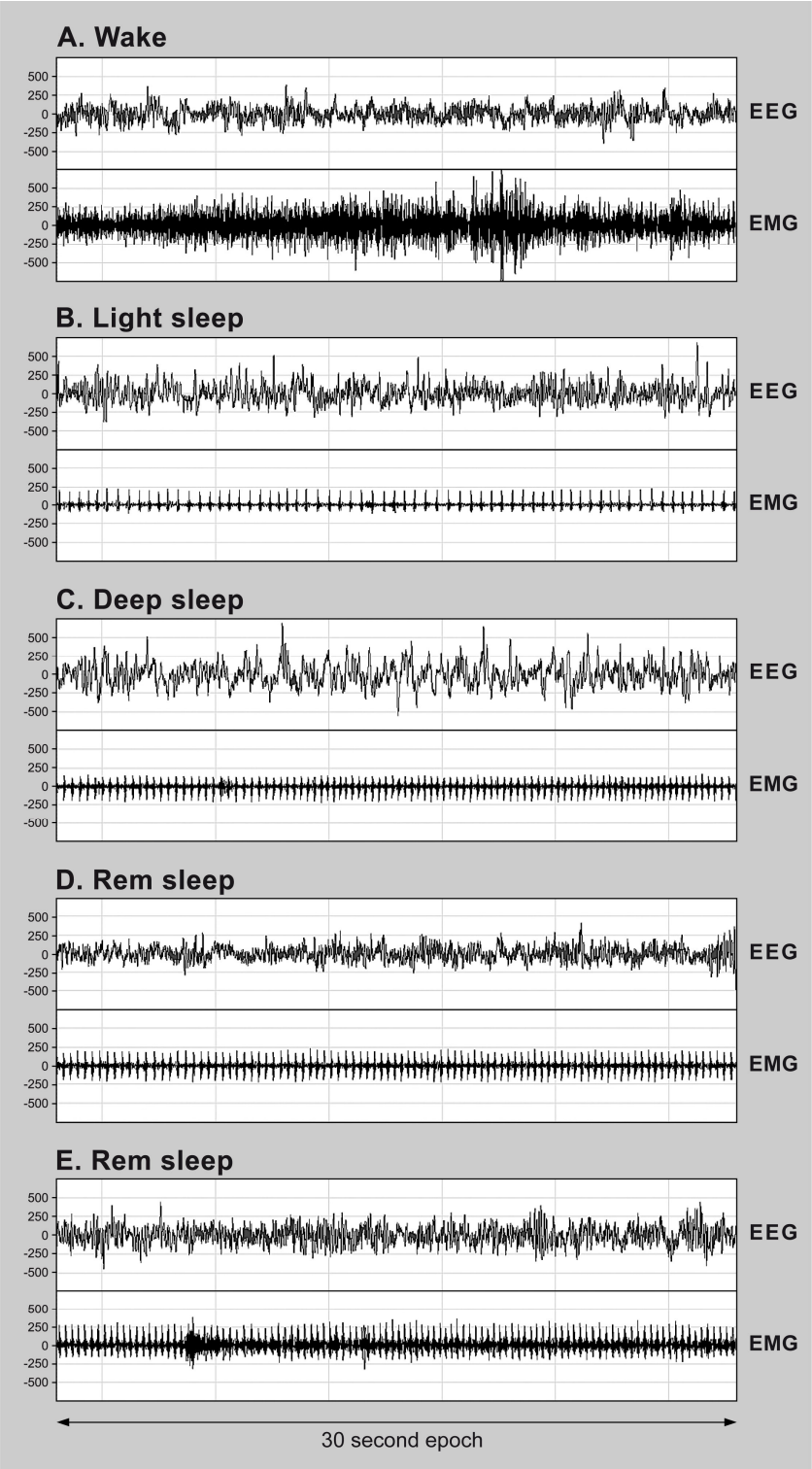

Figure 2. Recordings of sleep stages in marmoset monkeys. Images represent examples of 30 sec epochs of A. wake, B. light sleep, C. deep sleep, D. REM sleep with complete atonia and E. REM sleep with more than 50% of the time tonia.

Behavioral observations

Marmoset monkeys were scored at 8 A.M. and 6 P.M. using the dedicated clinical observation scale (van Vliet et al., 2006). This is a clinical condition-scoring list specified for Parkinsonian signs, which is used to monitor the pathological condition of MPTP-treated animals. Apathy, tremors, immobility and muscle rigidity were scored on a scale form zero (normal/healthy) to four (severely affected). The sum of these separate observations together formed a clinical score outcome.

Locomotor activity

An automated activity test was used to quantify locomotor behavior (Wolthuis et al., 1994; Philippens et al., 2000). The apparatus consisted of four equal compartments (23x23x23 cm) connected to each other by six non-transparent PVC tubes. The compartments were closed off on all sides except for a wire mesh roof. The animals were always placed in the same compartment before the start of a session and they could, thereafter, move freely from one compartment to another during a 20-min period. A video tracking system registered the movements and position of the animal within the apparatus. Locomotor activity was quantified as the number of compartment changes during this 20-min period.

Hand-eye coordination

Reward-related hand-eye coordination performance was tested with an automated robot-guided test setup (Philippens et al., 2000; van Vliet et al., 2006). In brief, the marmoset was placed in a test cage in front of a black plastic panel with a small window (5x8 cm). On the other side of the panel a robot arm presented 42 small marshmallow rewards, which the marmosets could reach through the window. The rewards were offered at three different rates: non-moving (0.0 m/s for a maximum of 30 sec); slow moving (0.04 m/s); and fast moving (0.08 m/s). Each test session consisted of 14 trials at each rate. A brief sound signal was used to alert the animal before each trial and in between each trial the window was closed by a sliding door. The percentage of correct hits was used as a criterion to judge the performance of each animal. Training started three months before the start of the study and all animals were trained to take > 80% of the presented rewards.

Statistical analysis

Data were assembled in Excel spreadsheets (Microsoft Corporation, Redmond, U.S.A.) and averaged to obtain a single value for both the baseline and the follow-up period for each animal. These averages were transferred to SPSS (Statistical Package for Social Sciences, version 15.0, SPSS (SPSS Inc, Chicago, USA). After testing for normality (Shapiro-Wilk), analyses consisted of either repeated measures ANOVA for data with normal distributions or Friedman tests for non-normal data to determine variation across time for each group separately, followed by a Mann-Whitney test for within or between group differences. Additionally, a stepwise discriminant analysis was performed to elucidate the sleep variable(s) that best discriminated between MPTP-treated and vehicle-treated animals. The level of probability for statistical significance was set at 0.05. Data are presented as means + SEM.

3.3 Results

The experimental timeline consisted of (1) surgical preparation followed by (2) a four-week recovery period and then by (3) seven weeks of experimental testing (fig. 1). The first two experimental weeks consisted of baseline measurements. After this baseline period a two-week Parkinsonism induction period started in the MPTP group. We first measured the average sleep macrostructure variables and wake motor function variables before and after saline or MPTP treatment (Table 1). Subsequently, average values of baseline and follow-up period measurements of time spent in different sleep stages were determined (fig. 3). Repeated measures ANOVA showed no treatment or treatment-time effects in the macrostructure of sleep between the two experimental groups ($p > 0.05$). MPTP-treated marmosets showed no reduction in total sleep time, time spent in REM sleep, light or deep sleep. Further, wake time after sleep onset was not significantly affected by the MPTP treatment. However, MPTP significantly increased endogenous muscle tone during REM sleep ($p < 0.05$).

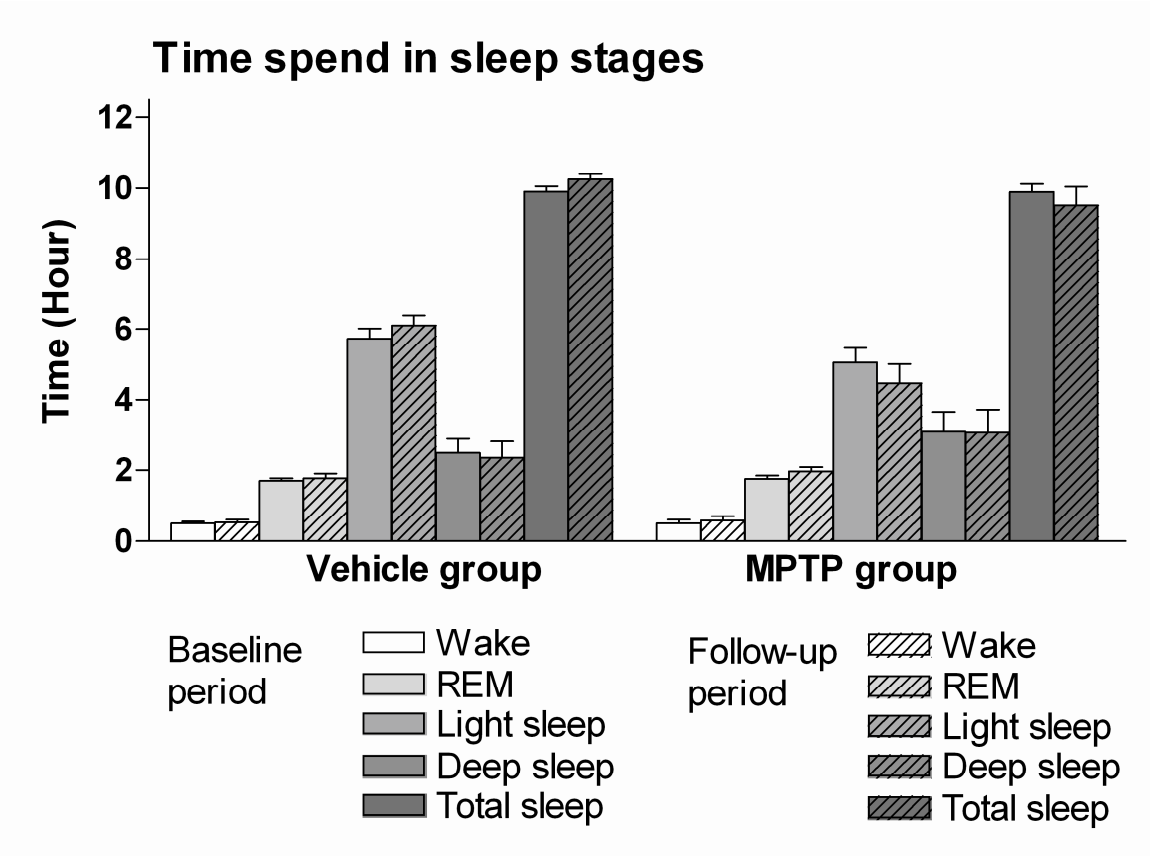

Figure 3. Sleep stages, wake time after sleep onset and total sleep time in the vehicle group and the MPTP group, before (Baseline, solid bars) and after Saline (n=4) or MPTP (n=5) treatment (Follow-up period, striped bars). No differences were found between baseline and follow-up period.

We also determined the distribution of muscle tone as a percentage of the total time in REM sleep (fig. 4). REM epochs were categorized according to muscle tone as either being absent or present in one of three predefined levels. For both the baseline and the follow-up period, epochs with muscle tone more than 10% of the time were scored most often. Epochs with muscle tone more than 50% of the time were found to be rare; however, they were more frequently scored in animals treated with MPTP. Friedman analysis showed a significant time effect within the MPTP group for the occurrence of epochs with muscle tone more than 10% of the time. MPTP, but not saline, increased the occurrence of epochs with muscle tone more than 10% of the time (32.8 ± 4.5 vs. 22.4 ± 2.9 % of REM epochs; $p < 0.05$). This repeated measures analysis also revealed an effect in the epochs with complete

atonia within the MPTP group, but not within the control group. There were significantly less epochs without atonia in the nights after MPTP treatment than in those before MPTP treatment (66.3 ± 9.1 vs. 38.3 ± 4.1±4.1, respectively; $p < 0.05$). Discriminant analysis showed that, in the follow-up period, the number of epochs with 10% muscle tone during REM sleep classified 80% of the animals correctly and the epochs with 3 or more twitches added the remaining 20% of this classification. These two variables together classified the MPTP treatment up to a maximum of 100% ($p < 0.05$).

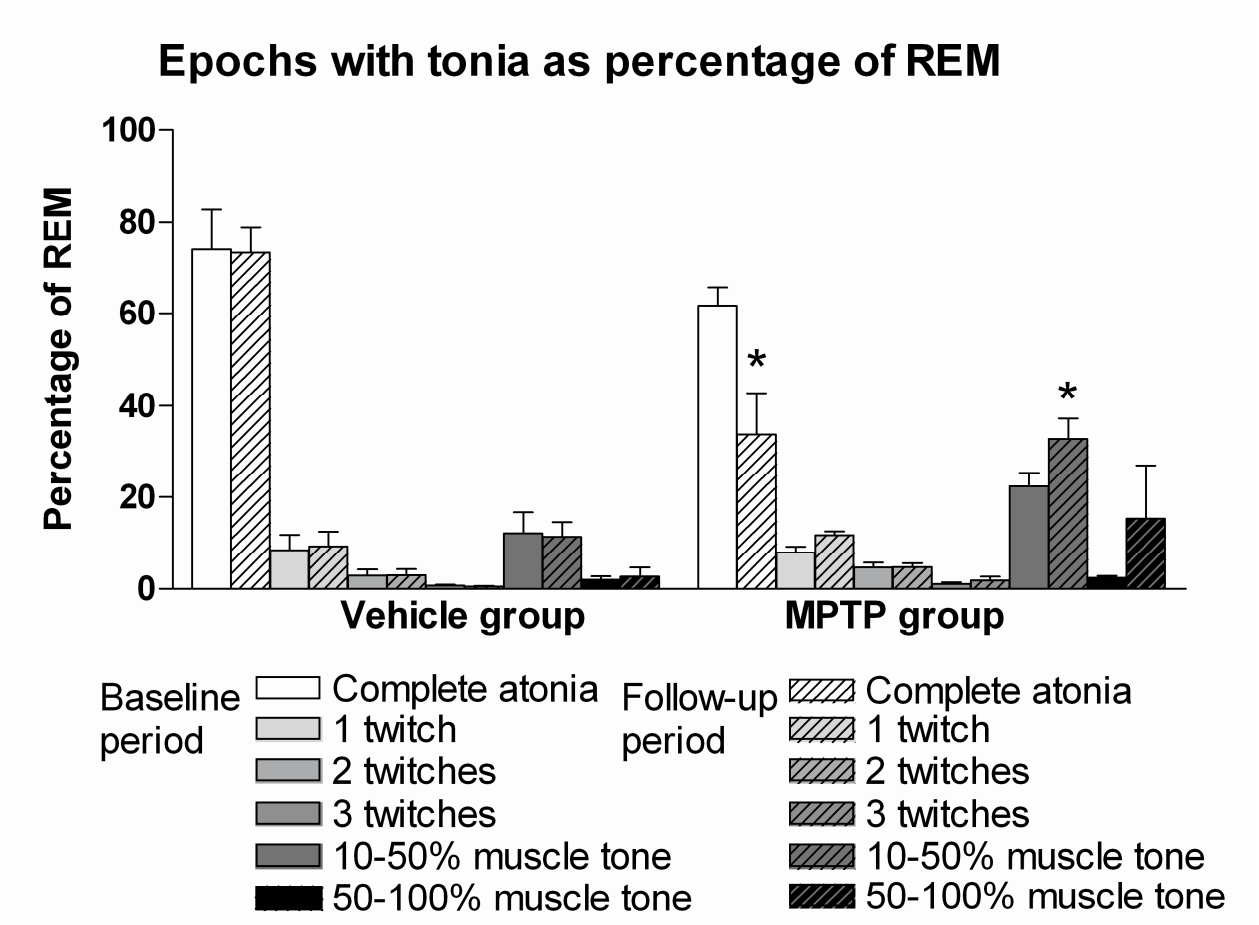

Figure 4. REM sleep epochs with muscle tone as a percentage of REM sleep in the vehicle group and the MPTP group before (Baseline, solid bars) and after Saline (n=4) or MPTP (n=5) treatment (Follow-up period, striped bars). Asterisks indicates significant differences between baseline and follow-up period (* $p < 0.05$ Friedman).

The PD-induction protocol also affected the motor behavior of the MPTP-treated marmosets while they were awake to a moderate extent. This protocol could therefore be described as a model of mildly Parkinsonian signs. Repeated measures analysis showed that the MPTP-treated marmosets' wake motor function was affected in terms of both clinical score and activity (Table 1). Friedman analysis showed that the clinical score was significantly increased within the MPTP-treated marmosets (cumulative score 8.0 ± 1.6 vs. 0.13 ± 0.1, respectively; $p < 0.05$). Repeated measures ANOVA revealed a significant treatment effect and a significant treatment-time interaction in spontaneous locomotor activity for the MPTP-treated group. In addition, the number of compartment changes was decreased after MPTP treatment (23.7 ± 8.8 vs. 64.4 ± 15.8; $p < 0.05$). In contrast to the spontaneous activity and rating scales, Friedman analysis showed that the specific motor function, in the hand-eye coordination task, was not significantly affected after MPTP treatment in the last two weeks of the experiment relative to the baseline values (number of correct trials, 23.40 ± 3.56 vs. 33.80 ± 3.79; $p = 0.22$).

Table 1. Sleep characteristics and wake motor function measurements before and after vehicle (saline) or MPTP treatments

	Vehicle group		MPTP group			
Treatment	**Baseline ± SEM**	**Saline ± SEM**	**Baseline ± SEM**	**MPTP ± SEM**	**F statistic/**	**P value**
Sex (male/female)	3/1	3/1	2/3	2/3	**Chi-square**	
Sleep efficiency (% of sleep time)	87.54±1.31	89.46±1.46	88.09±1.65	83.22±4.37	2.74	0.14[a]
Total Sleep time (min)	594.84±10.09	615.19±10.48	593.75±13.47	570.95±31.67	2.13	0.19[a]
Transitions (#)	259.63±6.77	259.88±11.65	258.00±23.54	253.70±10.66	0.04	0.85[a]
Wake bouts (#)	30.00±4.25	32.38±4.55	30.60±5.695	35.30±7.65	0.10	0.77[a]
Wake time (min)	66.09±2.60	72.5±8.75	67.98±4.16	144.25±29.68	2.78	0.14[a]
Time in REM (min)	102.19±5.60	107.13±8.72	105.35±5.98	118.35±7.73	0.70	0.43[a]
Time in light sleep (min)	343.13±20.93	366.50±20.48	303.30±24.68	268.50±31.77	2.79	0.14[a]
Time in deep sleep (min)	149.63±28.57	141.56±31.53	185.10±33.69	184.10±39.57	0.63	0.81[a]
Latency to first REM (min)	82.00±5.18	60.56±21.42	58.75±13.89	32.40±10.70	0.03	0.87[a]
Clinical Score (cum score)	0.20±0.29	0.21±0.35	0.13±0.08	8.03±1.60	5.0	0.025[b]
Activity (# changes)	85.75±16.79	94.88±9.74	64.40±15.80	23.70±8.80	7.67	0.028[a]
Hand-eye coordination (# trials)	34.13±4.12	36.13±5.88	33.80±3.79	23.40±3.56	1.0	0.32[b]

Repeated Measurements ANOVA interaction effect for parametric comparisons[a] and Friedman test for non-parametric MPTP versus Saline comparisons[b]

3.4 Discussion

The aim of the present study was to develop and to evaluate an animal model for RBD, in order to facilitate studies into the mechanisms and treatment of RBD and other sleep disturbances in the premotor stage of PD. Because of the direct link between RBD and PD (Schenck et al., 1996; Gagnon et al., 2002; Iranzo et al., 2005; Stiasny-Kolster et al., 2005; Iranzo et al., 2006; Postuma et al., 2006), we evaluated certain sleep components, particularly REM sleep chin muscle disturbances in an experimental model for PD, the marmoset MPTP model. Previous work using this preclinical animal model for idiopathic PD has focused mainly on motor behaviors. Since the focus in PD research has recently shifted from the motor phase to the premotor phase of the disease (Berg, 2008; Marek and Jennings, 2009; Stephenson et al., 2009; Tolosa et al., 2009; Tolosa and Poewe, 2009); it is of particular interest to investigate further the validity of the classic MPTP model for PD for

the study of premotor symptoms. To achieve this focus, we investigated general sleep characteristics and the EMG changes during REM sleep in the marmoset MPTP model of PD.

Rodent studies are frequently used for PD research. However, the nocturnal nature of these animals' behavior and the short sleep bouts (Monaca et al., 2004; Yi et al., 2007) make them less suitable as models for sleep in PD. Sleep changes have been reported in the rat (Lima et al., 2007), cat (Pungor et al., 1990) and cynomolgous macaque (Almirall et al., 1999) immediately following MPTP treatment. In rats, MPTP induces a temporary reduction of REM sleep and increases sleep efficiency (Lima et al., 2007). MPTP also induces a reduction in REM sleep in cats and monkeys (Pungor et al., 1990; Almirall et al., 1999). The temporary reduction of REM sleep after MPTP in these mammalian species can be interpreted as a sign of general discomfort. On the other hand, reductions in REM sleep appeared to remain in rhesus monkeys in a longitudinal follow-up study (Barraud et al., 2009). However, possible changes in muscle tone during REM sleep were not addressed in any of these studies. In our study, we induced a mild Parkinsonian state that did not significantly affect the sleep macrostructure and only mildly affected motor function, resembling an early phase of PD in humans. The animals were less active in a daytime locomotor activity test and they were mildly Parkinsonian, based on clinical scores in their home cages. Although the MPTP-treated animals were less active than control animals, they were not incapacitated as their hand-eye coordination was not affected.

The present study is the first report of selective abnormalities in REM muscle tone in non-human primates. The MPTP-treated marmoset model can be used for further studies into the mechanisms of RBD and sleep disturbances in the premotor symptom phase of PD (i.e., when patients can be diagnosed with RBD but not with PD) (Schenck et al., 1996; Gagnon et al., 2002; Iranzo et al., 2006). However, the stringent criteria for RBD described in human studies (50-100% muscle tone per epoch) are not met by the Parkinsonian marmosets in the present study. The International Classification of Sleep Disorders (2005) describes RBD as the presence of REM sleep without atonia, and disruptive behavior during sleep. The atonia, normally observed during REM sleep, is interrupted by either short (phasic, 2-3 sec) or long (tonic, 20-30 sec) episodes of EMG activity (Lapierre and Montplaisir, 1992; Gagnon et al., 2002; Iranzo et al., 2005). Then again, a significant change in tonic activity is definitely apparent in our experimental animals, which suggests an RBD-like phenomenon. Indeed, an

alternative and more suitable measure of RBD may be muscle activity as a percentage of REM sleep, given the variable outcome of polysomnogram (PSG) measurements from 62 diagnosed patients (Mayer et al., 2008). This interpretation would be supported by the parameters proposed by Mayer and colleagues (Mayer et al., 2008). For instance, the marmosets show a slight increase in phasic activity and a definite increase in tonic activity as a percentage of total REM sleep. The slight increase in phasic activity of EMG bursts or twitches (0.1-5 sec) was seen in the epochs with one single twitch and the increase in tonic EMG was seen in the epochs with more than 10% tone. This corresponds with EMG activity in at least one third of the 30-sec epochs.

The MPTP induced changes in REM sleep muscle tone are presumably due to changes in DA neurotransmission. Reduction in DA neurons in the *substantia nigra* caused by MPTP exposure results in a decrease DA signaling to its receptors localized within the *striatum* (Levey et al., 1993). In this regard knocking out the DA transporter (Dzirasa et al., 2006) or reducing DA signaling in the brain result in sleep changes in mice(Monaca et al., 2004). Similarly, a loss of DA neurons in the *substantia nigra* underlies some of the sleep changes in rats. Clinical conditions affecting DA, such as those seen in PD, also alter sleep architecture(Comella et al., 1993) which result in changes in REM sleep (Dahan et al., 2007) and muscle tone during REM sleep (Garcia-Borreguero et al., 2002; Fantini et al., 2003). Therefore, nigrostriatal neurons whose axons are located in the *striatum* are assumed to play a major role in the regulation of REM sleep. Additionally, a relatively small nigral lesion is enough to produce sleep changes(Lima et al., 2007) On the other hand, it has been suggested that the degenerative process in PD is initiated in the medulla, advances to the pons, and subsequently targets the midbrain (Braak et al., 2003). Thus, the presence of RBD might also reflect early involvement of non-DA medullary and/or pontine REM sleep-related structures(Iranzo et al., 2005). Therefore, it is suggested that these structures, which are closely connected to the *substantia nigra* pathways are affected by an imbalance of DA levels (Lai and Siegel, 1990) which would precede the actual neurodegeneration process.

In conclusion, the MPTP-treated marmoset provides a new opportunity for quantitative studies on the mechanisms and intervention strategies of RBD and the premotor phase of PD. Unlike the nocturnal preference and fragmented pattern of sleep in mice and rats, the architecture of marmoset monkeys' sleep resembles that of humans. Marmosets are diurnal

and, as in humans, their night sleep architecture consists of a recurring pattern of cycles with light, deep and REM sleep. Further, quantifying the different stages in marmoset monkeys can be performed with the classical sleep scoring system directly adapted from human scoring (Rechtschaffen and Kales, 1968). The observed increase in muscle tone during REM sleep is typical for RBD and one of the major symptoms preceding the classic motor problems in many PD patients. The fact that MPTP affects REM sleep atonia suggests a direct role for DA depletion as a cause for the increased REM sleep muscle tone in PD.

Acknowledgements

The authors would like to thank H.P.M. van Helden, D. Davis and G. Torres for critical reading of the manuscript. This study was supported by the U.S. Army Medical Research and Materiel Command, Award No. W81XH-05-1-0517.

4

Neuroprotective effects of riluzole in early phase Parkinson's disease on clinically relevant parameters in the marmoset MPTP model

P.S. Verhave M.J. Jongsma, R.M. van den Berg, R.A.P. Vanwersch, A.B. Smit and I.H.C.H.M. Philippens

Manuscript submitted

But although, at present, uninformed as to the precise nature of the disease, still it ought not to be considered as one against which there exists no countervailing remedy. On the contrary, there appears to be sufficient reason for hoping that some remedial process may ere long be discovered, by which, at least, the progress of the disease may be stopped. (Parkinson, 1817)

4 Abstract

The present study evaluates neuroprotection in a marmoset MPTP (1-methyl-1,2,3,6-tetrahydropyridine) model representing early Parkinson's disease (PD). The anti-glutamatergic compound riluzole is used as a model compound for neuroprotection. The compound is one of the few protective compounds used in the clinic for a neurodegenerative disorder.

