A Slow Progressive MPTP Marmoset Model for Idiopathic Parkinson's Disease

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GENERAL INTRODUCTION

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PARKINSON'S DISEASE

Clinical manifestation

Parkinson's Disease (PD) is the second most common neurodegenerative disorder [35, 174], with a prevalence of 0.3-1% that increases to 5-10