Three groups of six marmoset monkeys were used: 1) an MPTP group receiving a total MPTP dose of 7 mg/kg (4 injections in two weeks, s.c.) 2) a riluzole group receiving besides MPTP, twice daily a dose of riluzole (10 mg/kg, p.o.), starting one week before MPTP and up to one week after the last MPTP injection and 3) a control group receiving saline instead of MPTP and riluzole. The marmosets' Parkinsonian symptoms were scored daily and their activity level, hand-eye coordination, jumping behavior, axial turning and night sleep parameters were tested and recorded weekly. At three weeks after the MPTP challenge, brains were dissected and dopamine levels in the *striatum* and the tyrosine hydroxylase (TH) expressing dopamine (DA) neurons in the *substantia nigra* (SN) were compared. MPTP affected all behavioral parameters and sleep architecture and induced a relatively mild (50%) decline of DA neurons in the SN. Riluzole relieved the Parkinsonian signs, and improved the hand-eye coordination as well as turning ability. Moreover, riluzole prevented the impact of MPTP on sleep architecture and rapid eye movement behavioral disorder (RBD). Riluzole also imcreased the number of surviving DA neurons in MPTP-treated marmosets. However, riluzole did not prevent the MPTP-induced impairments on locomotor activity and jumping activity.

In conclusion, reduction of excitotoxicity by riluzole appeared to be effective in reducing progressive neurodegeneration and relieved several clinically relevant PD symptoms in an animal model representing the early phase of PD.

4.1 Introduction

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disorders worldwide especially in the aging societies. It is challenging today's scientific community in the search for new treatment strategies. Current priority in PD research is to move beyond symptom control to neuroprotective therapy (Jenner, 2008) in order to prevent the neurons from dying, and slowing the progression of PD. Crucial for this is the ability to evaluate neuroprotection in an early stage of the disease. Here we aim to show neuroprotection on behavior and pathology in a model for early PD.

One of the features in neurodegeneration is mitochondrial dysfunction (Betarbet et al., 2000) that affects cellular function via the accumulation of intracellular calcium levels (Greenamyre et al., 1999). This results in the production of free oxygen and nitrogen radicals (Fiskum et al., 2003; Nicholls, 2008) and puts neurons under stress and activates pathways leading to neuronal degeneration. Excitotoxicity has been implicated in relatively slowly progressing neurodegenerative disorders such as PD (Mandel et al., 2003) in which already vulnerable neurons may not survive elevated glutamate concentrations that would normally not be harmful. Therefore, reducing the effects of glutamate and calcium influx should prevent further progression of neuron deterioration. Indeed, counteracting the actions of glutamate, directly or through sodium or calcium channel manipulation (Song et al., 1997), are expected to have neuroprotective effects in neurodegenerative disorders (LeWitt and Taylor, 2008). To this end, the anti-glutamatergic compound riluzole is proposed to affect the calcium influx through various pathways, including the inhibition of glutamate in the synapse by the blockade of NMDA receptors (Doble, 1996) and voltage-dependent sodium channels (Hubert et al., 1994) on nerve endings and cell bodies. Indeed, riluzole appears to prevent neuronal damage at the start of neurodegeneration in MPTP-challenged mice, rhesus monkeys and marmosets (Araki et al., 2001; Benazzouz et al., 1995; Obinu et al., 2002) and also in other PD models (Barneoud et al., 1996; Fumagalli et al., 2006). Riluzole is an FDA-approved drug treatment because of life-prolonging effects in amyotrophic lateral sclerosis (ALS) (Bensimon et al., 1994). In this study we use riluzole to model neuroprotection and show the effects of the reduced neurodegeneration in a model for early PD.

Riluzole appears to be less effective in clinical studies when riluzole (50 mg, twice daily) treatment is started in the motor phase of PD when a large part of DA neurons has already

been degenerated (Jankovic and Hunter, 2002). Possibly complete prevention of neuronal cell loss may only be achieved if the treatment could be initiated before or simultaneously with the actual start of the apoptotic process. Patients would therefore benefit from premotor phase diagnosis of PD to start treatment at a stage where there are still neurons left to protect. Thus, in order to find new targets for neuroprotective therapies, animal models need to be employed that enable investigation of treatment options early in the pathogenesis of the neurodegenerative process.

In this study, we investigated the neuroprotective effects of riluzole in the marmoset model on several clinically relevant behavioral and sleep parameters which may be used as biomarkers for neuropathology. This translational integrative approach may contribute to the understanding of the mechanisms during PD progression. Additionally this approach offers a model for the early phase of PD to test new neuroprotective treatment strategies.

4.2 Material and Methods

Animals

Eighteen common marmoset monkeys of both sexes (*Callithrix jacchus*), between 2 and 3 years of age, purchased from the Biomedical Primate Research Centre in the Netherlands were used. The monkeys were housed in individual primate cages (61x61x41 cm) under controlled conditions of humidity (60%), temperature (23-25ºC) and lighting (12 hour light/dark cycles; lights on at 7:00 h). Marmosets were fed daily with pellet chow. Diet was enriched with peanuts, fruit and vegetables, raisins, sunflower seeds and an occasional grasshopper. Water was available *ad libitum*. All marmosets were provided with a varying cage environment. Experiments were conducted after approval by the Ethical Review Committee of the institute.

Drug treatment

The marmosets were divided into three groups semi-randomly based on their baseline activity level in the bungalow test. Two groups (n=6) received 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP, Sigma Aldrich, St. Louis, USA) by subcutaneous (sc) injections in the abdominal area. MPTP was administered in four injections on Mondays and Thursdays (subsequently 2, 2, 1.5, and 1.5 mg/kg) within two weeks till moderate Parkinsonian symptoms were established. A third group (n=6), received time and volume-

matched sc 0.9% saline injections (control group). One of the two MPTP-treated groups (n=6) received riluzole treatment (riluzole group). For oral administration riluzole was suspended in 2:1 water:syrup (Karvan Cevitam®) containing 0.5% methylcellulose (Sigma Aldrich, St Louis, USA), dosage was 10 mg/kg twice daily. The two other groups received time and volume-matched vehicle.

Study design

Treatment started one week before MPTP treatment and lasted until one week after last MPTP injection. All marmosets were observed in the morning and in the evening for Parkinsonian signs according to two observations scales. Functionality and activity tests were performed on Wednesdays and Thursdays between 13:00 and 17:00 h and sleep recordings were performed weekly. To make sure sickness behavior, a direct effect of MPTP, did not affect the analysis, only the last three weeks after the MPTP challenge were used for behavioral analysis. All parameters after MPTP treatment were tested for statistical significance with an ANOVA, followed by Dunnett's test for normal data, and a Friedman test followed by Mann-Whitney tests for non-normal data.

Observational scores

The observational tests were scored daily before treatment. Items were rated from 0 (=normal/healthy) to 4 (=severely affected). Abnormal Involuntary Movements Scale (AIMS) included scores for facial behavior items (jaw, facial muscles, tongue and lips) and full body behavior items (upper, lower and trunk). The scale also includes scores for: general severity of involuntary movements and incapacitation due to these movements. Clinical score includes scores for the items apathy (no interest in their surrounding), immobility, muscle rigidity (as measured by the stiffness of the legs and tail) and rest tremors. Immobility was also depicted separately to specify the home cage activity at rest to serve as a comparison to the out of cage activity tests.

Hand-eye coordination

The marmosets reward-related hand-eye coordination was tested with an automated test setup (van Vliet et al., 2006). During a session of 42 trials small pieces of marshmallow were presented behind a window (5x8 cm) at three different speeds, non-moving (0.0 m/s for a

maximum of 30 s) slow (0.04 m/s) and fast (0.08 m/s) moving. All trials started with a brief sound signal to alert the animal. Before the start of the study, all animals were trained until they grabbed 80% or more of the presented rewards. The percentage of correct hits was used as criterion to judge the performance of the animal.

Bungalow

Activity of the marmoset was tested in the so-called Bungalow test system, an automated test to evaluate the activity of the marmoset by compartment changes (van Vliet et al., 2006). The Bungalow consisted of four equal compartments (23x23x23 cm) connected to each other in which the marmoset could move freely from one compartment to the other, during a 20-min. period. A video tracking system registered the movement pattern and the position of the monkey in the apparatus. The number of compartment changes was used as a measure for activity.

Tower

In the Tower (Verhave et al., 2009) the marmosets jumping behavior and activity were evaluated. The trespa Tower (35x35x250 cm) contains 7 levels of horizontal crossbars with increasing distances. During each test the marmoset could move around freely on the 7 levels for 5 min. To motivate the marmoset to visit each level, a small piece of marshmallow was available on each level. All marmosets were habituated to the Tower before testing. The marmoset's location (level) was noted by non-automated video analysis.

Hourglass

In the Hourglass test the marmosets axial turning ability in a Plexiglas cylinder (11x27 cm) was evaluated (Verhave et al., 2009). One trial consisted of one manual 180º turn of the cylinder. A test consisted of five subsequent trials with intertrial intervals of 30 sec. The time the marmoset needed to get into the upright position, after the cylinder turn, was measured by means of non-automated video analysis by an observer unaware of the treatment. Maximum time noted was 30 sec (also for marmosets which did not turn upright at all). Only the three fastest turns were taken into account for statistical analyses.

Sleep

Under isoflurane/O_2 anesthesia combined with the local anesthetic lidocaine two stainless steel electroencephalogram (EEG) electrodes were placed into the skull on the intra-aural and 5 mm anterior from of the intra-aural and both 2 mm lateral (right hemisphere) from the *sutura sagitalis*, leaving the *dura mater* intact. To measure muscle activity one flexible electromyogram (EMG) electrode was placed into the chin muscle (*trigonum submandibularis*) and a second EMG electrode into the neck muscle (*trapezius*) and tunneled to the head of the animal. All electrodes were connected by a plug and fixed to the skull with dental cement (Fuji plus capsule, GC Corporation, Tokyo, Japan). Prophylactic antibiotic treatment was provided by 0.02 ml/kg im of 150 mg/ml ampicillin before and one day after surgery. All marmosets were extensively observed after surgery and were left for one week to recover.

Four weeks after the surgery the seven-week experiment started. During the experiment sleep recordings of all marmosets were performed weekly. During the EEG and EMG recordings the animals were kept one night a week in special sleep cages (40x20x30 cm) and a bioelectric two-channel transmitter (Data Sciences International, Transoma medical, Arden Hills, USA) was connected to their plug for telemetric registration of the EEG and the EMG. The signals were recorded with a sample frequency of 100 Hz using Data Sciences International software (Data Sciences International, Transoma medical, Arden Hills, USA). Somnologica software (Embla Inc, Broomfield, USA) was used for scoring of sleep in 30-sec epochs to obtain hypnograms. The sleep scoring was performed by an experienced sleep technologist, unaware to the treatment of the subjects, according to the criteria of Rechtschaffen and Kales adapted for marmosets (Edgar et al., 1993). Sleep macrostructure was described by wake time after sleep onset and three different sleep stages light sleep, deep sleep and rapid eye movement (REM) sleep. Additionally, 30-sec epochs assigned as REM sleep with muscle tension in more than 10% or more than 50% of the epoch time were scored.

Dopaminergic cells in the *substantia nigra*

Substantia nigra (SN) was analyzed for the presence of DA positive neurons with TH immunoreactive (TH-IR) staining. Part A7-A2 (Stephan et al., 1980) of the right hemisphere of the brain containing the SN was isolated at the beginning of week 8 and fixated in 4% paraformaldehyde at 4 °C and after 48h the brains were transferred to 0.5%

paraformaldehyde at 4 °C. The material was dehydrated in graded ethanol and xylene and subsequently embedded in paraffin.

Transversal sections of 5 µm were cut on a microtome and collected serially on aminosilane/acetone solution coated slides. The sections used for the TH-IR were deparaffinized and rehydrated in xylene and graded ethanol. Citrate buffer was used for antigen retrieval (10 min 100ºC). Sections were preincubated with 0.3% H2O2 in PBS and in PBS with 0.1% bovine serum albumin with 0.3% Triton X-100. Sections were left to incubate in anti-TH antibody (1:40000, Sigma Aldrich, St. Louis, USA) overnight at room temperature. The second antibody (1:2000, Santa Cruz Biotechnology Inc., CA, USA) was applied for 90 min. After thid the sections were left to incubate in Vector ABC (1:800, Vector Laboratories Inc) for 90-min incubation. The sections were thoroughly washed using PBS every time the sections were placed in an antibody containing solution. Sections were pre-incubated for 10 min with 0.025% 3'3'-diaminobenzidine containing 0.15% nickel ammonium sulfate (DAB-NI solution) followed by a 10-min incubation in the DAB-NI solution with 0.00015% H2O2. After a PBS-wash, the sections were dehydrated with alcohol cleared in xylol and overlaid with a coverslip with Malinol (Sigma Aldrich, St. Louis, USA). TH-IR positive neurons were counted in 3 sections of the SN in between 4 and 5.5 mm anterior of the external auditory meati (Stephan et al., 1980). Within each section, all TH-IR neurons in the SN *pars compacta* were counted manually and total area was estimated with a 10 mm × 10 mm eye-piece grid at a magnification of 200× using an Olympus light microscope.

Dopamine levels in the *striatum*

Striatum was weighed and homogenized by sonification (2x5 s) in 250 µL 0.4M perchloric acid containing 2 M sodium acetate. Homogenate was centrifuged at 20800 g for 30 min and the supernatant was stored at -70º. The internal standard, dihydroxybenzylamine (DHBA), was added to determine extraction efficiency. DA was determined by ion-pair reversed phase liquid chromatography. A 5-20 µL sample was injected using a SIL-10A auto-sampler (Shimadzu, Columbia, MD), on a RP18 column (150 mm x 4.6 mm i.d., 3 µm particle size; GRACE, Lokeren, Belgium). The mobile phase (32 mM citric acid, 54 mM sodium acetate, 0.074 mM EDTA, 0.215 mM octane sulphonic acid, 3% MeOH, 0.004% TFA adjusted to pH 3.8 with HAc) was pumped at a flow rate of 0.35 ml/min using a LC-10AD pump

(Shimadzu, Columbia, MD). The potential of the electrode was set at 600 mV and 0.2 nA/V sensitivity. Calibration plots were linear from 1 to 250 ng/ml for each of the compounds of interest. The lower limit of detection was 1 ng/ml. The intra-assay coefficient of variation was 2%.

4.3 Results

Parkinsonian Signs

Although the maximum level of the AIMS and the Clinical score was lower in the MPTP-riluzole group than in the MPTP group, there were no significant differences between the two groups (fig. 1). In the last three weeks of the experiment both groups were significantly affected in their Parkinsonian movements in comparison with controls. A trend for improved AIMS in the MPTP-riluzole group was found at the following time points: t=29, 30, 34, 36, 38 and 45 and for the Clinical score: t=36, 45 and 47 (fig. 1).

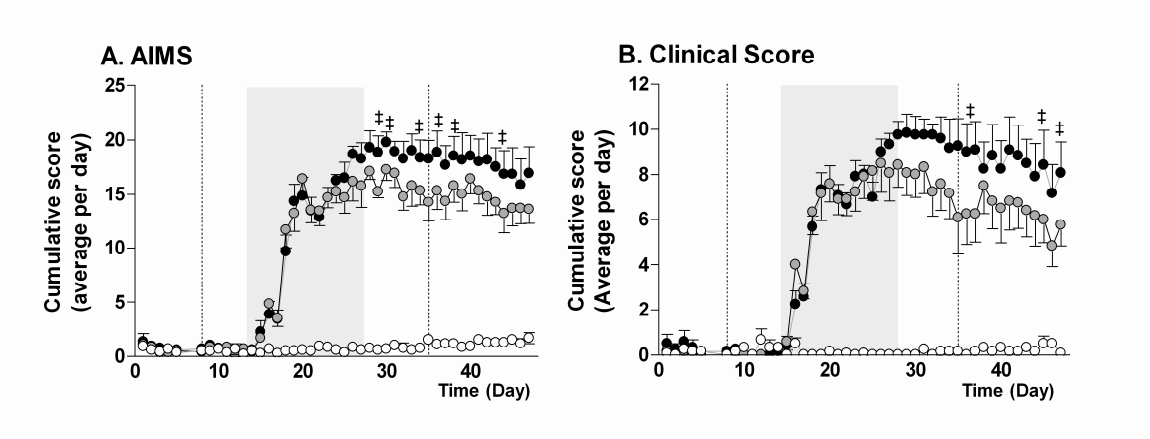

Figure 1. Behavioral observation scales: A. Abnormal Involuntary Movement Scale (AIMS) and B. Clinical score. Grey area indicates MPTP challenge and dotted lines indicate the treatment period with riluzole (20 mg/kg/day) or vehicle. Each data point represents mean ± SEM of observations for each group (n=6): control group (white circles), MPTP group (black circles) and the MPTP-riluzole group (grey circles). Symbols ‡ indicate differences in comparison to MPTP group, Friedman followed by selected Mann-Whitney tests (‡ $p = 0.093$ except for day 34 and day 35 $p = 0.063$).

Hand-Eye Coordination

At the start of the experiment all marmosets had an average of more than 75% correct performance per session, as observed in week 1. One week after the MPTP treatment, the number of correct hits was significantly reduced in the MPTP and MPTP-riluzole treated groups (fig. 2A). The MPTP-riluzole treated marmosets showed a significantly better hand-eye coordination performance than the MPTP group in week 5, the week following the MPTP treatment ($p < 0.05$). During week 6 and 7 the hand-eye coordination performance of the MPTP-treated animals returned gradually towards normal values.

Tower: Top level

All test groups showed a significantly lower top level after MPTP treatment in the Tower in comparison to the performance in week 1 (fig. 2B). Compared to the control group one week after MPTP treatment both the riluzole treated group and the untreated MPTP group had a significantly lower top level ($p < 0.05$). Both groups however considerably improved their performance after the MPTP treatment, and almost reached their original top level again at the end of the experiment.

Hourglass

Animals treated with MPTP needed significantly more time to turn upright in the Hourglass compared to the control group and did not recover to a normal level in the three week period after MPTP treatment (fig. 2C). The MPTP-riluzole group was less affected by MPTP. These riluzole treated animals did not show a significant decrease in their turning speed compared to the MPTP group and they recovered faster.

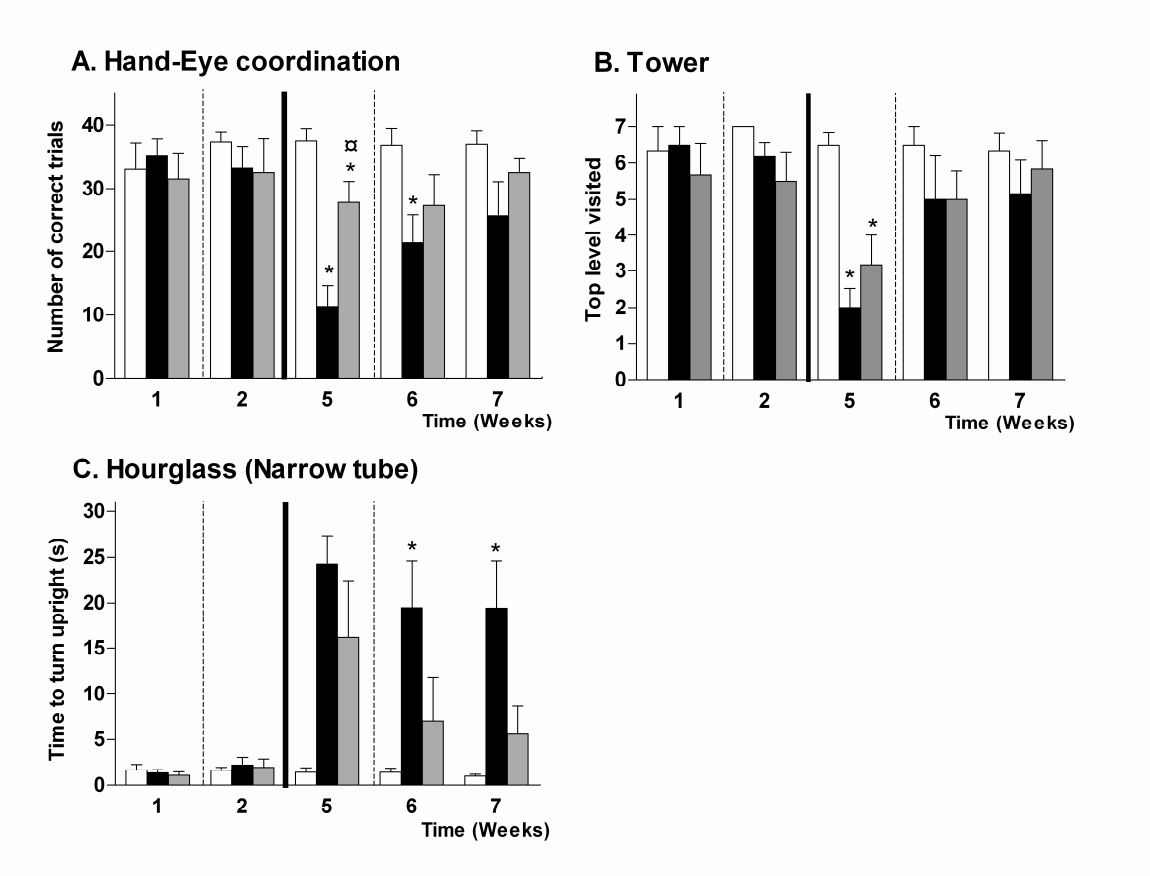

Figure 2. Functionality measures: A. Hand-Eye coordination, B. Tower- top level, and C. Hourglass. Each bar represents mean + SEM for each group (n=6); the control group (white bars), the MPTP group (black bars) and the MPTP-riluzole group (grey bars). The black vertical line indicates the MPTP challenge and dotted lines indicate the treatment period with riluzole (20 mg/kg/day) or vehicle. Stars indicate significant differences with the control group. Symbol ¤ indicates significant difference with MPTP, Friedman followed by selected Mann-Whitney tests (¤ and * $p < 0.05$)

Bungalow

The control animals showed a stable activity level of 75-120 compartment changes (fig. 3A). Activity was significantly reduced in both MPTP-treated groups to as few as 10 compartment changes in total ($p < 0.05$). This activity reduction was persistent for the remainder of the experiment. Marmosets of the MPTP group were the least active of all marmosets.

Tower: level changes

There was variability in the control group in regards to the number of level changes (10-30 level changes). Nevertheless, the activity was significantly decreased by the MPTP treatment and reduced in both groups to less than 5 level changes (riluzole $p < 0.05$ and MPTP $p < 0.01$). This activity reduction had significantly recovered in week 7 for the MPTP-riluzole treated marmosets compared to the monkeys from the MPTP group ($p < 0.05$).

Home cage immobility

After the MPTP treatment all treated marmosets were scored significantly less mobile in their home cage. A trend for a difference in home cage immobility between the MPTP-riluzole group and the MPTP group was found in week 7 at around t=35 days and t=45 days ($p < 0.1$). Hardly any immobility was scored in the control group.

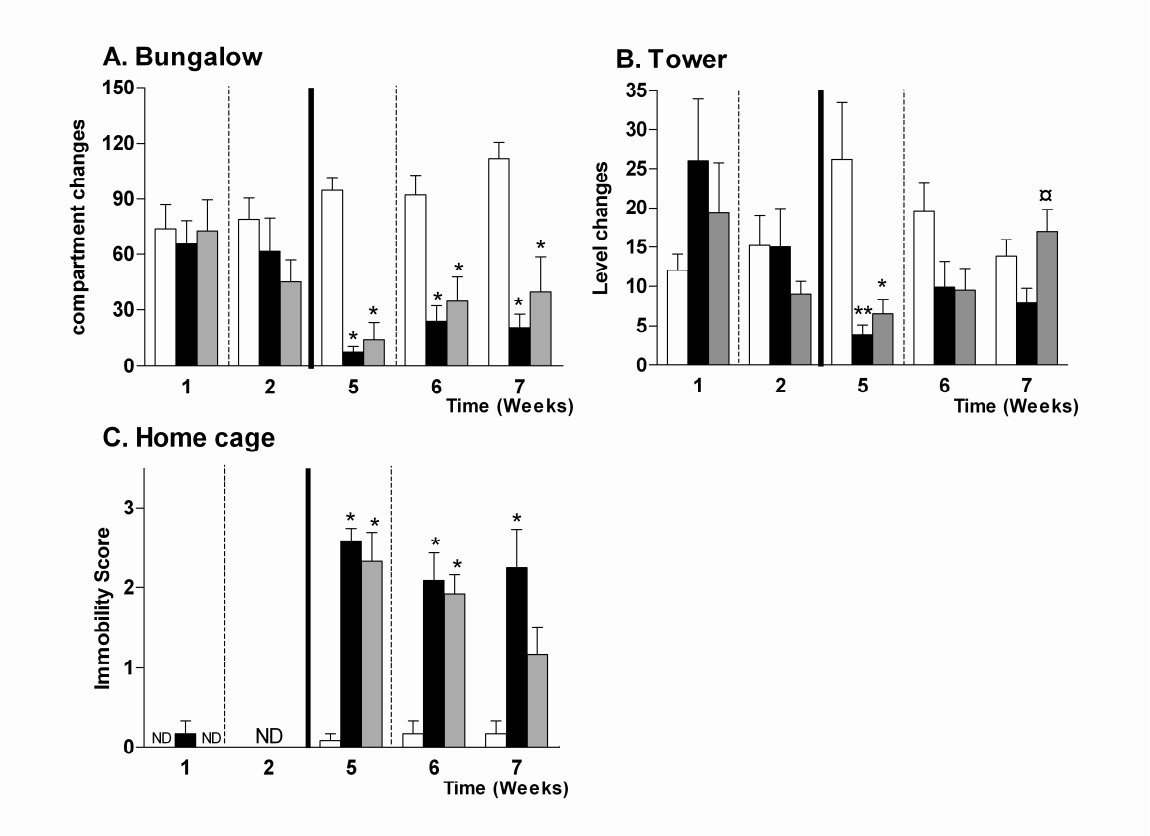

Figure 3. Activity measures: A. Bungalow, B. Tower and C. Home cage. Each bar represents mean + SEM for each group (n=6): control group (white bars), MPTP group (black bars) and the MPTP-riluzole group (grey bars). Black line indicates the MPTP challenge and dotted lines indicate the treatment period with riluzole (20 mg/kg/day) or vehicle. Stars indicate significant differences with the control group. Symbol ¤ indicates significant difference for difference in comparison to MPTP group. Friedman followed by selected Mann-Whitney tests (** $p < 0.01$, ¤ and * $p < 0.05$).

Sleep

The marmosets sleep macrostructure was constant over time as seen in the control group (fig. 4A-D) and MPTP treatment did not affect the sleep efficiency and total sleep time (data not shown). However, MPTP did affect the macrostructure over the whole night; light sleep was significantly changed when compared to the control group ($p < 0.05$) (fig. 4A) and deep sleep showed a non-significant increase in week 6 in the MPTP group. The decrease in light sleep was not apparent in the MPTP-riluzole treated marmosets. Riluzole alone did not

affect the sleep macrostructure as measured in week 2 (fig. 4A-D). Wake time after sleep onset (WASO) did show a not significant increase after MPTP treatment (fig. 4d).

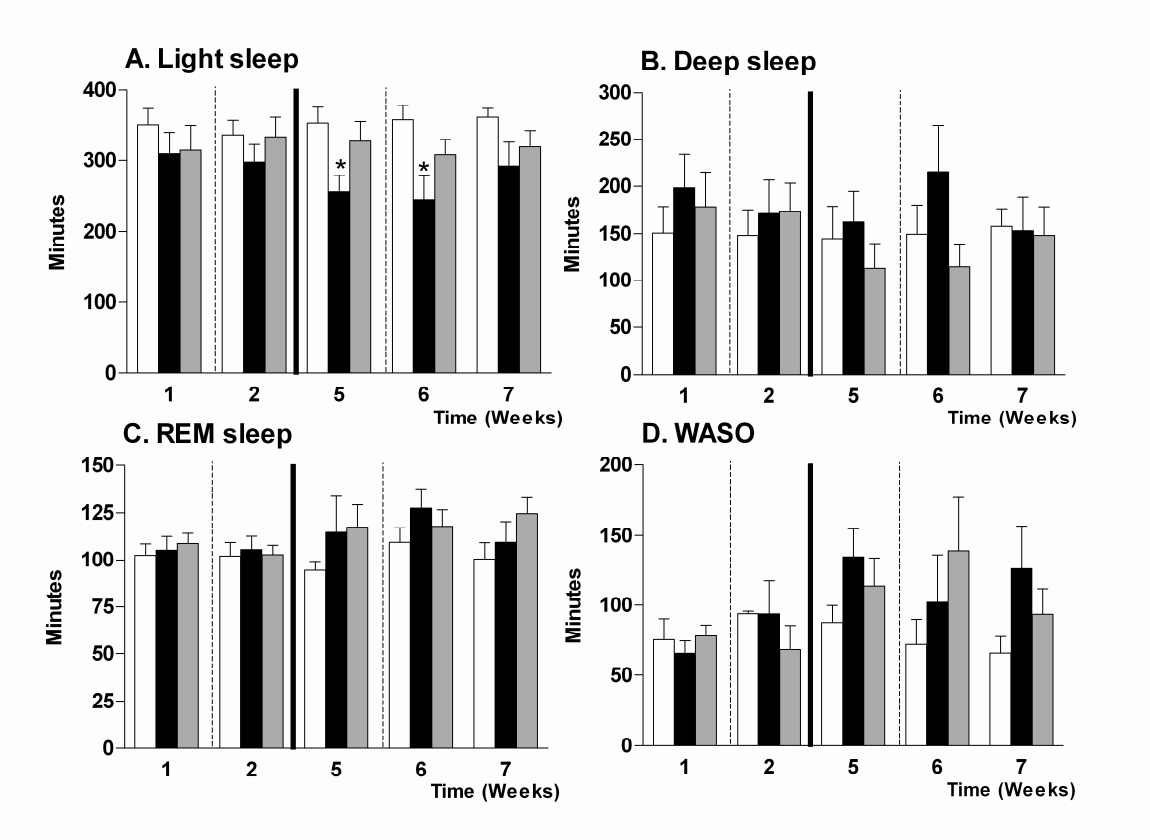

Figure 4. Time spent per sleep stage. A. Light sleep, B. Deep sleep, C. REM sleep and D.WASO (wake time during sleep). Each bar represents mean + SEM for each group (n=4-5): control group (white bars), MPTP group (black bars) and the MPTP-riluzole group (grey bars). Black line indicates the MPTP challenge and dotted lines indicate the treatment period with riluzole (20 mg/kg/day) or vehicle. Stars indicate significant differences with the control group. ANOVA followed by a Dunnett post hoc test (* $p < 0.05$).

Muscle tone during REM sleep increased significantly in the MPTP-treated marmosets (fig. 5A-B) as measured in epochs with more than 10% muscle tone in week 6 ($p < 0.05$) and in the riluzole group in week 7 ($p < 0.05$). A large difference between the MPTP group and the riluzole group is seen in the epochs with more than 50% muscle tone per epoch. Whereas almost none of these epochs were scored in the control group, the MPTP group showed an increase in these epochs. This stayed apparent until the end of the experiment. Despite the large variability between animals, there was a trend for an increase in week 5 in the MPTP group in comparison to the control group ($p < 0.10$) and a significant decrease in epochs

with more than 50% muscle tone in the MPTP-riluzole group compared to the MPTP group in week 6 ($p < 0.05$).

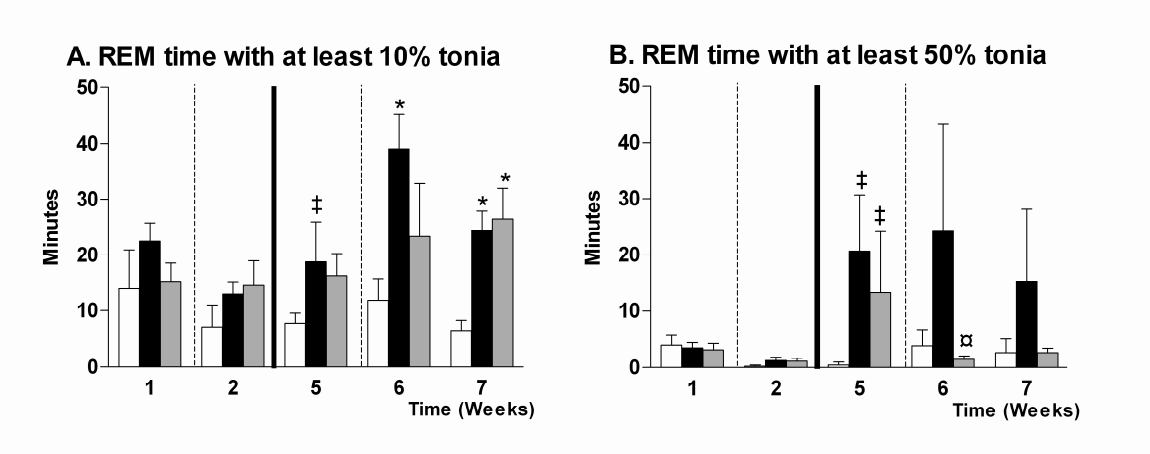

Figure 5. Muscle tone during REM sleep. A. REM time with at least 10% muscle tone and B. REM sleep with at least 50% muscle tone. Each bar represents mean + SEM of observations for each group (n=3-5): control group (white bars), MPTP group (black bars) and the MPTP-riluzole group (grey bars). Black line indicates the MPTP challenge and dotted lines indicate the treatment period with riluzole (20 mg/kg/day) or vehicle. Stars indicate significant differences and symbol ‡ a trend for a difference with the control group. Friedman followed by selected Mann-Whitney tests (‡ are $p = 0.63$ and ¤ or * $p < 0.05$).

Dopamine neurons

The MPTP group showed a 50% loss of TH-positive neurons in comparison to the control group ($p < 0.05$). The riluzole treatment caused a TH positive cell rate of more than 75%. This percentage was not significantly different from the MPTP group or the control group. The MPTP group had a significantly lower amount of DA in the *striatum* ($p < 0.001$). In the MPTP-riluzole treated marmosets the DA level was not significantly different from the control group.

Table 1. Dopamine neuron parameters

Brain region	Parameter (% of control)	Control		MPTP		Riluzole	
Substantia nigra	Neuron profiles	**100**	**±16.15**	**47.81**	**±3.00***	**76.76**	**±5.5**
Striatum	Dopamine	**100**	**±11.62**	**1.73**	**±0.63*****	**52.90**	**±2.53**

Values ± SEM measured three weeks after MPTP treatment. *$p < 0.05$ and *** $p < 0.001$ versus control (ANOVA; followed by Dunnett's post hoc tests).

4.4 Discussion

In this study, we have shown shown neuroprotection in an early model for PD. Riluzole riluzole partially prevents moderate neurodegeneration of DA neurons in SN and *striatum* caused by a mild PD induction protocol with MPTP. It also protects several motor-related functionalities and sleep-related aspects in early stage Parkinsonian marmosets.

The study design, in which pretreatment with riluzole started before PD induction, provides insight into the potential side-effects of riluzole on the behavioral readouts in this study. Apart from taste aversion to riluzole no significant side-effects were observed. We did, however, observe a slight decrease in locomotor activity in the Bungalow. Additionally immobility, scored within the clinical score, was increased in week 2: the first week of riluzole treatment. The fact that these changes were not significant might be due to the large variation between marmosets. This tendency towards a decrease in mobility is in line with previous reports on behavior in mice and rats, such as increased immobility after riluzole in rats and at higher dosages loss of righting reflex in mice (Doble, 1996; Irifune et al., 2007). The absence of side effects in this study might be due to the turnover and decrease in concentration of riluzole by the time testing occurred. The behavioral tests were performed 5-9 hours after the last dosage. This was within the estimated active period of riluzole. Tmax of riluzole was reported at 5 hours after dosage (p.o.) in macaque monkeys (Martinet et al., 1997). Additionally, in contrast with other literature (Stutzmann et al., 1988; Doble, 1996), no effects were found on sleep parameters. This might be explained by inter-species differences in anatomic sleep regulating structures (Spiegel et al., 2006). Sleep architecture in the marmosets did not change after riluzole although lights off was less than one hour after the riluzole administration. Additionally, no changes were found in the sleep onset or in the

latency to the first REM period (data not shown), which suggest that sleep induction was not changed by riluzole treatment.

The 50% reduction of the DA neurons after MPTP treatment points to early neurodegeneration at which time the clinical signs of PD in patients are just starting to occur (Fearnley et al., 1991). The large percentage of DA depletion compared to the cell loss in the SN during the early phase of the disease in this study can be explained by the fact that the neurodegeneration starts at the axonal terminal in the *striatum* (Duda, 2003; Li et al., 2009). Like the DA cell number, the DA levels in the MPTP-riluzole treated marmosets are restored or protected to a certain level in the *striatum* of the marmosets. This underlines that riluzole is neuroprotective in our study which is in line with previous neuroprotection studies in rat (Barneoud et al., 1996), mice (Araki et al., 2001) and marmosets (Obinu et al., 2002). The mechanisms by which riluzole protects against neurodegeneration are multiple. Riluzole, for example, reduces presynaptic glutamate release (Wang et al., 2004) and blocks activated sodium and calcium channels (Doble, 1996), thereby decreasing the degree of neuronal excitation. The fact that excitotoxicity may play a role in the pathogenesis of certain neurodegenerative disorders including PD, supports the predicted efficacy of glutamate inhibitors as neuroprotective agents. Indeed, riluzole tended to inhibit the deteriorative influence of MPTP on the majority of the measured parameters in the current study, that can be ascribed to the anti-excitotoxic mechanism and not to a direct effect on the MPP^+ accumulation within the cell (Boireau et al., 2000). Due to the relatively small dopaminergic lesions in this model for early PD and the natural variation of Parkinsonian marmosets, the neuroprotective effects are difficult to point out. Spontaneous behaviors, such as top level in the tower and the activity in the bungalow, were not positively affected by the riluzole treatment. On the other hand, the clinical score (e.g tremors), the AIMS, the hand-eye coordination (bradykinesia), turning ability (rigidity and posture problems) and the activity in the tower (akinesia) were less affected in the MPTP-riluzole treated Parkinsonian marmosets than in the non-treated animals. We noticed the effects of reward expectation on DA release (Verhave et al., 2009). The positive reinforced hand-eye coordination was normalized in all MPTP-treated marmosets at the end of the experiment (week 7). The non-rewarded reinforced turning behavior did not show this large improvement as seen in the hand-eye coordination. Therefore, this persistent decline of turning performance is possibly a direct effect of neurodegeneration. Positive reinforcement might also be the explanation

for the difference between the activity in the non-rewarded bungalow, in which the activity remains low until the end of the experiment in all MPTP-treated marmosets, and the positively reinforced activity in the tower, in which an improvement is found during the study. Measuring activity in or outside the home cage (fig. 3A and C) did not affect the results; the reduced activity was consistent in all non reinforced activity measures. All Parkinsonian marmosets showed an increase in immobility towards the end of the experiment.

The results on behavioral parameters obtained with riluzole in this study are not very strong but in agreement with previous studies using MPTP-treated mice and monkeys (Araki et al., 2001; Obinu et al., 2002; Diguet et al., 2005). An explanation for this may be that the reduction of DA neurons in the SN in the non-treated MPTP marmosets is comparable to the Obinu study in which a 65% reduction was seen (Obinu et al., 2002), instead of the 50% in our study with the early phase induction protocol. This might explain the difference between the behavioral effects of MPTP in both studies. Therefor in the early premotor phase of PD premotor aspects are more relevant than the motor related disturbances such as abnormal olfaction, mood, autonomic dysfunction and abnormal sleep (Berg, 2008; Tolosa et al., 2009). One of the sleep-related disturbances is an increased muscle tone during REM sleep. At least one third of the PD patients have increased and irregular chin muscle tone during REM sleep (Gagnon et al., 2002), which meet the criteria for RBD. The therapeutic effect of riluzole on tonic muscle tone during REM sleep, as shown in our study, implies an important contribution of riluzole in the treatment of early stage PD.

In conclusion, this study provides an extensive overview of the neuroprotective effects of riluzole in the early phase of neurodegeneration on motor behavior, motor function, sleep, and cell pathology. The current PD induction protocol and selected methods to evaluate the efficacy of the treatment underlines the therapeutic potency of riluzole on several aspects of early stage PD.

5

Striatal synaptic proteome in a mouse MPTP infusion model for early Parkinson's disease

P.S. Verhave, K.W. Li, R.E. van Kesteren, R.C. van der Schors, R.J. van der Loo, Y. Gouwenberg, W.D.J. van de Berg, D.M. Kimenai, I.H.C.H.M. Philippens and A.B. Smit

Manuscript submitted

Palsy, either consequent to compression of the brain, or dependent on partial exhaustion of the energy of that organ, may, when the palsied limbs become affected with tremulous motions, be confounded with this disease. (Parkinson, 1817)

5 Abstract

This study investigates the early molecular response of MPTP-challenged neurons in mouse *striatum*, thereby mimicking the early stages of neurodegeneration in Parkinson's disease (PD). We used a low-dose mini-pump infusion of the Parkinsonian toxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causing only a small, near-significant decrease in neurons in the *substantia nigra* after 1 week at a concentration of 80 mg/kg/day, but not at 20 mg/kg/day. MPTP infusions for 7 days at both concentrations did not cause any marked locomotor deficit. We used iTRAQ-based quantitative proteomics analysis to compare the molecular composition of striatal synaptosome fractions of MPTP-treated and riluzole-treated mice at 4 and 24 hours after the start of infusion. We found higher levels of mitochondrial proteins in 20 mg/kg MPTP-exposed mice not receiving riluzole treatment, only at 24 hours but not at 4 hours. This increase was observed for all mitochondrial proteins measured, reflecting either upregulation of mitochondrial activity or the recruitment of mitochondria into the synapse. Riluzole treatment prevented the MPTP-induced upregulation of mitochondrial proteins. These data not only show that riluzole acts benefically to the MPTP challenge, but they also indicate that the protective effects of riluzole are at least in part mediated at the level of synaptic mitochondrial activity or abundance.

5.1 Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder, affecting around 1.5% of Europeans over 65 years of age (von Campenhausen et al., 2005). It is foreseen that with the increasing lifetime expectancy the socio-economic burden of PD will increase (Dorsey et al., 2007). PD is characterized by selective degeneration of the dopamine (DA) neurons in the *substantia nigra* (Lang and Lozano, 1998). Despite intensive research efforts on dopaminergic neurodegeneration, the molecular mechanisms leading to PD are only partly understood, thereby preventing the design of effective treatment strategies for the disease. The early molecular and cellular stages of PD have been difficult to investigate in human post-mortem tissue as PD only displays clinical features when extensive cell death has already occurred. Therefore, molecular research of early stages of PD requires the usage of animal models.

The neurotoxic agent MPTP is well known for causing death of dopaminergic cells in the *substantia nigra* and consequently leading to PD in humans (Langston et al., 1983). Therefore, MPTP has been frequently used in animal studies to mimic aspects of the disease (Smeyne and Jackson-Lewis, 2005). Cell death caused by MPTP involves various processes, such as oxidative stress (Adams et al., 1993; Beal, 2003), inflammation (Beal, 2003) and glutamate toxicity (Robinson et al., 2003; Meredith et al., 2008), that are also associated with PD (Mandel et al., 2002). In order to counteract the PD-associated DA cell loss various potentially neuroprotective agents have been tested (Yacoubian and Standaert, 2009). One of these is riluzole, a compound shown to be neuroprotective in Amyotrophic Lateral Sclerosis (ALS) (Bensimon et al., 1994; Obinu et al., 2002) and in several cell, mouse and primate PD models (Bezard et al., 1998b; Boireau et al., 2000; Storch et al., 2000). Riluzole probably derives its neuroprotective properties via multiple direct and indirect routes that affect calcium transport (Lamanauskas and Nistri, 2008) as well as glutamate and GABA signaling events (Kretschmer et al., 1998).

Several studies have suggested that synaptic dysfunction may be an early causative process in neurodegeneration (Bagetta et al., 2010). Therefore synaptic proteins might be key targets for the development of novel diagnostic and therapeutic strategies in early PD. However, little is known about the molecular composition of nigrostriatal synapses in PD. In order to improve understanding of the early changes underlying PD, we investigated synaptic protein

levels in the *striatum*, directly affected by MPTP treatment in the normal and riluzole-treated synapse. MPTP injections are a widely used approach to model *substantia nigra* dopaminergic neuron degeneration, both in mice and in non-human primates (Jackson-Lewis et al., 1995; Jenner, 2003a). Here we exposed mice to a low-dose of MPTP using osmotic mini-pumps in order to mildly challenge DA neurons and closely mimic the early events in the progression of PD. This raises the opportunity to identify molecular mechanisms that might be present at the onset of neural degeneration rather than at the final stages of neuron loss. In addition, we combined MPTP exposure with riluzole treatment and investigated its protective properties. First, we investigated the effect of low-dose MPTP on neuronal survival and on the behavior of the animals. Subsequently, we evaluated the effect of riluzole on the protein expression levels in the MPTP challenged synaptosomes to identify processes involved in cell protection. Using iTRAQ labeling of proteins in different treatment groups, we determined condition-specific protein expression levels in striatal synaptosomes.

5.2 Material and Methods

Animals

Male C57Bl6 (Harlan, Horst, The Netherlands) mice, 8 weeks old at time of arrival, were housed individually in Macrolon cages under standard conditions with lights on from 7:00-19:00 h. All animals had a shelter, saw dust and extra paper bedding material. The experiments were approved by the Animal Users Care Committee of the Vrije Universiteit.

Drug Treatment

The MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride, Sigma Aldrich, St. Louis, USA) was dissolved in saline and stored at -20°C. Alzet minipumps (model 1004; Alza, Palo Alto, CA) containing saline or MPTP solution (releasing saline or MPTP in saline 20 or 80 mg/kg/day) were implanted during a short surgical procedure, under isoflurane anesthesia combined with the local anesthetic lidocaine. The pumps were implanted sc on the back of the mice through a small incision which was closed by a single suture. Pump insertion between 9 and 11 a.m. was considered to be t=0 and mice were subjected to either the 4 h, 24 h or 168 h protocol. An overview of the experimental setup is depicted in fig. 1. The riluzole (2-amino-6-trifluoromethoxybenzothiazole; Sigma Aldrich, St. Louis, USA) was stored at room temperature and was dissolved in 10% DMSO in saline at 10 mg/ml. Mice

were treated i.p. with riluzole dissolved in DMSO (10 mg/kg) solution, or DMSO solution alone at t=-0.5 h and for the mice in the longer exposure protocols also at t=11.5 h and t = 23.5 h. For the mice in the 168 h protocol the pumps were taken out by a short surgical procedure.

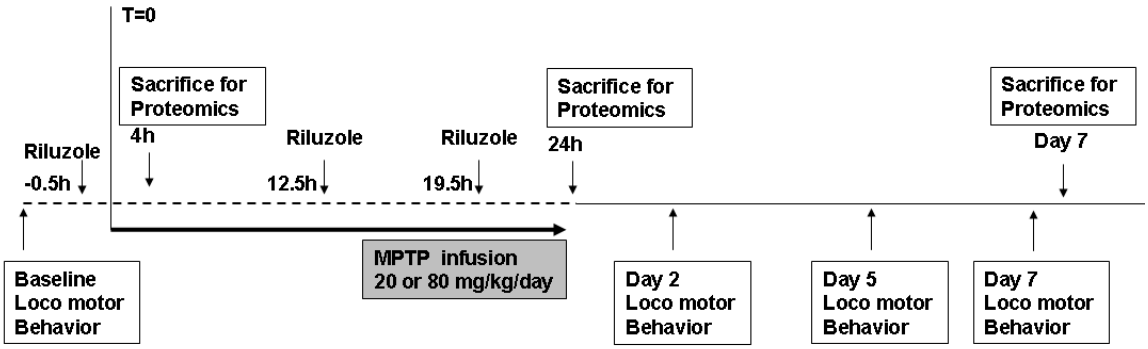

Figure 1. Schematic diagram depicting the experimental design. Mice receive either a saline or an MPTP filled mini-pump at t = 0. Mice receive either saline or riluzole injections at t=-0.5 h, t=12.5 h and 19.5 h. Protein expression was analyzed at t=4 h or t=24 h and neuron survival was analyzed at t=168 h. Locomotor behavior was tested at baseline, day 2, day 5 and day 7.

Behavioral analysis

Mice in the 168 h protocol were subjected to the behavioral tests at day 0 (before the surgical procedure) day 2, day 5 and day 7. Open field was used for measuring non-reinforced ambulant behavior or activity and rotarod was used as a measure for reinforced ambulant behavior.

Open-field: Activity 25x25 cm squared open arenas were used. Each animal was placed by hand into the corner of the arena and locomotion was recorded for 15 min. The arena was cleaned with a wet sponge after each run. A video tracking system placed 245 cm above the arena was used to register the locomotion and position of animals in the apparatus. Total distance moved in the 15 min trial was calculated per mouse.

Rotarod: Animals were placed on a stationary rod and were habituated to stay on it while the speed increased from 0 rpm to 40 rpm in 3 min. After habituation the average time spend on the rod was 60 sec. Five mice were trained and tested at each time point and in between trials the mice were left to rest for at least one minute. The mean time spent on the rotating rod during a 5-trial session was used to evaluate motor behavior.

Tissue preparation

At the designated time points, 4 h, 24 h, and 168 h, mice were quickly decapitated and brains were removed. Brains were either prepared for synaptosome isolation or immunocytochemistry and neuronal cell counts. For synaptosome isolation the *striatum* was dissected, rapidly frozen and stored at -80°C until further use.

Synaptosome preparation, protein isolation and labeling

Synaptosomes were isolated from *striatum* of individual animals as previously described (Li et al., 2007). In brief, samples were homogenized in 5 ml ice-cold 0.32 M sucrose and centrifuged at 1000 x g for 10 min. The supernatant was loaded on top of a sucrose gradient consisting of 0.8 M and 1.2 M sucrose. After centrifugation at 100.000 g for 2 h, the synaptosome fraction at the interface of 0.85/1.2 M sucrose was collected and protein concentrations were determined using a Bradford assay (BioRad). Of each sample 75 µg of protein was used for iTRAQ labeling, as described previously (Li et al., 2007). After drying overnight in a SpeedVac, proteins were resuspended in 28 µL of dissolution buffer and 2 µL of cleavage reagent (iTRAQ reagent kit, Applied Biosystems) with 0.85% RapiGest (Waters associates) for solubilization. After incubation for 1 h, 1 µL of cys blocking reagent was added and vortexed for 20 min. After that, trypsin (Promega) dissolved in 10 µL of water was added and incubated overnight at 37°C. The trypsinized peptides were then tagged with iTRAQ reagents dissolved in 85 µL of ethanol. The groups were labeled with iTRAQ reagents 113-121 Da. To obtain n=6, the iTRAQ experiment was repeated six times with independent samples from individual mice. After incubation for 3 h the samples were pooled and acidified with 10% trifluoroacetic acid to pH 2.5-3. After 1 h, the sample pools were centrifuged and again dried in the SpeedVac overnight.

Two-dimensional liquid chromatography

The sample pools were dissolved in 300 µl loading buffer (20% acetonitrile, 10 mM KH2PO4, pH 2.9) and loaded onto a polysulfoethyl A column (PolyLC). Peptides were then eluted with a linear gradient of 0-500 mM KCl in 20% acetonitrile, 10 mM KH2PO4, pH 2.9, over 25 min at a flow rate of 50 µl/min. Fractions were collected every minute. For the second dimensional separation, peptides were delivered with a FAMOS autosampler at 30 µl/min to a C18 trap column (1 mm x 300 µm i.d. column) and separated on a capillary

C18 column (150 mm x 100 μm i.d. column) at 400 nL/min using the LC-Packing Ultimate system. Peptides were separated using linearly increasing concentration of acetonitrile from 5-35% in 80 min, and to 45% in 7 min and finally to 90% in 2 min. The eluent was mixed with a matrix (7 mg α-cyano-hydroxycinnaminic acid in 1 ml 50% acetonitrile, 0.1% TFA, 10 mM dicitrate ammonium) and delivered at a flow rate of 1.5 μl/min and deposited off-line to the Applied Biosystems metal target every 15 s for a total of 384 spots using a robot (Dionex).

Mass spectrometry

MALDI plates were analyzed on a 4800 and a 5800 proteomics analyzer (AB Sciex). Peptide collision induced dissociation was performed at 1 kV with air as collision gas. MS/MS spectra were collected from 2500 laser shots. Peptides with signal to noise ratio above 50 at the MS mode were selected for MS/MS, at a maximum of 25 MS/MS per spot. The precursor mass window was set at a 200 relative resolution (FWHM).

Protein identification

MS/MS spectra were searched against the mouse database (Swissprot and NCBI) using GPS Explorer (AB Sciex) and Mascot (MatrixScience). After that a data list was generated containing all annotated peptides with a confidence interval score (C.I.) higher than 20%. Database redundancy and sequence redundancy were removed. Hence, quantification was performed only on those peptides that were annotated to a single protein, and are referred to as unique peptides. A protein was included for analysis if there were at least three unique peptides identified in each of the six experiments.

Protein quantification and annotation

The iTRAQ areas were extracted from the raw spectra and corrected for isotopic overlap using GPS explorer. The individual peak areas per sample of each iTRAQ signature peak were log2 transformed to obtain a normal distribution. After that the peaks were normalized to the mean peak area for each sample. Protein abundances in every age group were determined by taking the average iTRAQ peak area of all unique peptides annotated to a protein. Peptides with an iTRAQ spectrum larger than 10% of the average measured peak in all iTRAQ reagents were included. Proteins identified by mass spectrometry were annotated

according to protein function based on GO localization, Expasy information and/or literature.

Immunoblotting

Individual synaptosome samples (3 μg) were lysed in Laemmli buffer and loaded on 8-12% SDS-PAGE gels, depending on the size of the protein. Proteins were blotted onto a PVDF-membrane (Bio-Rad Laboratories, Hercules, CA). After blocking in TBS-Tween with 5% milkpowder and incubation of the first antibody (1:500-1:5000) in TBS-Tween with 0.5% milkpowder at 4°C the blot was washed and incubated for 1 h at RT with AP-conjugated secondary antibody (GE Healthcare, Diegem, Belgium, 1:10,000). Immunodetection was performed using the ECF immunoblotting detection system (GE Healthcare, Diegem, Belgium). For detection of fluorescence, blots were scanned with the FLA-5000 (Fuji Photo, GC corporation, Tokyo, Japan). Relative amounts of immunoreactivity were quantified using ImageJ (Bethesda, MD, USA). Protein loading was corrected by quantifying either the upper or lower half of the same gel stained with Coomassie Brilliant Blue.

Immunohistochemistry

For estimating the total number of neurons within the SNpc, the animals were sacrificed by decapitation one week after the start of MPTP-infusion and the brains were quickly removed from the skull and processed for immunohistochemistry and stereological analysis. The right hemisphere was dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h at 4°C and stored in 0.1 M PBS (pH 7.4) for a week. The tissue was cryoprotected using 30% sucrose in 0.5% paraformaldehyde at 4°C. The hemispheres were quickly frozen in isopentane at -40°C and stored at -80°C. The hemispheres were entirely cut into 40 μm-thick coronal free-floating sections using a cryostat and stored in 30% sucrose in PBS (PH 7.4) at -80°C. Every 8th section was processed for detection of tyrosine hydroxylase (TH) with immunohistochemistry. Sections were thoroughly washed with PBS and preincubated with 0.3% H2O2 in PBS to remove endogenous peroxidase activity. Thereafter, the sections were pre-incubated in PBS with 0.3% Triton X-100 (PBS-T) and 0.1% goat serum albumin. Sections were incubated with anti-TH antibody (1:10,000, Sigma Aldrich, St. Louis, USA) in PBS-T overnight at room temperature in a moist airtight environment. Then, the sections were washed three times in PBS for 10 min and

subsequently incubated with a biotinylated goat anti-mouse antibody (1:200, Santa Cruz Biotechnology Inc., CA, USA) 90 min. The sections were thoroughly washed using PBS every time the sections were placed in a solution-containing antibody. The ABC method (1:200, Vector Laboratories Inc., Burlingame, California, USA) for 90 min and 0.025% 3'3'-diaminobenzidine (DAB) with 0.00015% H2O2 for 10 min were used to visualize the peptides. After washing with TRIS-HCL, the sections were mounted on gelatine-coated slides and counter stained using Cresylviolet. Finally, the sections were dehydrated in alcohol series, cleared up in xylol and cover-slipped with DEPEX (Sigma Aldrich, St. Louis, USA).

Design based stereology

TH-IR neurons were counted in random but systematically sampled sections throughout the entire SNpc of eight mice per group using design-based stereology. The SNpc was delineated at low magnification (10x UplanApo objective) using the coordinates of the Paxinos atlas (2nd edition, 2001) using a computer-assisted morphometry system consisting of a Zeiss Axioplan photomicroscope with a CCD color video camera and a motorized stage controller for automatic sampling equipped with the StereoInvestigator software version 8 (MicroBrightfield Inc., Colchester, VT). A LEP XY motorized stage controller controlled the movements along the x- and y-axes, and a Heidenhain MT12 microcator attached to the stage measured the precise focal depth, with a resolution of ~0.5 μm. Estimates of the total number of TH-IR neurons were made using the Optical Fractionator method with an x40 objective. To prevent experimenter bias, all sections were coded. TH-IR neurons were counted if the nucleus came into focus within the counting frame. Analysis was performed starting with the first anterior appearance of TH-IR neurons (Bregma -2.5) and extending to the most caudal parts of the SNpc (Bregma -4). The following sampling parameters were used: ssf = 1/8, the height of the dissector = 8 μm, the guard height = 2 μm, the counting frame area = 2500 μm2 and asf = 0.444, as defined by the counting frame area multiplied by the reciprocal of the grid size (75x75 μm).

Data analyses and Statistics

iTRAQ-based proteomics was performed in six experiments using independent biological samples and analysis was done using the package Limma for the R computing environment (Smyth, 2004). Limma is part of the Bioconductor project at http://www.bioconductor.org

(Dudoit et al., 2003). Challenge, treatment, time and their interactions were included in the model. All the proteins that were affected by MPTP challenge, treatment or the interaction of the two or with time were used for post-hoc analyses. The false discovery rate (FDR) was calculated using the QVALUE package (Storey and Tibshirani, 2003) in R, with the list of p-values for the comparison between the treated samples and the saline samples 113 for the 4 hour time point and 117 for the 24 hour time point. After checking for normality, dopaminergic cell counts and immunoblotting data were analyzed with an ANOVA analysis with group and time as factors (SPSS). Separately the mitochondrial data was analyzed using a MANOVA with time and treatment as factors. The behavioral data was analyzed using a repeated measures ANOVA with treatment and MPTP challenge as factors. In case of statistically significant main effects, follow-up ANOVA's and if appropriate further post-hoc comparisons were conducted using Bonferroni post-hoc test. The level of probability for statistical significance was set at 0.05.

5.3 Results

We administered a low dose of MPTP to mice using a recently described osmotic mini-pump infusion protocol (Fornai et al., 2005), and we investigated the direct effects of this compound, in the presence or absence of riluzole, on neuronal survival, mouse behavior, and protein expression in striatal synapses.

Estimates of the total number of neurons SNpc

The design-based stereological estimates of the TH-IR neurons were determined in the SNpc of 6-8 mice per group (fig. 2). The mean (± SEM) estimated thickness of the sections after histological preparations was 10.9 ± 0.48. The average number of counted profiles per animal was 255 ± 77. We counted sections with the necessary less than 10% variability around the mean (Schmitz and Hof, 2005); this average predicted coefficient of error of the estimated total number of neurons CEpred was 0.063. The total unilateral number of TH-IR neurons in the SNpc from the systematically sampled sections was 7373 ± 699 in saline treated mice (n=8). A two-way ANOVA revealed a significant MPTP effect in the number of TH-IR neurons. Post-hoc analyses showed that in the groups without riluzole treatment, the 80 mg/kg MPTP infusion (n=8) led to a near significant decrease of the estimated total number of TH-IR neurons in comparison to saline treated mice (5133 ± 704 vs 7373 ± 699;

p = 0.053). No significant effects were found in the 20 mg/kg MPTP-infused mice (5876 ± 703 vs 7373 ± 699), and no significant effects of riluzole were found in the 80 mg/kg MPTP-infused group (5596 ± 557; n=7) and in the group infused with 20 mg/kg MPTP (7164 ± 697; n=8).

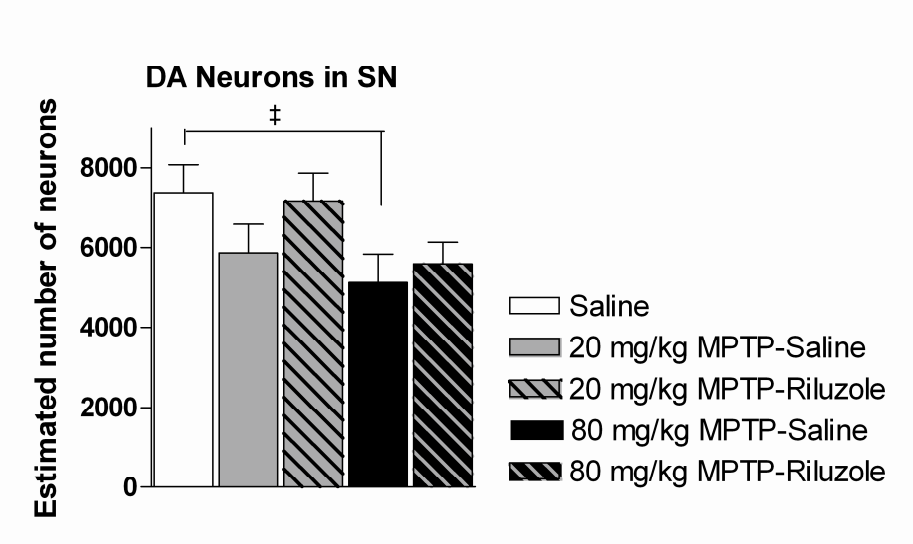

Figure 2. Dopaminergic cell counts in *substantia nigra*. Bars indicate cell numbers in the saline treated mice and in 20 or 80 mg/kg MPTP infused mice. Each bar represents mean + SEM of the saline group (white bar), MPTP treated groups (grey and black bars) and the riluzole treated groups (striped bars). Symbol ‡ indicates difference, ANOVA followed by Bonferroni corrected t-tests (‡ p = 0.053).

Locomotor behavior and activity

Mice were subjected to an open field test, i.e., a non-reinforced ambulant test setup and to the accelerating rotarod test, i.e., a reinforced ambulant test in order to test various aspects of locomotor behavior. Testing was performed just before the mini-pump insertion, and 2, 5 and 7 days after the pump insertion. The MPTP-treated groups of both 20 mg/kg and 80 mg/kg did not show any significant changes in both tests. Moreover, in both the riluzole- and the non-treated group, activity as measured in the open field paradigm was similar to that of saline-treated animals (fig. 3). The 80 mg/kg MPTP-infused mice showed a slight hyperactivity towards the end of the experiment at day 5 and day 7 as compared to the 20 mg/kg/day MPTP-infused saline and riluzole groups. This effect on spontaneous movements in the 80 mg/kg MPTP-infused mice has been described previously, in

particular when more chronic and high accumulative dose regimens were used (Luchtman et al., 2009). Rotarod performance was not significantly affected in any of the treatment conditions.

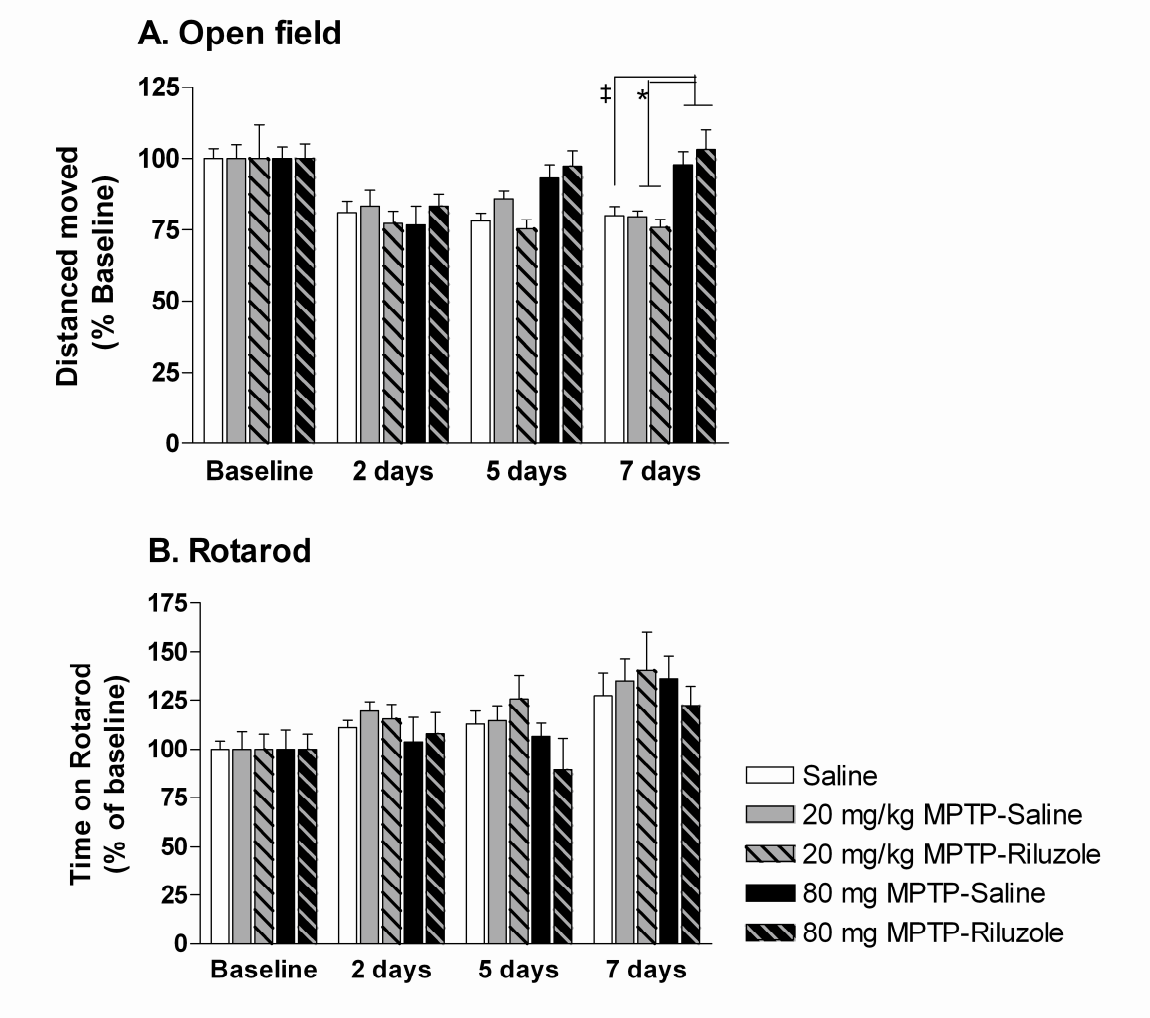

Figure 3. Behavioral performance of mice. A.Open field and B. Rotarod. Behavioral parameters measured in saline-treated mice and in 20 or 80 mg/kg MPTP infused mice. The latter two groups also combined with either saline or riluzole treatment. Each bar represents mean + SEM of the saline group (white bar), MPTP treated groups (grey and black bars) and the riluzole treated groups (striped bars). Star and symbol ‡ indicate differences, repeated measures ANOVA followed by One way anova per time point followed by Bonferroni corrected t-tests (‡ $p = 0.082$, * $p < 0.05$).

Quantitative Proteomics

Using mass spectrometric analysis, we identified 10,408 unique peptides in all MPTP infusion and riluzole iTRAQ experiments together. The protein abundances in every group were determined by taking the average normalized standardized iTRAQ peak area of all unique peptides annotated to a protein. Peptides with peak intensities larger than the 10% of the average intensities of all iTRAQ peaks were included in the analysis. On average this yielded 6500 peptides per experiment. These peptides were assigned with a confidence level of at least 95% to 909 unique proteins. In this group of 909 proteins, 404 fulfilled the criterion of the presence of 3 peptides or more identified in each experiment and were used for quantification. All quantified proteins were grouped in functional classes as previously described (Counotte et al., 2010). By far the largest functional group measured were the mitochondrial proteins (27%); other annotated proteins were assigned to groups of intracellular signal transduction (11%), structural plasticity (9.9%), protein synthesis/folding/breakdown (7.7%), cell metabolism (6.7%), excitotoxicity (6.4%) and ion balance (5.7%). An overview of all annotated proteins is depicted in table 1.

Table 1. Number of proteins per functional group

Functional protein Group	Number of proteins	Represented in data set (%)
Mitochondria	109	27.0
Intracellular signal transduction	46	11.4
Structural plasticity	40	9.9
Protein synthesis/folding/breakdown	31	7.7
Cell metabolism	27	6.7
Exocytosis	26	6.4
Ion balance/transport	23	5.7
Unknown	19	4.7
Cell adhesion/transsynaptic signaling	17	4.2
Endocytosis	17	4.2
Protein clustering	13	3.2
G-protein relay	11	2.7
Intracellular trafficking	10	2.5
Neurotransmitter metabolism	7	1.7
Peptide/neurotrophin signaling	3	0.7
GPCR signaling	2	0.5
LGIC signaling	2	0.5
Excitability	1	0.2
Tyrosine kinase signaling	0	0.0

All proteins analyzed given the set criteria per functional group. Proteins were assigned to one of the 18 classes based on functional description. Proteins with unknown functional annotation were assembled in the unknown group. Percentages indicate group size of total proteins.

Analysis of expression changes

All 404 proteins identified were used for multiple eBayes expression analysis. Because of our interest in the differential response in protein expression in MPTP-infused and riluzole-treated versus MPTP-infused only mice, we focused on protein levels in the MPTP and riluzole conditions, and their interaction. Additionally, we focused on the factor time, i.e., 4 h of infusion vs 24 h of infusion. This analysis yielded 62 proteins, which were differentially expressed by the factors treatment, MPTP challange or time. Direct comparison of the treatment groups to the corresponding saline control group revealed significant differences only at 4 hours in the MPTP-infused and the riluzole-treated MPTP-infused groups. Post-hoc analysis showed that within the 4 hours time point only 4 proteins were significantly regulated as compared to saline in the MPTP group. This relatively small effect of acute MPTP confirms our observations that the low-dose MPTP treatment did not cause any neuronal loss or locomotor deficit. Riluzole alone caused no significant protein expression changes. In contrast, 45 proteins were significantly affected in the riluzole-treated MPTP-infused group. This implicates a substantial acute interaction and more profound effect of MPTP and riluzole. Although the analysis showed this acute effect at 4 h, this effect could not be confirmed with immunoblotting. Additionally, this effect was subsided within 1 day, and does not allow drawing conclusions about the effect of MPTP or riluzole on synapse function.

A toxic challange that affects synapse function is expected to initiate processes which affect groups of proteins embedded in specific biological pathways. Whereas the fold change of individual proteins may be too low to be distinguished significantly, as is the case in the present study, functional group analysis might reveal regulation at the system level. We therefore used functional clustering as defined in table 1, and performed statistical analysis at a cluster level between MPTP with or without riluzole treatment and control animals. When analyzed as a cluster, mitochondrial proteins after 20 mg/kg MPTP infusion at 24 h were increased. The average fold change of the cluster is small, with an upregulation of 10% compared to the saline control group. ANOVA followed by Bonferroni-corrected t-tests taking into account all mitochondrial proteins showed that the small expression increase in MPTP infused mice in comparison to the riluzole-treated and the MPTP-infused plus riluzole protected mice is highly significant ($p < 0.001$) (fig. 4A). Mitochondrial proteins can be further divided into functional subgroups (Cotter et al., 2004). Most mitochondrial

proteins we identified belong to the inner membrane proteins (53%). The matrix is together with the non-localized mitochondrial proteins the second group (18% each), and the outer membrane and cytosolic proteins represent respectively 7% and 4% of the total mitochondrial protein pool. The large group of mitochondrial inner membrane proteins contains the electron transport chain proteins, which include complex I-IV. Increase in abundance due to MPTP treatment was observed equally for all classes of mitochondrial proteins (inner membrane, outer membrane and matrix (fig. 4B-D), indicating a protection by riluzole on the overall changes in mitochondrial activity or abundance induced by MPTP at 24 hours after the start of MPTP infusion.

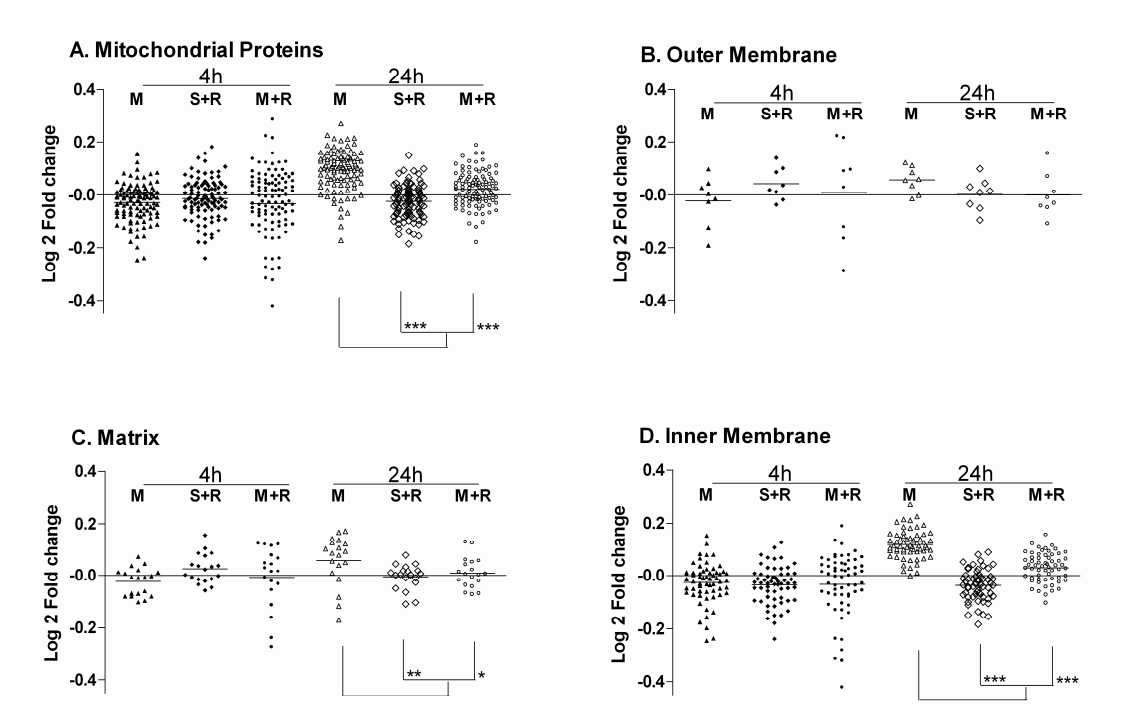

Figure 4. Normalized expression level (Log2) of mitochondrial proteins. A. All mitochondrial proteins, B. Outer Membrane proteins, C. Matrix proteins and D. Inner Membrane proteins (including complex I-V). M indicates MPTP-infused mice R indicates riluzole-treated mice and S indicates saline-infused mice. Stars indicate differences, repeated measures ANOVA followed by One way ANOVA per time point followed by Bonferroni corrected t-tests (*** $p < 0.001$, ** $p < 0.001$ and * $p < 0.05$).

Immunoblotting validation of iTRAQ regulated proteins

The validation of protein regulation with low-fold change is challenging. To independently confirm the regulation of mitochondria in MPTP and riluzole-treated animals, we performed quantitative immunoblotting analysis of the mitochondrial marker NDUFA6 (fig. 5). The immunoblot revealed a similar pattern of regulation as observed in the proteomics data across the treatments and time. Due to the small differences however, these changes were not statistically significant. As a control, to show that there are no changes in overall synapse size or numbers in the different treatment conditions, we also quantified the abundance of PSD-95 in striatal synaptosomes using immunoblotting. PSD-95 is a scaffolding protein in the postsynaptic density whose abundance in synaptosome is a good measure for changes in overall synapse size or number. In accordance with our proteomics data, no regulation of PSD-95 was observed in any of the experimental conditions (fig. 6).

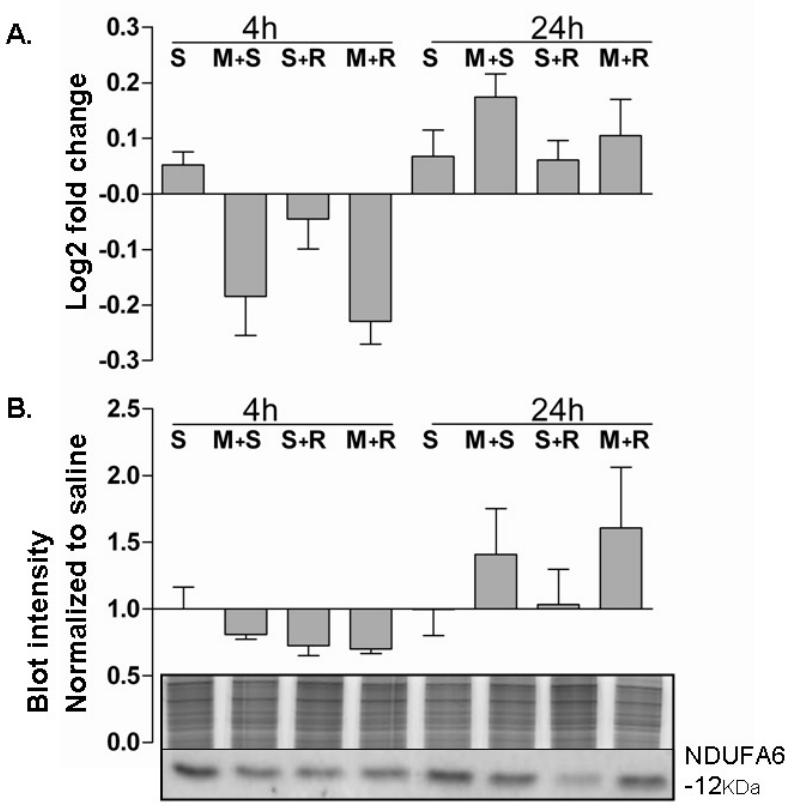

Figure 5. Expression data of NDUFA6 in the striatal synaptosome fraction. A. iTRAQ expression data (n=8; mean of log2 fold change ± SEM) B. Immunoblotting intensity normalized to 4 h or 24 h saline data (n=8; mean ± SEM) with representative image of an immunoblot and Coomassie stained gel.

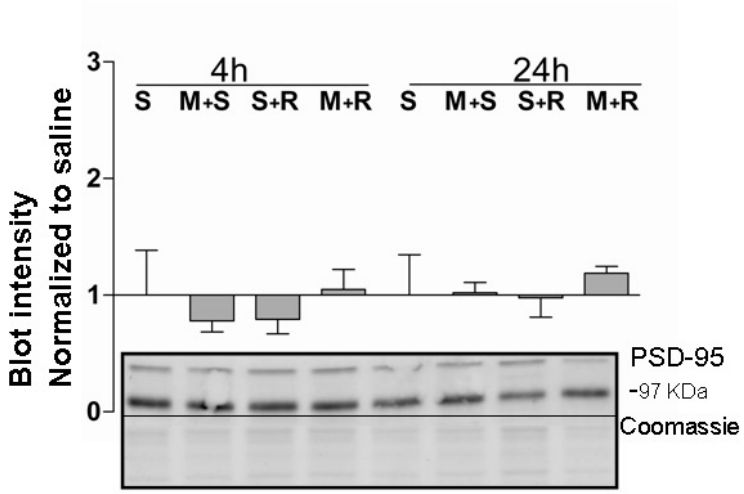

Figure 6. Expression data of PSD-95 in the striatal synaptosome fraction. A. Itraq expression data (n=7; mean of log2 fold change ± SEM) B. Immunoblotting intensity normalized to 4h or 24h saline data (n=6; mean ± SEM) with representative image of an immunoblot and coomassie stained gel.

5.4 Discussion

Changes in synaptic protein expression may be present at the onset of PD (Bagetta et al., 2010) but will be difficult to measure in the brains of human patients as these stages cannot yet be diagnosed in PD. Therefore we investigated changes in synaptic protein abundance in mice treated with MPTP, either with or without co-treatment of the neuroprotective agent riluzole. To mimic the early stages of neurodegeneration as closely as possible, we first evaluated the effect of the MPTP infusion on cell survival a week after the infusion, and motor behavior up to a week after the infusion.

Instead of peak dosing using i.p. injections we chose for the slow release of a low dose of 20 mg/kg MPTP from an osmotic mini-pump. This did not lead to any significant alterations in numbers of TH positive dopaminergic neurons or to behavioral abnormalities, as was previously observed with high doses acute MPTP protocols (Jackson-Lewis et al., 1995; Bezard et al., 1997). We found that a higher 80 mg/kg/day dose of MPTP, led to a significant decrease in neurons and to hyperactivity in the open field. Hyperactivity might be explained by decreased DA levels and increased DA turnover in the medial and anterior frontal cortex (Rousselet et al., 2003), or by hyperactivity of DA neurons as observed in early

stages of PD in humans and in animal models of PD (Bezard et al., 1998a). As such, the 20 mg/kg/day MPTP infusion protocol might provide a good model for the molecular responses during the early phase of PD, before the clinical symptoms arise, and is a preferred model for the initial challenge of the dopaminergic neurons without leading to immediate cell death. With this minimal MPTP challenge, no significant effects of riluzole were found on cell counts or on mouse behavior.

Effects of the MPTP challenge

Several studies have reported changes in total cellular protein or gene expression after single or multiple MPTP injections (Kuhn et al., 2003; Mandel et al., 2003; Jin et al., 2005). However the specific molecular mechanisms underlying the selective death of dopaminergic neurons are not well defined. Mitochondrial complex I dysfunction has been named as a key component in this process (Schapira et al., 1989; Abou-Sleiman et al., 2006; Perier et al., 2007). Recent studies however show alternative mechanisms. Deletion of a key complex I gene, which led to loss of complex I activity, had no effect on MPP+-induced dopaminergic neuron loss, suggesting that mitochondrial dysfunction is not primarily responsible for MPP+ toxicity within dopaminergic neurons (Choi et al., 2008). Our data show an MPTP-induced induction of mitochondrial protein levels. This is observed for proteins in all 5 complexes of the electron transport chain residing in the inner membrane, as well as for all other mitochondrial proteins. Similarly, a 24-hour incubation with MPP+ resulted in an accumulation of mitochondria in neurites of PC12 cells (Cartelli et al., 2010). In contrast however, MPP+ induced ATP depletion in dopaminergic neuroblastoma SH-SY5Y cell bodies (Storch et al., 2000), suggesting a reduction in mitochondrial activity or abundance. Taking together, our data and that of Cartelli and Storch might suggest a synaptic increase of mitochondrial activity which leads to a translocation of the mitochondria from the cell bodies via the axons into the synapse, and thus enrichment of mitochondria in synaptosomes isolated at 24 hours after the start of MPTP infusion.

Riluzole action and interaction

In vivo, riluzole does not interfere with MPTP metabolism, DA uptake (Samuel et al., 1992; Boireau et al., 1994a), or MAO-B activity (Boireau et al., 1994b). Central in all reported neuroprotective action of riluzole are its inhibitory effects on glutamate signaling, specifically

in active synapses. For instance, riluzole blocks glutamate release, but only when mEPSC (miniature excitatory postsynaptic current) frequencies are elevated (Lamanauskas and Nistri, 2008). Riluzole acts on glutamate release by affecting the presynaptic calcium influx (Wang et al., 2004) and postsynaptic membrane depolarization (Centonze et al., 1998). We could not find any significant changes in synaptic proteins that are related to glutamate release or glutamate transmission, suggesting that these earlier reported effects of riluzole are acute on pre-existing proteins, but do not change long-term molecular composition (and function) of the synapse. Instead, we do find that riluzole prevents the increase in mitochondrial proteins in the synapse, or the increase in mitochondria, induced by MPTP. In line with these findings, riluzole was previously shown to increase ATP availability in neuroblastoma cells (Storch et al., 2000). Taken together, this suggests a direct effect of riluzole treatment on mitochondrial toxicity induced by MPTP. This effect is distinct from the previously described effects of riluzole on glutamate signaling.

In conclusion, the low dose 20 mg/kg MPTP challenge used in this study did not affect the number of neurons and it did not lead to behavioral abnormalities. The 80 mg/kg dose of MPTP, however, showed a trend towards neuronal cell death and behavioral hyperactivity. At the protein level, slow release of 20 mg/kg MPTP did not show evidence for a specific mitochondrial complex I dysfunction. In contrast, we found evidence for an increase in the majority of mitochondrial proteins measured, or alternatively, mitochondrial organelle abundance, in the synapse. Riluzole treatment prevented the increased levels of mitochondrial proteins induced by MPTP. Thus our findings suggest that in a low dose MPTP challenge model of early PD, induction of mitochondrial abundance in the synapse is the primary response to low level neurodegenerative intoxication, and riluzole can prevent this induction.

Acknowledgements

A.B. Smit, K.W. Li and R.J. van der Schors received funding from the Center of Medical Systems Biology (CMSB). A.B. Smit and R.E. van Kesteren received funding from Top Institute-Pharma, project T5-207.

6

Discussion and concluding remarks

P.S. Verhave and I.H.C.H.M. Philippens

To such researches the healing art is already much indebted for the enlargement of its powers of lessening the evils of suffering humanity. Little is the public aware of the obligations it owes to those who, led by professional ardour, and the dictates of duty, have devoted themselves to these pursuits, under circumstances most unpleasant and forbidding. (Parkinson, 1817)

6 Parkinson's disease

Almost 200 years after the initial description of PD (Parkinson, 1817) and despite useful experimental insights, neither the cause of sporadic PD, nor a preventive method, nor an acceptable complete and lasting symptom treatment has been found to relieve PD pathalogy. The slow onset of the disease together with the variability in early and late symptoms makes clinical research a real challenge. Therefore, this thesis is focused on generating a new perspective in the changes that during the early phase of PD using new read-outs for animal models of PD, at the behavioral, physiological, pathological, biochemical and molecular level. We chose to work in MPTP-treated mice and marmosets with an open eye towards clinical validity. New behavioral measures were developed using the marmoset's natural behavior in a translational manner. Sleep parameters were measured to investigate the translational properties of the marmoset MPTP model for sleep disturbances as they occur in PD patients. The mouse MPTP infusion model was used to investigate the affected molecular pathways in the synapses of challenged DA neurons. Finally, riluzole was investigated in order to evaluate its early neuroprotective properties on DA neurons in the SN of both models. Here the results will be discussed and put into a clinical perspective.

6.1 Modeling Parkinson's disease with MPTP

The translation of fundamental research data into the clinic relies in the first place on appropriate animal models that show the pathological hallmarks and motor deficits of PD. It is of vital importance that these animal models actually mimic the clinical features of PD to an extent that makes the outcome relevant. This can be determined by evaluating the marmoset MPTP model and the mouse MPTP infusion model using the scientific-based criteria: face, predictive, construct and external validity offered by Van der Staay and et al. (2009).

Face validity: Based on literature and our own findings we can conclude that both the marmoset and the mouse models offer face validity in the sense that homologous neuro-anatomic structures are affected by MPTP like in PD, namely the *basal ganglia* (Jenner et al., 1984; Meredith et al., 2008), This results in neurodegeneration in the SN and accordingly reduced levels of DA in the *striatum*. The size of the lesion predicts the symptomatic

phenotype of the animals (van Vliet et al., 2008b). Therefore, a small lesion might explain that the Parkinsonism was not very pronounced (chapter 4). Limited symptomathology in combination with the restricted damage of the DA neurons in the SN suggests that this model mimics an early stage PD patient (Tolosa et al., 2009).

Predictive validity: Concerning the DA replacement therapy, predictive validity is certainly true for the marmoset models (Jenner, 2003b), and also for the mouse injection model (Kirchhoff et al., 2009) and the mouse infusion model (Fornai et al., 2005). However, for neuroprotection these models have not yet been very predictive. Neuroprotectiva showing promissing results in the lab, still have not led to succesful systematic treatment in patients (Olanow et al., 2008). This is mainly due to some shortcomings of the animal models currently in use, the unknown cause of the disease and the difficulty to accurately estimate the degree of neuroprotection in patients.

Construct validity: Because the nature of the disorder is not well understood, construct validity is the most difficult scientific criterion in modeling idiopathic PD. Therefore, animal models can only mimic the pathology and the symptomatology of the disease but not the etiology. However, the use of MPTP may be one of the few neurotoxic cases in which the neurodegeneration was first discovered in humans, whereby in human the presented symptoms were indistinguishable from those of idiopathic PD patients (Ballard et al., 1985). In both mouse and marmoset models, motor disabilities can be evaluated. In the mouse MPTP injection models (Petroske et al., 2001; Schmidt and Ferger, 2001; Mori et al., 2005) and also in the infusion model (Fornai et al., 2005), basic locomotor behaviors were measured. The MPTP-treated marmoset, however, is superior with regard to behavioral assessment, as it shows a complete range of behavioral characteristics associated with PD (Eslamboli, 2005; van Vliet et al., 2006; Jenner, 2009). In the present study the available behavioral testing methods were extended with two new test systems for motor impairment (chapter 2). Additionally, the results regarding non-motor symptoms such as sleep impairments (chapter 3), bring this model even closer to human PD. Therefore, the striking behavioral similarities between PD patients and MPTP-induced Parkinsonism in non-human primates (chapter 2 and 3) strongly points to an optimal construct validity. Nevertheless, MPTP-induced features diminish fast in mice and slow in non-human primates, while there is no report of symptom reduction over time not in the few human cases of MPTP use nor in actual PD patients.

External validity: Since both, the non-human primate and the mouse injection model, are very popular in preclinical research, a substantial number of reports appeared about the degeneration of DA neurons in combination with motor behavioral deficits (Speciale, 2002; Meredith et al., 2008; Philippens et al., 2010). The reproducibility of the mouse infusion model is, however, still disputed (Fornai et al., 2005; Alvarez-Fischer et al., 2008).
In the end, it is the scientific question that should be a predominant factor in deciding of which specific animal model to choose. In that sense, the marmoset model, with similar behavioral signs as in PD patients, has been chosen to investigate pathology combined with symptomatology in chapter 4 and the mouse infusion model has been chosen to examine the molecular changes of the neurodegeneration, at a stage in which assessment of the specific PD symptomatology was not the main goal in chapter 5. Both models offer a good opportunity to investigate neurodegeneration at different pathological stages and the impact of environmental factors on the process of degeneration.

6.2 Behavioral aspects in the MPTP models

6.2.1 Marmoset

The MPTP-treated marmoset was first proposed as a model for PD by Jenner and colleagues (Jenner et al., 1984). Since then the Parkinsonian behaviors reported for the marmoset, range from the typical behaviors like bradykinesia and tremors at rest, to dyskinesia and hallucinations after L-DOPA use. As presented in chapter 1, PD is diagnosed when patients display two typical Parkinsonian signs, one being bradykinesia, the other being either tremor, rigidity, akinesia, or postural instability. In the marmoset MPTP model all key motor symptoms of PD can be recognized to a certain extent. Increased activity of GPi neurons inhibit thalamocortical circuits, thereby reducing the cortical activation and thus leading to bradykinesia in PD (DeLong, 1990). This explains the slowness of movement in the locomotor tests, such as the Tower and the Bungalow tests. Indeed, shortly after the first MPTP exposure marmosets show bradykinesia in both tests. In the Tower- and the Bungalow tests the marmosets make significantly less transfers to other compartments or levels (chapter 4). Additionally using the Hand-eye coordination test it was found that directly after an MPTP challenge (chapter 3 and 4) the marmosets show disturbed hand-eye functionality. The marmosets take fewer fast moving rewards in the Hand-eye coordination

test (data not shown). As such, they displayed bradykinesia in this functional task. However, the hand-eye coordination task of the MPTP-treated marmosets recovered fast and the performance improves to almost normal levels. Because of this finding and also in comparison to other studies (van Vliet et al., 2006; van Vliet et al., 2008a), marmosets treated with only a mild PD induction protocol, did not show severe bradykinesia in this functional task.

The cause of tremor, rigidity and akinesia in PD can be traced back to increased firing rates and exaggerated responsiveness of neurons in the internal *globus pallidus* (GPi), due to a reduced input from the *striatum* (Filion et al., 1988; Miller and DeLong, 1988). The MPTP-treated marmosets also have this reduced input and scored mildly on tremors at rest and rigidity from of the time of treatment until the end of the experiment indicating a mild induction of PD (rigidity and tremors are scored as part of the clinical score).

In the Hourglass test the time is measured it takes the marmosets to turn, in order to restore their upright position. However, immediately after the challenge with MPTP we observed that some marmosets did not attempt to move at all while none of the control marmosets were akinetic in the Hourglass test-setup.

Postural instability is hard to quantify, we did observe postural instability in the home cage, in the Hourglass test and in the Tower test. Postural instability is suggested to be caused by non-dopaminergic extra-nigral lesions (Poewe and Mahlknecht, 2009). However, involvement of the *basal ganglia* is suggested by the long-term improvement of postural instability after a pallidotomy in PD patients (Gross et al., 1999).

Although the combination of scoring and behavioral test setups for marmosets used in this thesis covers clinically relevant parameters, direct translation to a clinical situation remains a challenge because of: 1) the semi-chronic induction protocol with MPTP whereas PD development is a slow process lasting several years 2) the natural recovery of the marmosets in the MPTP model (chapter 2 and 4) (Boulet et al., 2008), and 3) the fact that non-motor pathways can positively affect animal motor behavior due to anticipation of getting a reward as found in the Tower and Hand-eye coordination tests (de la Fuente-Fernandez et al., 2002). Nonetheless, it is concluded that the MPTP-induced pathology can underlie all the hallmarks of PD and the MPTP-treated marmosets do show some of the marked motor symptoms.

6.2.2 Mice

Because mice show a relatively restricted PD-like symptomatology (Luchtman et al., 2009) this model is mainly of interest for neuropathology and investigating the molecular and cellular changes coinciding neurodegeneration of the DA neurons. The most prevalent behavioral measures in mice are spontaneous locomotor behavior (Luchtman et al., 2009) and forced locomotor behavior (Diguet et al., 2005). These frequently used motor-behavior tests can distinguish only full body motor-behaviors that recover very fast after the MPTP challenge (Schmidt and Ferger, 2001). This might be due to the plasticity of the TH-positive neurons in the *striatum* (Sager et al., 2010), as they show potential for spontaneous regenerative sprouting (Mitsumoto et al., 1998).

A lesion of at least 56%, induces behavioral problems in mice (Kirchhoff et al., 2009). The relatively small MPTP-induced lesion, as showed in chapter 5, in line with literature did not show impairment in motor behaviors. Therefore, it is concluded that this mouse MPTP model is not suited for extensive research into clinically-based symptomatology.

6.3 Sleep in the marmoset MPTP model

The non-motor symptoms of PD have only recently started to become of interest (Park and Stacy, 2009) to the scientific community. Studying non-motor parameters in animal models for PD is a very new field of research. And non-motor parameters are generally not part of the symptom description of an animal PD model. However, there are reports on olfaction problems in the MPTP-treated marmoset (Miwa et al., 2004), and several studies on sleep disturbances in non-human primates (Almirall et al., 1999; Barraud et al., 2009), as well as on constipation (Anderson et al., 2007). We studied sleep disturbances in this early phase model for PD (chapter 2) and concluded that the MPTP-treated marmosets provide a new opportunity for quantitative studies on the mechanisms and intervention possibilities for RBD-like sleep disturbances. RBD relates to the involvement of non-dopaminergic *medullary* and *pontine* structures relevant for REM sleep (Iranzo et al., 2005), suggesting that RBD is not only dependent on DA neurodegeneration. However, the fact that these structures are closely related to the SN pathways (Lai and Siegel, 1990) and that the SNpc is involved in sleep (Dzirasa et al., 2006), specifically REM sleep (Lima et al., 2007), suggests that DA

neurodegeneration induced by MPTP, caused the RBD-like phenomenon in the marmosets. Thus, this parameter significantly adds value to the validity of this preclinical model of PD.

6.4 Molecular events after an MPTP challenge

There are several issues to consider when discussing the effects of MPTP on experimental animals. Firstly, the specific mechanisms underlying the selective DA neuron degeneration are not completely defined, although mitochondrial complex I dysfunction, excitotoxicity, oxidative stress, protein dysfunction and inflammation are pointed out as key processes in PD (chapter 1). Secondly, since MPTP is administered by repeated injections or during a certain period of time by infusion, the MPTP induction of PD is generally considered to be not progressive, whereas in PD patients slow progression of the disease has been indicated as a key issue in the neurodegeneration process (Schapira et al., 1989; Abou-Sleiman et al., 2006; Perier et al., 2007).

Concerning the first remark, our data suggest a key role for mitochondria in the early events caused by MPTP. A marked increase in protein levels of all 5 complexes of the electron transport chain located in the inner membrane, is present in these data set. Also, we found a general upregulation of other mitochondrial proteins. In line with this general change of mitochondrial proteins, recent studies discart the traditional dysfunction theory of the involvement of only mitochondrial complex I to the DA neuordegeneration (Choi et al., 2008). Deletion of a key complex I gene led to loss of complex I activity. However, this deletion had no effect on MPP^+-induced DA neuron loss, suggesting that the mitochondrial dysfunction is not the main mechanism responsible for MPP^+ toxicity within DA neurons (Choi et al., 2008). Because 24 hours after incubation with MPP^+, an accumulation of mitochondria in the axons was found in PC12 cells (Cartelli et al., 2010), an upregulation of the mitochondria in the synaptosomes at 24 hours after the start of MPTP infusion might be expected. This increase in the number of mitochondria will lead to an increased level of mitochondrial proteins and can be a direct response to the energy demanding processes caused by the chronic low levels of MPTP infusion (Gu et al., 2003).

6.5 Riluzole

6.5.1 Riluzole effects in mice

The 80 mg/kg MPTP infusion challenge in mice did affect the number of neurons in mice (chapter 5), but did not lead to motor dysfunction in the mice. It did, however cause hyperactivity in the Open field test. At the protein expression level, the slow release of 20 mg/kg MPTP, did not show proteins dysregulated specifically for complex I. In contrast, several mitochondrial proteins were upregulated, which might be linked to an increased energy demand of the synapse. Moreover, the neuroprotective treatment with riluzole did not induce an overall upregulation of mitochondrial proteins.

The mechanisms underlying normalization of the cellular response by riluzole are diverse. The anti-excitotoxic nature of riluzole reduces the amount of calcium influx in the synapse (Cousins et al., 2008). This is achieved via a blockage of the calcium or ion transport which in turn affects glutamate and GABA levels in the synapse. Because the nerve cell energy demands are too large for maintaining a ion gradient across the cell membrane (up to 70% of cellular ATP) (Pedersen, 2007), specifically the decrease in ion transport across ion channels might cause a decrease in energy demand of the terminal. This might explain the reduction of mitochondria abundance and/or the mitochondrial activity in this early stage PD model. However this subtle challenge did not lead to an actual change in proteins directly involved in the regulation of ion balance at synapse level in the *striatum*.

6.5.2 Functional neuroprotection with riluzole

A neuroprotective therapy, such as riluzole, can be very promising when started at a phase of the disease at which there are still enough neurons left to be protected. Therefore essential for clinical treatment with e.g. riluzole is an accurate early diagnosis of PD. In preclinical studies riluzole appeared to be effective when commenced simultaneously with the MPTP treatment (Araki et al., 2001; Obinu et al., 2002). Riluzole does not interfere with MPTP metabolism and DA uptake (Samuel et al., 1992; Boireau et al., 1994b), and it does not diminish the MAO-B activity (Boireau et al., 1994b), it also does not interfere with the Parkinson induction by MPTP. This implies that the neuroprotective efficacy of riluzole is not with the interference of MPTP toxicity. The clinical score, the abnormal involuntary movements, the hand-eye coordination, the turning ability, and the activity in the Tower were less affected in the MPTP-riluzole-treated Parkinsonian marmosets than in the MPTP

marmosets not treated with riluzole. On the other hand, spontaneous behaviors, such as top level in the Tower test and the activity in the Bungalow, were not positively affected by riluzole treatment. It is therefore concluded that treatment with riluzole realizes an improvement but not complete neuroprotection. This partial neuroprotection in combination with the timing of riluzole treatment is what might underline the fact that in clinical trials riluzole is not effective in PD (Bensimon et al., 2009). This may be due to the fact that at the start of treatment, PD patients were in an advanced stage of neurodegeneration. Riluzole is therefore more likely to be effective when started in the early phase of neurodegeneration and should be given in the premotor phase of PD. The premotor phase of PD can be accompanied by non-motor symptoms such as abnormal olfaction, mood, autonomic dysfunction and abnormal sleep (Berg, 2008; Tolosa et al., 2009). One of the sleep-related problems that occur early in PD is increased muscle tone during REM sleep. At least one third of the PD patients have increased and irregular chin muscle tone during REM sleep (Comella et al., 1993; Gagnon et al., 2002), which meet the criteria for REM Sleep Behavior Disorder (RBD). The therapeutic effect of riluzole on tonic muscle tone during REM sleep, as shown in chapter 3, implies an important contribution of riluzole in the treatment of early stage PD. Improvement of tonic muscle tone can be regarded as a novel diagnostic marker of the early stage neuroprotection. It is therefore of interest to study on the effectiveness of the well-tolerated compound riluzole (Lacomblez et al., 2002) in the premotor phase of PD.

6.6 Implications for further research

Today, patients, family members and physicians struggle with the fact that there is no cure or satisfactory treatment for PD. The nature of the disorder is suggested to be a combination of endogenous and exogenous factors starting years before the actual diagnosis and causing unnoticed but progressive neurodegeneration. This finally underlies the symptomatology of PD. *But although, at present, uninformed as to the precise nature of the disease, still it ought not to be considered as one against which there exists no countervailing remedy* (Parkinson, 1817). Therefore, it is our notion that research should go towards a combination of early diagnosis and development of neuroprotective treatments. As a first step, a shift should be made from diagnosis that is completely based on motor complications (Pahwa and Lyons, 2010) to early

diagnosis that is supported by premotor symptoms. Besides sleep disturbances relatively simple but informative diagnostic tools are the Parkinson's disease Sleep Scale (Dhawan et al., 2006) and the University of Pennsylvania Smell Identification Test (Deeb et al., 2010). Insight into potential diagnostic tools for the early phase of the disease and the mechanisms for neuropathology and subsequently neuroprotection in early neurodegeneration will rely on models for this early stage of the disease. One of the aims of this thesis was to evaluate new possible behavioral markers for dopaminergic neurodegeneration in a preclinical model of PD, the marmoset MPTP model. Therefore, we designed two new behavioral setups to quantify jumping behavior as a measure for akinesia and the righting reflex as a measure for rigidity and axial turning. Furthermore, we found that an RBD-like phenomenon takes place in marmosets with moderate neurodegeneration. This underlines the fact that this MPTP model entails features of the premotor phase of PD. Both the behavioral- and sleep markers were evaluated in a neuroprotective study using the compound riluzole. This validation with riluzole increases the insight in neuroprotection at an early stage neurodegeneration. We suggest that when riluzole is used for treatment of PD patients, it has to be combined with early diagnosis and sensitive neurodegeneration markers. Another aim of this thesis was to investigate the mechanisms involved in early neurodegeneration with the classic compound MPTP. A low dose osmotic mini-pump paradigm was used in the mouse to gain insight in the proteins involved in the initiation of DA neuron specific neurodegeneration. We showed the prominent contribution of mitochondrial dysfunction even at the start of a cell challenge, suggesting that this is the key factor in DA neurodegeneration and an important focus for further research.

Summary

Summary

Parkinson's disease (PD) is one of the main chronic and progressive neurodegenerative disorders characterized by extensive loss of dopaminergic neurons, in particular in the *substantia nigra* (SN). Most patients are in the prime of their life, when they start suffering from the classic symptoms of motor dysfunction. To relieve their symptoms these patients are dependent on dopamine (DA) replacement treatment. Before the display of motor deterioration, they report non-motor problems, such as olfactory dysfunction, mood changes and sleep problems that can start years before the actual diagnosis. Although the ultimate clinical goal is to limit neurodegeneration, this is only possible by an early identification of individuals at risk and early start of neuroprotective treatment before progressive loss of neurons is achieved. Therefore, this research project was initiated as a response to the increasing interest in early PD and the initiation of the neurodegenerative process based on the hypothesis that neuroprotection at an early stage of PD will limit its progression and that protection will reduce the functional deterioration and pathology. This thesis aims to give additional insight in functional and molecular markers of DA neurodegeneration using animal models for PD.

In **chapter 1**, PD is introduced as a multi-factorial disorder, caused by the combined effect of age, environmental factors, genetic susceptibility and complex genetic-environmental interactions. The processes affected by these factors are: mitochondrial dysfunction, glutamate excitotoxicity, oxidative stress, inflammatory responses and proteasome dysfunction. Finally, this results in a loss of dopaminergic neurons in the SN. These neurons are the key players in the motor domains, responsible for the fine-tuning of movements. At the time of diagnosis, which to date is mostly based on the classic motor symptoms, PD patients have already lost at least 50% of the DA neurons in the SN. Together with the loss of these neurons the amount of released DA is reduced.

Unfortunately, there is still no cure or satisfactory treatment for PD that can stop the neurodegenerative process due to the multi-factorial nature of PD and the slow induction proces. The initiation of the disorders is caused by a combination of endogenous and exogenous factors, which lead to neurodegeneration already years before the actual diagnosis based on symptomatology. Therefore research efforts should be focused on a combination of markers for early diagnosis and neuroprotective treatment.

Studying PD neurobiology in combination with disease manifestation in humans is limited to clinical trials and post-mortem material. Therefore, in order to find new targets for neuroprotective therapies, animal models are a great asset in PD research. Animal models should ideally mimic the main features of the disease pathology and additionally show the typical Parkinsonian syndrome. The multi-factorial aspects of the disease dictate the need for PD-like animal models that couple disease manifestation to the underlying pathology. The ultimate animal model for PD has not yet been described; however there are several experimental models used worldwide that model PD-like neurodegeneration in combination with motor symptoms. One of these induction models is based on the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the compound of choice for this thesis, which causes selective cell death in the DA neurons highly specific for the SN in humans, monkeys and mice.

In **Chapter 2** we present two new clinically relevant biomarkers for DA neurodegeneration in an animal model closely related to humans. Two new behavioral test systems were developed for marmoset monkeys to quantify jumping behavior as a measure for akinesia (Tower test) and the righting reflex as a measure for rigidity and axial turning (Hourglass test). The marmoset's righting reflex in the Hourglass test remained significantly impaired during the period after the MPTP intoxication. In the Tower test, the marmosets were not able to jump the largest distances one week after MPTP and showed a persistent reduction in activity after the MPTP intoxication. Because not all aspects of motor behavior are similarly affected by MPTP, a complete behavioral sketch of Parkinsonian marmosets should preferably include a range of motor behavioral functions to create an overview of the full range of motor impairments. Both the Hourglass and Tower test provide important behavioral information in a clinically-relevant approaches for testing motor dysfunction.

Besides the motor function tests, symptoms related to sleep are evaluated as a possible marker for moderate neurodegeneration in the marmoset MPTP model in **chapter 3.** This is a relevant parameter for the clinical sleep problems in the premotor phase of PD. We describe changes during rapid eye movements (REM) sleep, analogous to REM sleep behavior disorder (RBD) in patients; one of the suggested premotor symptoms of PD. MPTP increased the number of epochs with high-amplitude muscle contractions during

REM sleep. Of all sleep measures, RBD-like measures discriminated best MPTP-treated versus control animals. At this stage of sleep disturbances, the marmoset monkeys did not show any changes in functional motor behavior as measured by the Hand-eye coordination test. This REM sleep specific change, in the absence of profound changes in wake motor behavior, suggests that the MPTP marmoset model is of high potential for studies into mechanisms and treatment of RBD and sleep disturbances in the early phase of PD.

To validate neuroprotection in early PD models the anti-excitotoxic compound riluzole was tested for its neuroprotective efficacy in marmosets in **chapter 4** and in mice in **chapter 5**. Riluzole is effective in ALS because of its life prolonging effects. However, riluzole appeared to be less or completely ineffective in clinical trials when started in the motor phase of PD. Cell loss can only be prevented if started before or at the same time as the actual initiation of the apoptotic process. Therefore, the effect of pretreatment with the riluzole is examined in **chapter 4** in the MPTP-treated marmoset model for the early phase of PD. Not only the traditional observational scoring and histo-pathological evidence for neuroprotection is included in the analysis, but also a complete range of motor behavioral tests and sleep aspects, in order to find the most efficient way of predicting the therapeutic value of a compound in a preclinical trials. MPTP affected all behavioral parameters and sleep architecture and induced a relatively mild (50%) decline of DA neurons in the SN. Riluzole relieved the Parkinsonian signs, and improved the hand-eye coordination as well as turning ability. Moreover, riluzole prevented the impact of MPTP on sleep architecture and RBD. Riluzole also improved the number of surviving DA neurons. However, riluzole did not prevent the MPTP-induced impairments on locomotor activity and jumping activity. In conclusion, reduction of excitotoxicity by riluzole appeared to be effective in reducing progressive neurodegeneration and in minimizing several clinically relevant PD symptoms in an animal model representing the early phase of PD.

Insights into the multiple effects produced by riluzole on MPTP-challenged neurons and their synaptic transmission can also help us to understand why this drug might be clinically useful. In **chapter 5** the effect of riluzole was evaluated for the early molecular changes of neurodegeneration by means of proteomics analysis in the synaptosome of DA neurons. The changes in protein expression in the brain cannot be measured in humans for medical

ethical reasons. Besides this, the actual start of the neurodegenerative process cannot be estimated or predicted for idiopathic PD. Therefore the changes in protein expression were investigated in a chronic low level MPTP mouse model. MPTP was slowly infused in mice to generate a chronic progressive cell challenge. By sampling brain tissue at different time points, neurodegenerative as well as neuroprotective processes on protein expression were studied directly after the initiation of DA neuron specific neurodegeneration. The low dose 24 h MPTP challenge used in this mouse study did not affect the number of neurons and it did not lead to behavioral abnormalities. On the other hand, mitochondrial proteins showed a general increase of abundance at 24 hours after the start of infusion, reflecting either upregulation of mitochondrial activity or the recruitment of mitochondria into the synaptic fraction. Riluzole treated MPTP-exposed mice did not show this upregulation at 24 hours. This rescue effect of riluzole suggests a general inhibition of the mitochondrial activity in the synapse, which may act protective against the MPTP challenge. We showed the mitochondrial dysfunction at the start of a cell challenge, suggesting that this is the key factor in DA neurodegeneration and an important focus for further research. This relevant mechanistic and neuroprotective information could be used to delay or perhaps even arrest the disease before the typical symptoms emerge and the damage due to the neurodegenerative state becomes irreversible.

Today, patients, family members and physicians struggle with the fact that there is no cure or satisfactory treatment for PD. Therefore in **chapter 6** we emphasize that research should focus on a combination of early diagnosis supported by premotor symptoms and the further development of neuroprotective treatment. Insights into neuroprotection and the mechanisms of neuropathology in early neurodegeneration will rely on animal models for the early stage of PD. Both the marmoset MPTP model and the mouse MPTP infusion model used for this thesis are congruent with the scientific criteria of face, predictive, construct and external validity. The general conclusion is that the specific scientific question raised should match the model and that the two models used in this study offer a good opportunity to investigate neurodegeneration at different stages of pathology. The behavioral tests and the RBD-like sleep changes in the marmoset monkeys add to the range of PD hallmarks in the marmoset model. The protective effect of riluzole on tonic muscle tone during REM sleep implies an important contribution of riluzole in the treatment of

early stage PD. Improvement in tonic muscle tone during REM sleep can be seen as a novel diagnostic marker of the early stage neuroprotection. However, direct translation of features of the marmoset model to a clinical situation remains a rather difficult challenge because of the chemical induction of PD used, the natural recovery in marmosets from MPTP intoxication and the involvement of non-motor pathways in marmosets' measured motor behavior. The increased abundance of mitochondrial proteins in the early stage PD-like MPTP mouse model demonstrates that this is a main mechanism by which neurons cope with this neuronal challenge. The validation with riluzole in this thesis raises the insight in the possibilities for riluzole as a treatment against early neurodegeneration. In conclusion, the etiology of idiopathic PD is suggested to be influenced by a combination of endogenous and exogenous factors starting years before the actual diagnosis. Therefore, this thesis emphasizes that research should focus on new means of early diagnosis and the development of neuroprotective treatments during the early phase of PD.

Samenvatting

Samenvatting

De ziekte van Parkinson (PD) is één van de belangrijkste chronische en progressieve neurodegeneratieve aandoeningen gekenmerkt door het verlies van dopaminerge neuronen in het bijzonder in de *substantia nigra* (SN). De meeste patiënten zijn in de bloei van hun leven wanneer de typische bewegingsproblemen starten. De behandeling van PD is nog steeds afhankelijk van dopamine (DA) vervangende medicatie. Patiënten rapporteren ook beweging onafhankelijke problemen zoals reuk, depressie maar ook bijvoorbeeld problemen met slapen, soms jaren voor de eigenlijke diagnose op basis van de bewegingsproblemen. Het uiteindelijk klinische doel is het minimaliseren van de neurodegeneratie, maar dit is alleen mogelijk wanneer risico groepen vroeg kunnen worden geïdentificeerd zodat neuroprotectieve behandeling kan worden gestart nog voordat de progressieve neurodegeneratie in gang is gezet. Dit onderzoeksproject richt zich op onderzoek naar de vroege fase van PD en het begin van neurodegeneratie, met de gedachte dat neuroprotectie in een vroeg stadium, de voortzetting van PD kan voorkomen en daarmee de functionele achteruitgang en pathologie van patiënten kan verminderen. Dit proefschrift heeft als doel om meer inzicht te geven in de functionele en moleculaire markers van DA neurodegeneratie met behulp van diermodellen voor de ziekte van Parkinson.

In **hoofdstuk 1**, wordt het multifactoriële karakter van de ziekte van Parkinson benadrukt, veroorzaakt door het gecombineerde effect van leeftijd, omgevingsfactoren, genetische vatbaarheid en complexe genen-omgevings interacties. De processen die daarin een rol spelen zijn: mitochondriale dysfunctie, glutamaat excitotoxiciteit, oxidatieve stress, inflammatoire reacties en proteasoom dysfunctie. Dit resulteert uiteindelijk in een verlies van dopamine neuronen in de SN. Neuronen in de SN zijn essentieel in het bewegingsapparaat en verantwoordelijk voor het reguleren van beweging. Op het moment van de diagnose, welke tot op heden voornamelijk is gebaseerd op de typische bewegingsproblematiek, hebben PD patiënten al meer dan 50% van de DA neuronen in de SN verloren. Tegelijk met het verlies van de neuronen neemt de hoeveelheid afgegeven DA af.
Helaas is er nog geen genezing of aanvaardbare behandeling beschikbaar voor PD waarmee neurodegeneratie kan worden gestopt. Dit komt door de complexe multifactoriële oorzaak van PD welke wordt gestuurd door zowel interne en externe processen. Dit zorgt voor de start van de neurodegeneratie meerdere jaren voor de eigenlijke PD diagnose. Onderzoek is

dus idealiter gericht op een combinatie van markers voor vroege diagnose en neuroprotectieve behandeling.
Het onderzoeken van de neurobiologische processen in patiënten met PD is beperkt tot niet-invasieve klinische studies en post-mortem materiaal. Daarom is het gebruik van diermodellen nog steeds essentieel in het onderzoek naar PD. Diermodellen hebben idealiter de belangrijkste kenmerken van de PD pathologie en de daaraan gerelateerde typische Parkinson symptomen. De multifactoriële achtergrond van de ziekte onderstreept de noodzaak van deze modellen om de manifestatie van de ziekte te kunnen koppelen aan de onderliggende pathologie. Het ultieme PD diermodel is nog niet beschreven, maar er zijn wereldwijd verschillende experimentele modellen in gebruikt die een PD gelijkende neurodegeneratie vertonen in combinatie met bewegingsproblemen. Een voorbeeld is de stof 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), gebruikt voor dit proefschrift, welke selectief toxisch is voor DA neuronen, zeer specifiek is voor de SN, en Parkinsonisme veroorzaakt in mens, aap en muis.

In **Hoofdstuk 2** presenteren we twee nieuwe klinisch relevante biomarkers voor DA neurodegeneratie in een aan de mens gerelateerd primaten diermodel. Twee nieuwe gedrags-testsystemen werden ontwikkeld voor marmosetaapjes, met springen als een maat voor akinesia (Tower test), en het zich kunnen oprichten als een maat voor stijfheid en axiaal draaien (Hourglass test). De marmosets hadden een significant verminderde opricht reflex in de Hourglass testopstelling in de periode na de MPTP intoxicatie. In de Toren testopstelling waren de marmosets direct na de MPTP niet in staat om de grootste afstanden te springen en lieten ze een aanhoudende vermindering van de activiteit na de MPTP intoxicatie zien. Omdat niet alle aspecten van het motorisch gedrag op dezelfde manier worden beïnvloed door MPTP omvat een fenotypering bij voorkeur een scala aan motorische gedragingen en functies om een overzicht te verkrijgen van de motorische problemen. Zowel de Hourglass als de Tower test zijn waardevolle aanvullingen op een dergelijke fenotypering.

Naast de motorische symptomen wordt er in **hoofdstuk 3** gekeken naar slaap gerelateerde symptomen in het marmoset MPTP model als een marker voor gematigde neurodegeneratie. Dit is een relevante parameter voor klinische slaapproblemen in de premotorische fase van PD. In dit hoofdstuk beschrijven we veranderingen tijdens de rapid eye movements (REM)

slaap welke vergelijkbaar zijn aan REM sleep behavior disorder bij patiënten, één van de voorgestelde premotorische symptomen van PD. In de MPTP behandelde dieren is tijdens de REM slaap een significante toename van het aantal epochs met hoge amplitude spiersamentrekkingen. Van alle slaap parameters zijn de RBD metingen bruikbaar om te bepalen welke dieren MPTP hebben gehad en welke niet. In dit stadium hadden de dieren een milde vorm van Parkinsonisme zonder problemen met functionele motorische gedragingen, zoals gemeten in de Hand-oog coördinatie test. Deze REM-slaap specifieke verandering zonder daaraan gepaard gaande ingrijpende bewegingsveranderingen suggereert dat het MPTP marmoset model interessant is voor studies naar de mechanismen en de behandeling van RBD en slaapstoornissen in premotor PD.

De anti-excitotoxische stof riluzole wordt gebruikt om het effect als neuroprotectieve stof te valideren in modellen voor vroege PD, in de marmoset in **hoofdstuk 4** en in de muis in **hoofdstuk 5**. Riluzole werkt levensverlengend in amyotrofische laterale sclerose (ALS). Maar riluzole was niet effectief in klinische studies gestart in motorische fase van PD. De behandeling om neuron verlies te voorkomen kan alleen effectief zijn als deze wordt ingezet vóór of op hetzelfde moment als het neuronale apoptose proces. Daarom wordt de voorbehandeling met riluzole onderzocht in MPTP behandelde marmosetten in **hoofdstuk 4**. Neuroprotectie werd vervolgens op verschillende niveaus gemeten: de dieren zijn gedragsmatig geobserveerd, histo-pathologisch werd er gekeken naar celoverleving en daarnaast is er een serie aan motor gedragstesten uitgevoerd en is er gekeken naar slaap aspecten. Op deze manier werd er gekeken naar de meest efficiënte manier om de therapeutische waarde van een stof te bepalen in een preklinisch proef. De MPTP behandeling had effect op alle gedragsparameters en ook op de slaap architectuur. Dit werd veroorzaakt door een relatief milde vorm van Parkinsonisme (50% daling van DA neuronen in de SN). Riluzole zorgde voor een gedeeltelijke verbetering van de Parkinson symptomen. Er was een verbetering van de hand-oog coördinatie en de opricht reflex in de Hourglass. Daarnaast had riluzole een positief effect op de slaap architectuur en RBD symptomen gedurende de REM slaap. Riluzole beschermde ook het aantal overlevende DA neuronen. Er was geen verbetering op spontane activiteit. Daarmee concluderen we dat de behandeling met riluzole effectief is in het verminderen progressieve neurodegeneratie en meerdere

klinisch relevante PD symptomen in het marmoset MPTP model voor de vroege fase van de PD.

De effecten van riluzole op MPTP behandelde neuronen en hun synaptische transmissie geeft inzicht in de mogelijkheden voor riluzole in de kliniek. In **hoofdstuk 5** worden de vroege moleculaire veranderingen van neurodegeneratie en de effecten van riluzole onderzocht door middel van eiwitanalyse van het synaptosoom van DA cellen. De veranderingen in eiwitexpressie tijdens de start van PD kan niet worden gemeten in humane hersenen om ethische redenen en bovendien omdat dit moment niet kan worden gediagnosticeerd of voorspeld. Daarom is er voor gekozen om de veranderingen in eiwitexpressie te onderzoeken in een langzaam chronische MPTP muismodel. MPTP werd hiervoor langzaam geïnfuceerd bij muizen met behulp van een osmotisch minipompje zodat de cellen een langzame progressieve intoxicatie ondervonden. Daarna werd hersenweefsel op verschillende tijdstippen onderzocht. De 24 uur behandeling met een lage hoeveelheid MPTP in deze muizenstudie had geen invloed op het aantal neuronen en het veroorzaakte geen motorische beperkingen. Als gevolg van een verhoogde mitochondriale activiteit of de verplaatsing van mitochondriën naar de synaps was er een algemene verhoging van mitochondriale eiwitten op 24 uur na de start van de MPTP infusie. Riluzole behandelde dieren vertoonde deze verhoging van expressie niet. Dit regulerende effect van riluzole suggereert een inhibitie van de mitochondriale activiteit in de synaps, welke wellicht neuroprotectief werkt op de MPTP behandeling. Deze studie laat dus mitochondriale dysfunctie tijdens het begin van MPTP intoxicatie zien. Dit suggereert dat dit een belangrijke factor is in DA neurodegeneratie en een belangrijk aandachtspunt vormt voor verder onderzoek. Deze relevante mechanistische en neuroprotectieve informatie kan worden gebruikt om de schade als gevolg van PD te stoppen of te vertragen zodat de klassieke symptomen niet of later ontstaan.

Patiënten, hun familieleden en hun artsen kampen nog steeds met het feit dat er geen genezing of bevredigende behandeling is voor PD. Daarom benadrukken we in **hoofdstuk 6** dat onderzoek gefocust zou moeten zijn op vroege diagnose op basis van premotor symptomen in combinatie met een verdere ontwikkeling van neuroprotectieve behandeling. Meer inzicht in de mechanismen in de vroege fase van PD en neurodegeneratie zal

afhankelijk zijn van diermodellen voor deze fase van de pathologie. Zowel het marmoset MPTP model en het muizen MPTP infusie model, zoals gebruikt in dit proefschrift, werden gespiegeld aan vier wetenschappelijke criteria voor goede modellen: vergelijkende, voorspellende constructieve en externe waarde. De conclusie is dat de specifieke wetenschappelijke vraag bepalend is voor het te gebruiken model. De hier gebruikte modellen zijn zeer geschikt om neurodegeneratie te onderzoeken in verschillende stadia van de pathogenese. We concludeerden dat de gedragsmetingen en de RBD-overeenkomstige slaap veranderingen in de marmoset bijdragen aan de PD markers. Het protectieve effect van riluzole op spierspanning tijdens de REM slaap betekent een belangrijke bijdrage van riluzole in de behandeling van vroege PD. Het verbeteren van de spierspanning tijdens de REM slaap zou hiermee een nieuwe diagnostische marker kunnen zijn voor vroege neuroprotectie. Maar de directe vertaling van deze effecten in het marmoset model naar de klinische situatie blijft een uitdaging vanwege de chemische inductie van PD, het natuurlijke herstel van de marmosetten en de betrokkenheid van niet-motorische trajecten in het gemeten bewegingsgedrag van de marmosets. De toegenomen expressie van mitochondriale eiwitten in het vroege stadium van het MPTP muismodel geeft aan dat dit een belangrijke response is in de synaps tijdens vroege neurodegeneratie. De validatie met riluzole in marmoset verhoogt het inzicht in de mogelijkheden voor riluzole als een behandeling in het begin van neurodegeneratie. Concluderend, idiopatische PD wordt waarschijnlijk veroorzaakt door een combinatie van endogene en exogene factoren die neurodegeneratieve processen opgang brengen jaren vóór de daadwerkelijke diagnose. Daarom benadruk ik in mijn proefschrift dat onderzoek naar PD een combinatie moet zijn van nieuwe methoden voor vroege diagnose en het ontwikkelen van neuroprotectieve behandelingen.

Abbreviations

A	Anterior
AD	Alzheimer's Disease
AIMS	Abnormal Involuntary Movements Score
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
AMPA	α-amino-3-hydroxy-5-methylisoxazolepropionic acid
BBB	Blood–brain barrier
COMT	Catechol-O-methyl transferase
DA	Dopamine
DAB-NI	Nickel ammonium sulfate
DAR	Dopamine receptor
DAT	Dopamine transporter
DaTSCAN	Dopamine transporter scan
DBS	Deep Brain Stimulation
D1	Dopamine type 1
D2	Dopamine type 2
EAATs	Astrocytic glutamate transporters
EEG	Electroencephalogram
EMG	Electromyogram
F	Female
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
GDNF	Glial cell-derived neurotrophic factor
GPe	External *globus pallidus*
GPi	Internal *globus pallidus*
Im	Intra Muscular
L-DOPA	L-dihydroxy-phenylalanine

M	Male
Min	Minute(s)
MPP^+	1-methyl-4-phenylpyridinium
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA	N-methyl-D-aspartic acid
MOA-B	Monoamine oxidase B
PD	Parkinson's disease
PKC	Protein kinase C
p.o.	Oral dosage
PSG	Polysomnogram
RBD	REM sleep behavior disorder
REM	Rapid eye movements
rpm	Raps per minute
sec	Second(s)
SEM	Standard error of the mean
sc	Subcutaneous
SN	*Substantia nigra*
STN	*Subthalamic nucleus*
SNpc	*Substantia nigra pars compacta*
SNr	S*ubstantia nigra reticularis*
TH-IR	Tyrosine hydroxylase immunoreactivity
vs	Versus
WASO	Wake time after sleep onset
6-OHDA	6-hydroxidopamine

References

AASM (2005) International classification of sleep disorders: Diagnostic and Coding manual, 2nd ed. In: (Westchester, ed), pp 148-152. IL: American Academy of Sleep Medicine.

Abou-Sleiman PM, Muqit MM, Wood NW (2006) Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat Rev Neurosci 7:207-219.

Adams JD, Jr., Klaidman LK, Leung AC (1993) MPP+ and MPDP+ induced oxygen radical formation with mitochondrial enzymes. Free Radic Biol Med 15:181-186.

Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR (2001) Protein oxidation in the brain in Alzheimer's disease. Neuroscience 103:373-383.

Albanese A, Jenner P, Marsden CD, Stephenson JD (1988) Bladder hyperreflexia induced in marmosets by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurosci Lett 87:46-50.

Alexander GE, Crutcher MD (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci 13:266-271.

Alexi T, Borlongan CV, Faull RL, Williams CE, Clark RG, Gluckman PD, Hughes PE (2000) Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. Prog Neurobiol 60:409-470.

Almirall H, Pigarev I, de la Calzada MD, Pigareva M, Herrero MT, Sagales T (1999) Nocturnal sleep structure and temperature slope in MPTP treated monkeys. J Neural Transm 106:1125-1134.

Alvarez-Fischer D, Guerreiro S, Hunot S, Saurini F, Marien M, Sokoloff P, Hirsch EC, Hartmann A, Michel PP (2008) Modelling Parkinson-like neurodegeneration via osmotic minipump delivery of MPTP and probenecid. J Neurochem 107:701-711.

Anderson G, Noorian AR, Taylor G, Anitha M, Bernhard D, Srinivasan S, Greene JG (2007) Loss of enteric dopaminergic neurons and associated changes in colon motility in an MPTP mouse model of Parkinson's disease. Exp Neurol 207:4-12.

Andres-Mateos E, Perier C, Zhang L, Blanchard-Fillion B, Greco TM, Thomas B, Ko HS, Sasaki M, Ischiropoulos H, Przedborski S, Dawson TM, Dawson VL (2007) DJ-1 gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase. Proc Natl Acad Sci U S A 104:14807-14812.

References

Annett LE, Martel FL, Rogers DC, Ridley RM, Baker HF, Dunnett SB (1994) Behavioral assessment of the effects of embryonic nigral grafts in marmosets with unilateral 6-OHDA lesions of the nigrostriatal pathway. Exp Neurol 125:228-246.

Araki T, Kumagai T, Tanaka K, Matsubara M, Kato H, Itoyama Y, Imai Y (2001) Neuroprotective effect of riluzole in MPTP-treated mice. Brain Res 918:176-181.

Bagetta V, Ghiglieri V, Sgobio C, Calabresi P, Picconi B (2010) Synaptic dysfunction in Parkinson's disease. Biochem Soc Trans 38:493-497.

Ballard PA, Tetrud JW, Langston JW (1985) Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): seven cases. Neurology 35:949-956.

Barneoud P, Mazadier M, Miquet JM, Parmentier S, Dubedat P, Doble A, Boireau A (1996) Neuroprotective effects of riluzole on a model of Parkinson's disease in the rat. Neuroscience 74:971-983.

Barraud Q, Lambrecq V, Forni C, McGuire S, Hill M, Bioulac B, Balzamo E, Bezard E, Tison F, Ghorayeb I (2009) Sleep disorders in Parkinson's disease: the contribution of the MPTP non-human primate model. Exp Neurol 219:574-582.

Beal MF (1992) Mechanisms of excitotoxicity in neurologic diseases. Faseb J 6:3338-3344.

Beal MF (2003) Mitochondria, oxidative damage, and inflammation in Parkinson's disease. Ann N Y Acad Sci 991:120-131.

Belforte JE, Pazo JH (2004) Turning behaviour induced by stimulation of the 5-HT receptors in the subthalamic nucleus. Eur J Neurosci 19:346-355.

Beltran-Parrazal L, Charles A (2003) Riluzole inhibits spontaneous Ca2+ signaling in neuroendocrine cells by activation of K+ channels and inhibition of Na+ channels. Br J Pharmacol 140:881-888.

Benavides J, Camelin JC, Mitrani N, Flamand F, Uzan A, Legrand JJ, Gueremy C, Le Fur G (1985) 2-Amino-6-trifluoromethoxy benzothiazole, a possible antagonist of excitatory amino acid neurotransmission--II. Biochemical properties. Neuropharmacology 24:1085-1092.

Benazzouz A, Boraud T, Dubedat P, Boireau A, Stutzmann JM, Gross C (1995) Riluzole prevents MPTP-induced parkinsonism in the rhesus monkey: a pilot study. Eur J Pharmacol 284:299-307.

Bensimon G, Lacomblez L, Meininger V (1994) A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. N Engl J Med 330:585-591.

Bensimon G, Ludolph A, Agid Y, Vidailhet M, Payan C, Leigh PN (2009) Riluzole treatment, survival and diagnostic criteria in Parkinson plus disorders: the NNIPPS study. Brain 132:156-171.

Berg D (2008) Biomarkers for the early detection of Parkinson's and Alzheimer's disease. Neurodegener Dis 5:133-136.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3:1301-1306.

Bezard E, Imbert C, Gross CE (1998a) Experimental models of Parkinson's disease: from the static to the dynamic. In: Rev Neurosci, pp 71-90.

Bezard E, Dovero S, Bioulac B, Gross C (1997) Effects of different schedules of MPTP administration on dopaminergic neurodegeneration in mice. Exp Neurol 148:288-292.

Bezard E, Stutzmann JM, Imbert C, Boraud T, Boireau A, Gross CE (1998b) Riluzole delayed appearance of parkinsonian motor abnormalities in a chronic MPTP monkey model. Eur J Pharmacol 356:101-104.

Birkmayer W, Hornykiewicz O (1961) Der L-3, 4-Dioxyphenylalanin (= DOPA)-Effekt bel der Parkinson-Akinese. Wien Klin Wochenschr 73:787-788.

Blanchet PJ, Calon F, Morissette M, Hadj Tahar A, Belanger N, Samadi P, Grondin R, Gregoire L, Meltzer L, Di Paolo T, Bedard PJ (2004) Relevance of the MPTP primate model in the study of dyskinesia priming mechanisms. Parkinsonism Relat Disord 10:297-304.

Blandini F, Levandis G, Bazzini E, Nappi G, Armentero MT (2007) Time-course of nigrostriatal damage, basal ganglia metabolic changes and behavioural alterations following intrastriatal

injection of 6-hydroxydopamine in the rat: new clues from an old model. Eur J Neurosci 25:397-405.
Boireau A, Miquet JM, Dubedat P, Meunier M, Doble A (1994a) Riluzole and experimental parkinsonism: partial antagonism of MPP(+)-induced increase in striatal extracellular dopamine in rats in vivo. Neuroreport 5:2157-2160.
Boireau A, Dubedat P, Bordier F, Imperato A, Moussaoui S (2000) The protective effect of riluzole in the MPTP model of Parkinson's disease in mice is not due to a decrease in MPP(+) accumulation. Neuropharmacology 39:1016-1020.
Boireau A, Dubedat P, Bordier F, Peny C, Miquet JM, Durand G, Meunier M, Doble A (1994b) Riluzole and experimental parkinsonism: antagonism of MPTP-induced decrease in central dopamine levels in mice. Neuroreport 5:2657-2660.
Botella JA, Bayersdorfer F, Gmeiner F, Schneuwly S (2009) Modelling Parkinson's disease in Drosophila. Neuromolecular Med 11:268-280.
Boulet S, Mounayar S, Poupard A, Bertrand A, Jan C, Pessiglione M, Hirsch EC, Feuerstein C, Francois C, Feger J, Savasta M, Tremblay L (2008) Behavioral recovery in MPTP-treated monkeys: neurochemical mechanisms studied by intrastriatal microdialysis. J Neurosci 28:9575-9584.
Bove J, Prou D, Perier C, Przedborski S (2005) Toxin-induced models of Parkinson's disease. NeuroRx 2:484-494.
Braak H, Rub U, Gai WP, Del Tredici K (2003) Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. J Neural Transm 110:517-536.
Brownell AL, Canales K, Chen YI, Jenkins BG, Owen C, Livni E, Yu M, Cicchetti F, Sanchez-Pernaute R, Isacson O (2003) Mapping of brain function after MPTP-induced neurotoxicity in a primate Parkinson's disease model. Neuroimage 20:1064-1075.
Buchanan-Smith HM, Shand C, Morris K (2002) Cage use and feeding height preferences of captive common marmosets (Callithrix j. jacchus) in two-tier cages. J Appl Anim Welf Sci 5:139-149.
Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ (1983) A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Proc Natl Acad Sci U S A 80:4546-4550.
Cartelli D, Ronchi C, Maggioni MG, Rodighiero S, Giavini E, Cappelletti G (2010) Microtubule Dysfunction Precedes Transport Impairment and Mitochondria Damage in Mpp-Induced Neurodegeneration. J Neurochem.
Castellani R, Smith MA, Richey PL, Kalaria R, Gambetti P, Perry G (1995) Evidence for oxidative stress in Pick disease and corticobasal degeneration. Brain Res 696:268-271.
Centonze D, Calabresi P, Pisani A, Marinelli S, Marfia GA, Bernardi G (1998) Electrophysiology of the neuroprotective agent riluzole on striatal spiny neurons. Neuropharmacology 37:1063-1070.
Chan DK, Woo J, Ho SC, Pang CP, Law LK, Ng PW, Hung WT, Kwok T, Hui E, Orr K, Leung MF, Kay R (1998) Genetic and environmental risk factors for Parkinson's disease in a Chinese population. J Neurol Neurosurg Psychiatry 65:781-784.
Chan F, Armstrong IT, Pari G, Riopelle RJ, Munoz DP (2005) Deficits in saccadic eye-movement control in Parkinson's disease. Neuropsychologia 43:784-796.
Chau KY, Ching HL, Schapira AH, Cooper JM (2009) Relationship between alpha synuclein phosphorylation, proteasomal inhibition and cell death: relevance to Parkinson's disease pathogenesis. J Neurochem 110:1005-1013.
Chen CC, Chen TF, Hwang YC, Wen YR, Chiu YH, Wu CY, Chen RC, Tai JJ, Chen TH, Liou HH (2009) Different prevalence rates of Parkinson's disease in urban and rural areas: a population-based study in Taiwan. Neuroepidemiology 33:350-357.
Choi WS, Kruse SE, Palmiter RD, Xia Z (2008) Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. Proc Natl Acad Sci U S A 105:15136-15141.

Clarke CE (2004) Neuroprotection and pharmacotherapy for motor symptoms in Parkinson's disease. Lancet Neurol 3:466-474.
Comella CL, Tanner CM, Ristanovic RK (1993) Polysomnographic sleep measures in Parkinson's disease patients with treatment-induced hallucinations. Ann Neurol 34:710-714.
Cookson MR (2009) alpha-Synuclein and neuronal cell death. Mol Neurodegener 4:9.
Cotter D, Guda P, Fahy E, Subramaniam S (2004) MitoProteome: mitochondrial protein sequence database and annotation system. Nucleic Acids Res 32:D463-467.
Counotte DS, Li KW, Wortel J, Gouwenberg Y, Van Der Schors RC, Smit AB, Spijker S (2010) Changes in molecular composition of rat medial prefrontal cortex synapses during adolescent development. Eur J Neurosci 32:1452-1460.
Couratier P, Sindou P, Esclaire F, Louvel E, Hugon J (1994) Neuroprotective effects of riluzole in ALS CSF toxicity. Neuroreport 5:1012-1014.
Cousins SL, Papadakis M, Rutter AR, Stephenson FA (2008) Differential interaction of NMDA receptor subtypes with the post-synaptic density-95 family of membrane associated guanylate kinase proteins. J Neurochem 104:903-913.
Crenna P, Carpinella I, Rabuffetti M, Calabrese E, Mazzoleni P, Nemni R, Ferrarin M (2007) The association between impaired turning and normal straight walking in Parkinson's disease. Gait Posture 26:172-178.
Dahan L, Astier B, Vautrelle N, Urbain N, Kocsis B, Chouvet G (2007) Prominent burst firing of dopaminergic neurons in the ventral tegmental area during paradoxical sleep. Neuropsychopharmacology 32:1232-1241.
Danielson SR, Andersen JK (2008) Oxidative and nitrative protein modifications in Parkinson's disease. Free Radic Biol Med 44:1787-1794.
Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889-909.
de la Fuente-Fernandez R, Stoessl AJ (2002) The placebo effect in Parkinson's disease. Trends Neurosci 25:302-306.
de la Fuente-Fernandez R, Phillips AG, Zamburlini M, Sossi V, Calne DB, Ruth TJ, Stoessl AJ (2002) Dopamine release in human ventral striatum and expectation of reward. Behav Brain Res 136:359-363.
Debono MW, Le Guern J, Canton T, Doble A, Pradier L (1993) Inhibition by riluzole of electrophysiological responses mediated by rat kainate and NMDA receptors expressed in Xenopus oocytes. Eur J Pharmacol 235:283-289.
Deeb J, Shah M, Muhammed N, Gunasekera R, Gannon K, Findley LJ, Hawkes CH (2010) A basic smell test is as sensitive as a dopamine transporter scan: comparison of olfaction, taste and DaTSCAN in the diagnosis of Parkinson's disease. Qjm.
Del Zompo M, Piccardi MP, Ruiu S, Quartu M, Gessa GL, Vaccari A (1993) Selective MPP+ uptake into synaptic dopamine vesicles: possible involvement in MPTP neurotoxicity. Br J Pharmacol 109:411-414.
DeLong MR (1990) Primate models of movement disorders of basal ganglia origin. Trends Neurosci 13:281-285.
DeLong MR, Wichmann T (2001) Deep brain stimulation for Parkinson's disease. Ann Neurol 49:142-143.
Dhawan V, Dhoat S, Williams AJ, Dimarco A, Pal S, Forbes A, Tobias A, Martinez-Martin P, Chaudhuri KR (2006) The range and nature of sleep dysfunction in untreated Parkinson's disease (PD). A comparative controlled clinical study using the Parkinson's disease sleep scale and selective polysomnography. J Neurol Sci 248:158-162.
Di Monte DA, McCormack A, Petzinger G, Janson AM, Quik M, Langston WJ (2000) Relationship among nigrostriatal denervation, parkinsonism, and dyskinesias in the MPTP primate model. Mov Disord 15:459-466.
Diguet E, Fernagut PO, Scherfler C, Wenning G, Tison F (2005) Effects of riluzole on combined MPTP + 3-nitropropionic acid-induced mild to moderate striatonigral degeneration in mice. J Neural Transm 112:613-631.
Doble A (1996) The pharmacology and mechanism of action of riluzole. Neurology 47:S233-241.

Dorsey ER, Constantinescu R, Thompson JP, Biglan KM, Holloway RG, Kieburtz K, Marshall FJ, Ravina BM, Schifitto G, Siderowf A, Tanner CM (2007) Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. Neurology 68:384-386.

Douhou A, Debeir T, Murer MG, Do L, Dufour N, Blanchard V, Moussaoui S, Bohme GA, Agid Y, Raisman-Vozari R (2002) Effect of chronic treatment with riluzole on the nigrostriatal dopaminergic system in weaver mutant mice. Exp Neurol 176:247-253.

Duda J (2003) Is the initial insult in Parkinson's Disease and dementia with lewy bodies a neuritic dystrophy?

Dudoit S, Gentleman RC, Quackenbush J (2003) Open source software for the analysis of microarray data. Biotechniques Suppl:45-51.

Dzirasa K, Ribeiro S, Costa R, Santos LM, Lin SC, Grosmark A, Sotnikova TD, Gainetdinov RR, Caron MG, Nicolelis MA (2006) Dopaminergic control of sleep-wake states. J Neurosci 26:10577-10589.

Edgar DM, Dement WC, Fuller CA (1993) Effect of SCN lesions on sleep in squirrel monkeys: evidence for opponent processes in sleep-wake regulation. J Neurosci 13:1065-1079.

Ehringer H, Hornykiewicz O (1960) [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system.]. Klin Wochenschr 38:1236-1239.

Ekstrand MI, Galter D (2009) The MitoPark Mouse - an animal model of Parkinson's disease with impaired respiratory chain function in dopamine neurons. Parkinsonism Relat Disord 15 Suppl 3:S185-188.

Emborg ME (2004) Evaluation of animal models of Parkinson's disease for neuroprotective strategies. J Neurosci Methods 139:121-143.

Eslamboli A (2005) Marmoset monkey models of Parkinson's disease: which model, when and why? Brain Res Bull 68:140-149.

Fahn S (2005) Does levodopa slow or hasten the rate of progression of Parkinson's disease? J Neurol 252 Suppl 4:IV37-IV42.

Fahn S (2008) The history of dopamine and levodopa in the treatment of Parkinson's disease. Mov Disord 23 Suppl 3:S497-508.

Fantini ML, Gagnon JF, Filipini D, Montplaisir J (2003) The effects of pramipexole in REM sleep behavior disorder. Neurology 61:1418-1420.

Fearnley JM, Lees AJ, Revesz T, Brooks DJ, Frackowiak RS (1991) Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain 114 (Pt 5):2283-2301.

Ferini-Strambi L, Zucconi M (2000) REM sleep behavior disorder. Clin Neurophysiol 111:S136-140.

Filion M, Tremblay L, Bedard PJ (1988) Abnormal influences of passive limb movement on the activity of globus pallidus neurons in parkinsonian monkeys. Brain Res 444:165-176.

Fornai F, Schluter OM, Lenzi P, Gesi M, Ruffoli R, Ferrucci M, Lazzeri G, Busceti CL, Pontarelli F, Battaglia G, Pellegrini A, Nicoletti F, Ruggieri S, Paparelli A, Sudhof TC (2005) Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein. Proc Natl Acad Sci U S A 102:3413-3418.

Fox SH, Visanji NP, Johnston TH, Gomez-Ramirez J, Voon V, Brotchie JM (2006) Dopamine receptor agonists and levodopa and inducing psychosis-like behavior in the MPTP primate model of Parkinson disease. Arch Neurol 63:1343-1344.

Fulda S, Gorman AM, Hori O, Samali A (2009) Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010:214074.

Fumagalli E, Bigini P, Barbera S, De Paola M, Mennini T (2006) Riluzole, unlike the AMPA antagonist RPR119990, reduces motor impairment and partially prevents motoneuron death in the wobbler mouse, a model of neurodegenerative disease. Exp Neurol 198:114-128.

Gagnon JF, Bedard MA, Fantini ML, Petit D, Panisset M, Rompre S, Carrier J, Montplaisir J (2002) REM sleep behavior disorder and REM sleep without atonia in Parkinson's disease. Neurology 59:585-589.

Garber PA (1992) Vertical clinging, small body size, and the evolution of feeding adaptations in the Callitrichinae. Am J Phys Anthropol 88:469-482.

Garcia-Borreguero D, Larrosa O, Bravo M (2003) Parkinson's disease and sleep. Sleep Med Rev 7:115-129.

Garcia-Borreguero D, Caminero AB, De La Llave Y, Larrosa O, Barrio S, Granizo JJ, Pareja JA (2002) Decreased phasic EMG activity during rapid eye movement sleep in treatment-naive Parkinson's disease: effects of treatment with levodopa and progression of illness. Mov Disord 17:934-941.

Gatto NM, Cockburn M, Bronstein J, Manthripragada AD, Ritz B (2009) Well-water consumption and Parkinson's disease in rural California. Environ Health Perspect 117:1912-1918.

Goetz CG (1986) Charcot on Parkinson's disease. Mov Disord 1:27-32.

Gold BG, Nutt JG (2002) Neuroimmunophilin ligands in the treatment of Parkinson's disease. Curr Opin Pharmacol 2:82-86.

Goldman SM, Tanner CM, Olanow CW, Watts RL, Field RD, Langston JW (2005) Occupation and parkinsonism in three movement disorders clinics. Neurology 65:1430-1435.

Gross RE, Lombardi WJ, Lang AE, Duff J, Hutchison WD, Saint-Cyr JA, Tasker RR, Lozano AM (1999) Relationship of lesion location to clinical outcome following microelectrode-guided pallidotomy for Parkinson's disease. Brain 122 (Pt 3):405-416.

Gu G, Deutch AY, Franklin J, Levy S, Wallace DC, Zhang J (2003) Profiling genes related to mitochondrial function in mice treated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Biochem Biophys Res Commun 308:197-205.

Guy W (1979) ECDEU assessment manual for psychopharmacology. In, pp 543-547. Washington D.C.: U.S. Departement of Health, Education and Welfare,.

Hantraye P, Varastet M, Peschanski M, Riche D, Cesaro P, Willer JC, Maziere M (1993) Stable parkinsonian syndrome and uneven loss of striatal dopamine fibres following chronic MPTP administration in baboons. Neuroscience 53:169-178.

Hardman CD, Henderson JM, Finkelstein DI, Horne MK, Paxinos G, Halliday GM (2002) Comparison of the basal ganglia in rats, marmosets, macaques, baboons, and humans: volume and neuronal number for the output, internal relay, and striatal modulating nuclei. J Comp Neurol 445:238-255.

Hilker R, Voges J, Weber T, Kracht LW, Roggendorf J, Baudrexel S, Hoevels M, Sturm V, Heiss WD (2008) STN-DBS activates the target area in Parkinson disease: an FDG-PET study. Neurology 71:708-713.

Holdorff B (2006) Fritz Heinrich Lewy (1885-1950). J Neurol 253:677-678.

Hubert JP, Delumeau JC, Glowinski J, Premont J, Doble A (1994) Antagonism by riluzole of entry of calcium evoked by NMDA and veratridine in rat cultured granule cells: evidence for a dual mechanism of action. Br J Pharmacol 113:261-267.

Iannello RC, Crack PJ, de Haan JB, Kola I (1999) Oxidative stress and neural dysfunction in Down syndrome. J Neural Transm Suppl 57:257-267.

Iranzo A, Molinuevo JL, Santamaria J, Serradell M, Marti MJ, Valldeoriola F, Tolosa E (2006) Rapid-eye-movement sleep behaviour disorder as an early marker for a neurodegenerative disorder: a descriptive study. Lancet Neurol 5:572-577.

Iranzo A, Santamaria J, Rye DB, Valldeoriola F, Marti MJ, Munoz E, Vilaseca I, Tolosa E (2005) Characteristics of idiopathic REM sleep behavior disorder and that associated with MSA and PD. Neurology 65:247-252.

Iravani MM, Jackson MJ, Kuoppamaki M, Smith LA, Jenner P (2003) 3,4-methylenedioxymethamphetamine (ecstasy) inhibits dyskinesia expression and normalizes motor activity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated primates. J Neurosci 23:9107-9115.

Irifune M, Kikuchi N, Saida T, Takarada T, Shimizu Y, Endo C, Morita K, Dohi T, Sato T, Kawahara M (2007) Riluzole, a glutamate release inhibitor, induces loss of righting reflex, antinociception, and immobility in response to noxious stimulation in mice. Anesth Analg 104:1415-1421.

Jackson-Lewis V, Jakowec M, Burke RE, Przedborski S (1995) Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurodegeneration 4:257-269.

Jankovic J (2005) Motor fluctuations and dyskinesias in Parkinson's disease: clinical manifestations. Mov Disord 20 Suppl 11:S11-16.

Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH (1985) Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci U S A 82:2173-2177.

Jellinger KA (2008) Neuropathological aspects of Alzheimer disease, Parkinson disease and frontotemporal dementia. Neurodegener Dis 5:118-121.

Jenner P (2003a) The contribution of the MPTP-treated primate model to the development of new treatment strategies for Parkinson's disease. Parkinsonism Relat Disord 9:131-137.

Jenner P (2003b) The MPTP-treated primate as a model of motor complications in PD: primate model of motor complications. Neurology 61:S4-11.

Jenner P (2009) From the MPTP-treated primate to the treatment of motor complications in Parkinson's disease. Parkinsonism Relat Disord 15 Suppl 4:S18-23.

Jenner P, Rupniak NM, Rose S, Kelly E, Kilpatrick G, Lees A, Marsden CD (1984) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in the common marmoset. Neurosci Lett 50:85-90.

Jin J, Meredith GE, Chen L, Zhou Y, Xu J, Shie FS, Lockhart P, Zhang J (2005) Quantitative proteomic analysis of mitochondrial proteins: relevance to Lewy body formation and Parkinson's disease. Brain Res Mol Brain Res 134:119-138.

Jin LJ, Schlesinger F, Song YP, Dengler R, Krampfl K (2010) The interaction of the neuroprotective compounds riluzole and phenobarbital with AMPA-type glutamate receptors: a patch-clamp study. Pharmacology 85:54-62.

Joshua M, Adler A, Bergman H (2009) The dynamics of dopamine in control of motor behavior. Curr Opin Neurobiol 19:615-620.

Junn E, Mouradian MM (2002) Human alpha-synuclein over-expression increases intracellular reactive oxygen species levels and susceptibility to dopamine. Neurosci Lett 320:146-150.

Kalaria RN, Mitchell MJ, Harik SI (1987) Correlation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity with blood-brain barrier monoamine oxidase activity. Proc Natl Acad Sci U S A 84:3521-3525.

Kirchhoff J, Mork A, Brennum LT, Sager TN (2009) Striatal extracellular dopamine levels and behavioural reversal in MPTP-lesioned mice. Neuroreport 20:482-486.

Koh JY, Kim DK, Hwang JY, Kim YH, Seo JH (1999) Antioxidative and proapoptotic effects of riluzole on cultured cortical neurons. J Neurochem 72:716-723.

Kretschmer BD, Kratzer U, Schmidt WJ (1998) Riluzole, a glutamate release inhibitor, and motor behavior. Naunyn Schmiedebergs Arch Pharmacol 358:181-190.

Kuhn K, Wellen J, Link N, Maskri L, Lubbert H, Stichel CC (2003) The mouse MPTP model: gene expression changes in dopaminergic neurons. Eur J Neurosci 17:1-12.

Lacomblez L, Dib M, Doppler V, Faudet A, Robin V, Salachas F, Bensimon G, Meininger V (2002) [Tolerance of riluzole in a phase IIIb clinical trial]. Therapie 57:65-71.

Lai YY, Siegel JM (1990) Muscle tone suppression and stepping produced by stimulation of midbrain and rostral pontine reticular formation. J Neurosci 10:2727-2734.

Lakke JP (1985) Axial apraxia in Parkinson's disease. J Neurol Sci 69:37-46.

Lamanauskas N, Nistri A (2008) Riluzole blocks persistent Na+ and Ca2+ currents and modulates release of glutamate via presynaptic NMDA receptors on neonatal rat hypoglossal motoneurons in vitro. Eur J Neurosci 27:2501-2514.

Lang AE, Lozano AM (1998) Parkinson's disease. First of two parts. N Engl J Med 339:1044-1053.

Langston JW, Ballard P, Tetrud JW, Irwin I (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219:979-980.

Lapierre O, Montplaisir J (1992) Polysomnographic features of REM sleep behavior disorder: development of a scoring method. Neurology 42:1371-1374.
Le Couteur DG, Muller M, Yang MC, Mellick GD, McLean AJ (2002) Age-environment and gene-environment interactions in the pathogenesis of Parkinson's disease. Rev Environ Health 17:51-64.
Lees AJ, Selikhova M, Andrade LA, Duyckaerts C (2008) The black stuff and Konstantin Nikolaevich Tretiakoff. Mov Disord 23:777-783.
Levey AI, Hersch SM, Rye DB, Sunahara RK, Niznik HB, Kitt CA, Price DL, Maggio R, Brann MR, Ciliax BJ (1993) Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. Proc Natl Acad Sci U S A 90:8861-8865.
Lewitt PA (2008) Levodopa for the treatment of Parkinson's disease. N Engl J Med 359:2468-2476.
Li KW, Miller S, Klychnikov O, Loos M, Stahl-Zeng J, Spijker S, Mayford M, Smit AB (2007) Quantitative proteomics and protein network analysis of hippocampal synapses of CaMKIIalpha mutant mice. J Proteome Res 6:3127-3133.
Li LH, Qin HZ, Wang JL, Wang J, Wang XL, Gao GD (2009) Axonal degeneration of nigra-striatum dopaminergic neurons induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. J Int Med Res 37:455-463.
Lima MM, Andersen ML, Reksidler AB, Vital MA, Tufik S (2007) The role of the substantia nigra pars compacta in regulating sleep patterns in rats. PLoS ONE 2:e513.
Luchtman DW, Shao D, Song C (2009) Behavior, neurotransmitters and inflammation in three regimens of the MPTP mouse model of Parkinson's disease. Physiol Behav 98:130-138.
Mandel S, Weinreb O, Youdim MB (2003) Using cDNA microarray to assess Parkinson's disease models and the effects of neuroprotective drugs. Trends Pharmacol Sci 24:184-191.
Mandel S, Grunblatt E, Maor G, Youdim MB (2002) Early and late gene changes in MPTP mice model of Parkinson's disease employing cDNA microarray. Neurochem Res 27:1231-1243.
Marek K, Jennings D (2009) Can we image premotor Parkinson disease? Neurology 72:S21-26.
Marshall JW, Ridley RM (1996) Primate models for studying functional recovery after drug treatment in stroke. European neuropsychopharmacology 9:S191-S192.
Martens DJ, Whishaw IQ, Miklyaeva EI, Pellis SM (1996) Spatio-temporal impairments in limb and body movements during righting in an hemiparkinsonian rat analogue: relevance to axial apraxia in humans. Brain Res 733:253-262.
Marti J, Santa-Cruz MC, Bayer SA, Ghetti B, Hervas JP (2007) Generation and survival of midbrain dopaminergic neurons in weaver mice. Int J Dev Neurosci 25:299-307.
Martin GM (2000) Molecular mechanisms of late life dementias. Exp Gerontol 35:439-443.
Martinet M, Montay G, Rhodes G (1997) Pharmacokinetics and metabolism of riluzole. Drugs of today 33:587-594.
Mattay VS, Fera F, Tessitore A, Hariri AR, Das S, Callicott JH, Weinberger DR (2002) Neurophysiological correlates of age-related changes in human motor function. Neurology 58:630-635.
Mayer G, Kesper K, Ploch T, Canisius S, Penzel T, Oertel W, Stiasny-Kolster K (2008) Quantification of tonic and phasic muscle activity in REM sleep behavior disorder. J Clin Neurophysiol 25:48-55.
Melamed E, Rosenthal J, Globus M, Cohen O, Uzzan A (1985) Suppression of MPTP-induced dopaminergic neurotoxicity in mice by nomifensine and L-DOPA. Brain Res 342:401-404.
Meredith GE, Totterdell S, Potashkin JA, Surmeier DJ (2008) Modeling PD pathogenesis in mice: Advantages of a chronic MPTP protocol. Parkinsonism Relat Disord.
Migliore L, Coppede F (2009) Genetics, environmental factors and the emerging role of epigenetics in neurodegenerative diseases. Mutat Res 667:82-97.
Miller WC, DeLong MR (1988) Parkinsonian symptomatology. An anatomical and physiological analysis. Ann N Y Acad Sci 515:287-302.
Mitsumoto Y, Watanabe A, Mori A, Koga N (1998) Spontaneous regeneration of nigrostriatal dopaminergic neurons in MPTP-treated C57BL/6 mice. Biochem Biophys Res Commun 248:660-663.

Miwa T, Watanabe A, Mitsumoto Y, Furukawa M, Fukushima N, Moriizumi T (2004) Olfactory impairment and Parkinson's disease-like symptoms observed in the common marmoset following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Acta Otolaryngol Suppl:80-84.

Mizoule J, Meldrum B, Mazadier M, Croucher M, Ollat C, Uzan A, Legrand JJ, Gueremy C, Le Fur G (1985) 2-Amino-6-trifluoromethoxy benzothiazole, a possible antagonist of excitatory amino acid neurotransmission--I. Anticonvulsant properties. Neuropharmacology 24:767-773.

Mizuno Y, Sone N, Saitoh T (1987) Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on activities of the enzymes in the electron transport system in mouse brain. J Neurochem 48:1787-1793.

Monaca C, Laloux C, Jacquesson JM, Gele P, Marechal X, Bordet R, Destee A, Derambure P (2004) Vigilance states in a parkinsonian model, the MPTP mouse. Eur J Neurosci 20:2474-2478.

Mori A, Ohashi S, Nakai M, Moriizumi T, Mitsumoto Y (2005) Neural mechanisms underlying motor dysfunction as detected by the tail suspension test in MPTP-treated C57BL/6 mice. Neurosci Res 51:265-274.

Moussaoui S, Obinu MC, Daniel N, Reibaud M, Blanchard V, Imperato A (2000) The antioxidant ebselen prevents neurotoxicity and clinical symptoms in a primate model of Parkinson's disease. Exp Neurol 166:235-245.

Nicklas WJ, Vyas I, Heikkila RE (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci 36:2503-2508.

Nicoletti G, Arabia G, Pugliese P, Torchia G, Pucci F, Gambardella A, Quattrone A, Zappia M (2005) Movement time and aging: a normative study in healthy subjects with the "Movement Time Analyzer". Aging Clin Exp Res 17:207-210.

Nieuwboer A, De Weerdt W, Dom R, Lesaffre E (1998) A frequency and correlation analysis of motor deficits in Parkinson patients. Disabil Rehabil 20:142-150.

Noh KM, Hwang JY, Shin HC, Koh JY (2000) A novel neuroprotective mechanism of riluzole: direct inhibition of protein kinase C. Neurobiol Dis 7:375-383.

Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, Laws ER, Jr., Lozano AM, Penn RD, Simpson RK, Jr., Stacy M, Wooten GF (2003) Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 60:69-73.

Nuytemans K, Theuns J, Cruts M, Van Broeckhoven C (2010) Genetic etiology of Parkinson disease associated with mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 genes: a mutation update. Hum Mutat.

Obinu MC, Reibaud M, Blanchard V, Moussaoui S, Imperato A (2002) Neuroprotective effect of riluzole in a primate model of Parkinson's disease: behavioral and histological evidence. Mov Disord 17:13-19.

Oerlemans WG, de Weerd AW (2002) The prevalence of sleep disorders in patients with Parkinson's disease. A self-reported, community-based survey. Sleep Med 3:147-149.

Olanow CW, Kieburtz K, Schapira AH (2008) Why have we failed to achieve neuroprotection in Parkinson's disease? Ann Neurol 64 Suppl 2:S101-110.

Ondo WG, Dat Vuong K, Khan H, Atassi F, Kwak C, Jankovic J (2001) Daytime sleepiness and other sleep disorders in Parkinson's disease. Neurology 57:1392-1396.

Pahwa R, Lyons KE (2010) Early diagnosis of Parkinson's disease: recommendations from diagnostic clinical guidelines. Am J Manag Care 16 Suppl Implications:S94-99.

Park A, Stacy M (2009) Non-motor symptoms in Parkinson's disease. J Neurol 256 Suppl 3:293-298.

Parkinson J (1817) An essay on the shaking palsy. journal of clinical neuroscience 14:223-230.

ParkinsonStudyGroup (1993) Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease. The Parkinson Study Group. N Engl J Med 328:176-183.

ParkinsonStudyGroup (2002) Dopamine transporter brain imaging to assess the effects of pramipexole vs levodopa on Parkinson disease progression. Jama 287:1653-1661.

Patil N, Cox DR, Bhat D, Faham M, Myers RM, Peterson AS (1995) A potassium channel mutation in weaver mice implicates membrane excitability in granule cell differentiation. Nat Genet 11:126-129.

Pearce RK, Collins P, Jenner P, Emmett C, Marsden CD (1996) Intraventricular infusion of basic fibroblast growth factor (bFGF) in the MPTP-treated common marmoset. Synapse 23:192-200.

Pearce RK, Owen A, Daniel S, Jenner P, Marsden CD (1997) Alterations in the distribution of glutathione in the substantia nigra in Parkinson's disease. J Neural Transm 104:661-677.

Pedersen PL (2007) Transport ATPases into the year 2008: a brief overview related to types, structures, functions and roles in health and disease. J Bioenerg Biomembr 39:349-355.

Perier C, Bove J, Wu DC, Dehay B, Choi DK, Jackson-Lewis V, Rathke-Hartlieb S, Bouillet P, Strasser A, Schulz JB, Przedborski S, Vila M (2007) Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease. Proc Natl Acad Sci U S A 104:8161-8166.

Petroske E, Meredith GE, Callen S, Totterdell S, Lau YS (2001) Mouse model of Parkinsonism: a comparison between subacute MPTP and chronic MPTP/probenecid treatment. Neuroscience 106:589-601.

Philippens IH, Hart BA, Torres G (2010) The MPTP marmoset model of Parkinsonism: a multi-purpose non-human primate model for neurodegenerative diseases. Drug Discov Today.

Philippens IH, Melchers BP, Roeling TA, Bruijnzeel PL (2000) Behavioral test systems in marmoset monkeys. Behav Res Methods Instrum Comput 32:173-179.

Poewe W, Mahlknecht P (2009) The clinical progression of Parkinson's disease. Parkinsonism Relat Disord 15 Suppl 4:S28-32.

Postuma RB, Lang AE, Massicotte-Marquez J, Montplaisir J (2006) Potential early markers of Parkinson disease in idiopathic REM sleep behavior disorder. Neurology 66:845-851.

Przedborski S, Vila M (2003) The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. Ann N Y Acad Sci 991:189-198.

Przedborski S, Jackson-Lewis V, Naini AB, Jakowec M, Petzinger G, Miller R, Akram M (2001) The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. J Neurochem 76:1265-1274.

Przybyszewski AW, Sosale S, Chaudhuri A (2006) Quantification of three-dimensional exploration in the cylinder test by the common marmoset (Callithrix jacchus). Behav Brain Res 170:62-70.

Pungor K, Papp M, Kekesi K, Juhasz G (1990) A novel effect of MPTP: the selective suppression of paradoxical sleep in cats. Brain Res 525:310-314.

Ramsay RR, Krueger MJ, Youngster SK, Gluck MR, Casida JE, Singer TP (1991) Interaction of 1-methyl-4-phenylpyridinium ion (MPP+) and its analogs with the rotenone/piericidin binding site of NADH dehydrogenase. J Neurochem 56:1184-1190.

Rascol O, Payoux P, Ory F, Ferreira JJ, Brefel-Courbon C, Montastruc JL (2003) Limitations of current Parkinson's disease therapy. Ann Neurol 53 Suppl 3:S3-12; discussion S12-15.

Ravina BM, Fagan SC, Hart RG, Hovinga CA, Murphy DD, Dawson TM, Marler JR (2003) Neuroprotective agents for clinical trials in Parkinson's disease: a systematic assessment. Neurology 60:1234-1240.

Rechtschaffen A, Kales A (1968) A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects. In. Los Angeles: Brain Information Service/Brain Research Institute, University of California.

Robinson S, Freeman P, Moore C, Touchon JC, Krentz L, Meshul CK (2003) Acute and subchronic MPTP administration differentially affects striatal glutamate synaptic function. Exp Neurol 180:74-87.

Rousselet E, Joubert C, Callebert J, Parain K, Tremblay L, Orieux G, Launay JM, Cohen-Salmon C, Hirsch EC (2003) Behavioral changes are not directly related to striatal monoamine levels, number of nigral neurons, or dose of parkinsonian toxin MPTP in mice. Neurobiol Dis 14:218-228.

Ruiz PJ, Bernardos VS, Bartolome M, Torres AG (2007) Capit timed tests quantify age-related motor decline in normal subjects. J Neurol Sci 260:283-285.
Russ H, Mihatsch W, Gerlach M, Riederer P, Przuntek H (1991) Neurochemical and behavioural features induced by chronic low dose treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the common marmoset: implications for Parkinson's disease? Neurosci Lett 123:115-118.
Sager TN, Kirchhoff J, Mork A, Van Beek J, Thirstrup K, Didriksen M, Lauridsen JB (2010) Nest building performance following MPTP toxicity in mice. Behav Brain Res 208:444-449.
Samuel D, Blin O, Dusticier N, Nieoullon A (1992) Effects of riluzole (2-amino-6-trifluoromethoxy benzothiazole) on striatal neurochemical markers in the rat, with special reference to the dopamine, choline, GABA and glutamate synaptosomal high affinity uptake systems. Fundam Clin Pharmacol 6:177-184.
Sawada M, Imamura K, Nagatsu T (2006) Role of cytokines in inflammatory process in Parkinson's disease. J Neural Transm Suppl:373-381.
Schapira AH (2009) Etiology and pathogenesis of Parkinson disease. Neurol Clin 27:583-603, v.
Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD (1989) Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1:1269.
Schenck CH, Bundlie SR, Mahowald MW (1996) Delayed emergence of a parkinsonian disorder in 38% of 29 older men initially diagnosed with idiopathic rapid eye movement sleep behaviour disorder. Neurology 46:388-393.
Scherfler C, Sather T, Diguet E, Stefanova N, Puschban Z, Tison F, Poewe W, Wenning GK (2005) Riluzole improves motor deficits and attenuates loss of striatal neurons in a sequential double lesion rat model of striatonigral degeneration (parkinson variant of multiple system atrophy). J Neural Transm 112:1025-1033.
Schmidt N, Ferger B (2001) Neurochemical findings in the MPTP model of Parkinson's disease. J Neural Transm 108:1263-1282.
Schmidt WJ, Alam M (2006) Controversies on new animal models of Parkinson's disease pro and con: the rotenone model of Parkinson's disease (PD). J Neural Transm Suppl:273-276.
Schmitz C, Hof PR (2005) Design-based stereology in neuroscience. Neuroscience 130:813-831.
Schule B, Pera RA, Langston JW (2009) Can cellular models revolutionize drug discovery in Parkinson's disease? Biochim Biophys Acta 1792:1043-1051.
Sethi KD, O'Brien CF, Hammerstad JP, Adler CH, Davis TL, Taylor RL, Sanchez-Ramos J, Bertoni JM, Hauser RA (1998) Ropinirole for the treatment of early Parkinson disease: a 12-month experience. Ropinirole Study Group. Arch Neurol 55:1211-1216.
Sherer TB, Kim JH, Betarbet R, Greenamyre JT (2003) Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol 179:9-16.
Shults CW, Oakes D, Kieburtz K, Beal MF, Haas R, Plumb S, Juncos JL, Nutt J, Shoulson I, Carter J, Kompoliti K, Perlmutter JS, Reich S, Stern M, Watts RL, Kurlan R, Molho E, Harrison M, Lew M (2002) Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline. Arch Neurol 59:1541-1550.
Smeyne RJ, Jackson-Lewis V (2005) The MPTP model of Parkinson's disease. Brain Res Mol Brain Res 134:57-66.
Smith D, Trennery P, Farningham D, Klapwijk J (2001) The selection of marmoset monkeys (Callithrix jacchus) in pharmaceutical toxicology. Lab Anim 35:117-130.
Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3:Article3.
Speciale SG (2002) MPTP: insights into parkinsonian neurodegeneration. Neurotoxicol Teratol 24:607-620.
Spiegel J, Storch A, Jost WH (2006) Early diagnosis of Parkinson's disease. J Neurol 253 Suppl 4:IV2-7.
Stack E, Ashburn A, Jupp K (2005) Postural instability during reaching tasks in Parkinson's disease. Physiother Res Int 10:146-153.

Stadtman ER, Berlett BS (1998) Reactive oxygen-mediated protein oxidation in aging and disease. Drug Metab Rev 30:225-243.
Steiger MJ, Thompson PD, Marsden CD (1996) Disordered axial movement in Parkinson's disease. J Neurol Neurosurg Psychiatry 61:645-648.
Stephan H, Baron G, Schwerdtfeger W (1980) The Brain of the Common Marmoset: Callthrix jacchus. New York: Springer Verlag.
Stephenson R, Siderowf A, Stern MB (2009) Premotor Parkinson's disease: clinical features and detection strategies. Mov Disord 24 Suppl 2:S665-670.
Stiasny-Kolster K, Doerr Y, Moller JC, Hoffken H, Behr TM, Oertel WH, Mayer G (2005) Combination of 'idiopathic' REM sleep behaviour disorder and olfactory dysfunction as possible indicator for alpha-synucleinopathy demonstrated by dopamine transporter FP-CIT-SPECT. Brain 128:126-137.
Storch A, Burkhardt K, Ludolph AC, Schwarz J (2000) Protective effects of riluzole on dopamine neurons: involvement of oxidative stress and cellular energy metabolism. J Neurochem 75:2259-2269.
Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 100:9440-9445.
Stutzmann JM, Lucas M, Blanchard JC, Laduron PM (1988) Riluzole, a glutamate antagonist, enhances slow wave and REM sleep in rats. Neurosci Lett 88:195-200.
Sydow O (2008) Parkinson's disease: recent development in therapies for advanced disease with a focus on deep brain stimulation (DBS) and duodenal levodopa infusion. Febs J 275:1370-1376.
Tandberg E, Larsen JP, Karlsen K (1998) A community-based study of sleep disorders in patients with Parkinson's disease. Mov Disord 13:895-899.
Tolosa E, Poewe W (2009) Premotor Parkinson disease. Neurology 72:S1.
Tolosa E, Gaig C, Santamaria J, Compta Y (2009) Diagnosis and the premotor phase of Parkinson disease. Neurology 72:S12-20.
Umemiya M, Berger AJ (1995) Inhibition by riluzole of glycinergic postsynaptic currents in rat hypoglossal motoneurones. Br J Pharmacol 116:3227-3230.
van der Staay FJ, Arndt SS, Nordquist RE (2009) Evaluation of animal models of neurobehavioral disorders. Behav Brain Funct 5:11.
van Hilten B, Hoff JI, Middelkoop HA, van der Velde EA, Kerkhof GA, Wauquier A, Kamphuisen HA, Roos RA (1994) Sleep disruption in Parkinson's disease. Assessment by continuous activity monitoring. Arch Neurol 51:922-928.
van Vliet SA, Vanwersch RA, Jongsma MJ, Olivier B, Philippens IH (2008a) Therapeutic effects of Delta9-THC and modafinil in a marmoset Parkinson model. Eur Neuropsychopharmacol 18:383-389.
van Vliet SA, Vanwersch RA, Jongsma MJ, van der Gugten J, Olivier B, Philippens IH (2006) Neuroprotective effects of modafinil in a marmoset Parkinson model: behavioral and neurochemical aspects. Behav Pharmacol 17:453-462.
van Vliet SA, Blezer EL, Jongsma MJ, Vanwersch RA, Olivier B, Philippens IH (2008b) Exploring the neuroprotective effects of modafinil in a marmoset Parkinson model with immunohistochemistry, magnetic resonance imaging and spectroscopy. Brain Res 1189:219-228.
Verhave PS, Vanwersch RA, van Helden HP, Smit AB, Philippens IH (2009) Two new test methods to quantify motor deficits in a marmoset model for Parkinson's disease. Behav Brain Res 200:214-219.
Visanji NP, Gomez-Ramirez J, Johnston TH, Pires D, Voon V, Brotchie JM, Fox SH (2006) Pharmacological characterization of psychosis-like behavior in the MPTP-lesioned nonhuman primate model of Parkinson's disease. Mov Disord 21:1879-1891.
von Campenhausen S, Bornschein B, Wick R, Botzel K, Sampaio C, Poewe W, Oertel W, Siebert U, Berger K, Dodel R (2005) Prevalence and incidence of Parkinson's disease in Europe. Eur Neuropsychopharmacol 15:473-490.

Wang SJ, Wang KY, Wang WC (2004) Mechanisms underlying the riluzole inhibition of glutamate release from rat cerebral cortex nerve terminals (synaptosomes). Neuroscience 125:191-201.
Ward NS, Frackowiak RS (2003) Age-related changes in the neural correlates of motor performance. Brain 126:873-888.
Wichmann T, DeLong MR (2003) Pathophysiology of Parkinson's disease: the MPTP primate model of the human disorder. Ann N Y Acad Sci 991:199-213.
Wichmann T, Kliem MA, DeLong MR (2001) Antiparkinsonian and behavioral effects of inactivation of the substantia nigra pars reticulata in hemiparkinsonian primates. Exp Neurol 167:410-424.
Willner P (1986) Validation criteria for animal models of human mental disorders: learned helplessness as a paradigm case. Prog Neuropsychopharmacol Biol Psychiatry 10:677-690.
Wolthuis OL, Groen B, Philippens IH (1994) A simple automated test to measure exploratory and motor activity of marmosets. Pharmacol Biochem Behav 47:879-881.
Wolthuis OL, Groen B, Busker RW, van Helden HP (1995) Effects of low doses of cholinesterase inhibitors on behavioral performance of robot-tested marmosets. Pharmacol Biochem Behav 51:443-456.
Xi J, Zhang SC (2008) Stem cells in development of therapeutics for Parkinson's disease: a perspective. J Cell Biochem 105:1153-1160.
Yacoubian TA, Standaert DG (2009) Targets for neuroprotection in Parkinson's disease. Biochim Biophys Acta 1792:676-687.
Yi PL, Tsai CH, Lu MK, Liu HJ, Chen YC, Chang FC (2007) Interleukin-1beta mediates sleep alteration in rats with rotenone-induced parkinsonism. Sleep 30:413-425.

About the author

Nelleke Verhave was born on January 18th, 1981 in Nijmegen. She grew up in Malden near Nijmegen as the youngest of four girls and a daughter of two biologists. In 1999 she obtained her Gymnasium diploma at the Nijmeegse Scholen Gemeenschap Groenewoud. Nelleke studied Animal Science at Wageningen University (WUR). Here she learned to use animals for human use in the broadest sense of the word. She specialized in using animals for experiments in neuroscience and behavior. For this she studied the anticipation behavior of the catfish as a measure for animal welfare, supervised by dr. Dinand Ekkel. Thereafter she studied the changes in metabotropic Glutamate receptor expression in several brain regions in the stress-sensitized rat as a model for post traumatic stress disorder at the Rudolf Magnus Institute in Utrecht, supervised by dr. Bo de Lange. She finished her duties for a Master's degree with a practical period, studying reproduction and stress in sheep and lambs at Massey University, New Zealand. Besides her study, Nelleke was active in the Animal Science student community in several committees and in the Student Chaplaincy in committees and as a board member. She also participated in discussions on the use and welfare of all animal experiments done at WUR, in the university's Animal Ethics Committee. She worked as an education assistant and as a research technician in the Ethology department and in the Adaptation Physiology Department at WUR. In 2006, Nelleke started her PhD project at TNO Defence, Security and Safety, Rijswijk. Her studies

on animal models for Parkinson's disease were supervised by dr. Ingrid Philippens and prof. dr. Guus Smit. Together with fellow graduate students Nelleke discussed and added to several research projects at TNO and organized leisure activities after work. Besides her interest in neuroscience and behavior which resulted in this thesis, she stayed active in the field of animal ethics. She now is a member of both the TNO and the ABC (University and Medical Centre Utrecht) Animal Ethics Committees. While looking for a full time position in the animal experimentation field, she is coordinating a decommissioning project for Merck. Meanwhile, Nelleke became an experienced aunt to 13 nieces and nephews and she married to Alex Bruijnis. Together they enjoy cycling and spending time with familiy and friends, they live in Delfgauw and are expecting their first child in August 2011.

Publications

Verhave, PS, Li KW, Van Kesteren RE, Van der Schors RC, Van der Loo RJ, Gouwenberg Y, Van de Berg, WDJ. Kimenai DM, Philippens, IHCHM, Smit AB. Striatal synaptic proteome in a mouse MPTP infusion model for early Parkinson's disease. *Manuscript submitted*

Verhave PS, Jongsma MJ, Van den Berg RM, Vanwersch RAP, Smit AB, Philippens IHCHM. Neuroprotective effects of riluzole in early phase Parkinson´s disease on clinically relevant parameters in the marmoset MPTP model *Manuscript submitted*

Verhave PS, Jongsma MJ, Van den Berg RM, Vanwersch RAP, Vis JC, Smit AB, Van Someren EJW Philippens IHCHM. REM Sleep Behavior Disorder in the marmoset MPTP model for early Parkinson's disease Sleep *Manuscript in press*

Verhave PS, Vanwersch RAP, van Helden HP, Smit AB, Philippens, IHCHM (2009). Two new test methods to quantify motor deficits in a marmoset model for Parkinson's disease. Behav Brain Res 200(1):214-219.

Stam R, de Lange RP, Graveland H, Verhave PS, Wiegant VM (2007). Involvement of group II metabotropic glutamate receptors in stress-induced behavioural sensitization. Psychopharmacology 191(2):365-375.

Dankwoord

De afgelopen jaren heb ik met veel plezier tijd besteed aan dit proefschrift. Tijdens mijn werk maar vooral ook daarnaast heb ik steun, gezelligheid en afleiding ervaren van collega's vrienden en familie. Ik voel mij gezegend met een rijk sociaal leven en ik geniet met volle teugen van jullie!

Ingrid, je bent in praktisch ieder opzicht mijn tegenpool, maar we delen het enthousiasme in het onderzoek. Ik heb van jou geleerd hoe je je staande kan houden in een werkomgeving, zowel tussen de onderzoekers als tussen de managers. Ik ben heel blij met jou als copromotor!

Guus, bedankt voor de gelegenheid die je me bood om in jouw lab mijn weg te vinden. Ik heb veel geleerd van de discussies over mijn werk. Met jou zat er vaart in mijn werk, bedankt voor je rol als promotor!

Marjan en Roland, niemand anders kan er aan mijn zijde zitten als jullie het niet zijn. Marjan, ik heb met veel plezier van je geleerd, maar ik heb je met nog meer plezier weggestuurd. Ik heb diepe respect voor je lef! Roland, bedankt voor je leergierigheid en je inzet. We hebben heel wat stagiaires zien komen en gaan, maar met veel plezier heb ik het Wolthuis tot op de laatste snik met je bemand.

Sanneke, het is een gespreid bedje als er een aio al zoveel werk heeft gedaan. Ik ben heel erg dankbaar voor al je tips, maar vooral voor alle gezelligheid. Ik ben blij dat het contact

gebleven is! Raymond, bedankt voor je eindeloze technische ideeën, maar vooral ook je gevoel voor humor.
Een aantal co-auteurs wil ik graag bedanken omdat zij zich erg in hebben gezet voor de artikelen. Herman, bedankt voor je hulp bij TNO, bij het Parkinson rapport en het artikel dat daar als eerste uit voort kwam. Eus, bedankt voor je inzichten en inzet voor de slaap-data. José, bedankt voor je enorme enthousiasme bij het uitwerken van deze data. Dat heeft aanstekelijk gewerkt. Ronald en Ka Wan, bedankt voor de inleiding in de eiwitten van de synaps. Wilma, bedankt voor je gastvrijheid en hulp met de stereologie. Dorien, een betere stagiair had ik me niet kunnen wensen.
Aio zijn doe je niet alleen. Marloes, Dorien en Margo lang leve de aio-meeting, die vervolgens veranderde in het Beren-eten. Ik weet dat we elkaar nog regelmatig zullen blijven zien! Cathrin, heerlijk om tijdens de laatste loodjes samen te lunchen in het zonnetje.
Collega's TNO bedankt! Roos, Helma, Marcel, Marcel, Jan, Daan, Jitse, Esther, Rose, Netty, Madelon, Linda, Wendy, Daan, Ton, Willem, Dory, Ingeborg, Pascalle, Jeroen, Stijn, Jeroen, Martijn, Duurt, Annemieke, Maarten en Judith.
Collega's MCN bedankt! Roel, Rolinka, Yvonne, Pryanka, Nutabi, Pieter, Marlène, Eva, Ka Wan, Ronald, René, Sabine, Maarten, Danielle, Harold, Patricia, Michel, Jorn, Remco, Marion, Tariq, Ning, Anya, Nikel, Josephine, Loek, Bart, Pim, Jochem en Brigitte.
Vriendinnen uit Wageningen: Marieke, Karianne, Erna en mijn studievrienden Freerk, Lia, Marieke, Anke, Jehan, Pieter en Danitsja, fijn dat ik jullie ook nu nog regelmatig zie! En al vanaf peuter en puber lieve vriendinnen, bedankt Martine en Bregje.
Lieve Familie Bruijnis een intense periode vol verdriet met Ralph nu in ons hart. Agnes, Astrid, Coen, Karin, Thijs, Else, Carlijn, Anouk, Iris, Lars en Niels. Ik voel me thuis in jullie midden en als Bruijnis.
En bedankt, hartstikke bedankt! Pap en Mam, Willemijn, Jeroen, Jacobien, José, Johanneke, Andreas, Janne, Gijs, Dorus, Mats, Nathan, Tamar en Henrik. Grootgebracht door fantastische ouders met drie lieve zussen waar ik enorm tegenop kijk. Ik ben er trots op dat ik jullie dochter, kleine zusje, schoonzusje en tante ben!
Alex, je hebt ooit gezegd: "jij de d ik de r". Je hebt ze allebei verdiend. Je geeft me een berg aan vertrouwen en je maakt mijn leven leuk, uitdagend, interessant en vol van liefde.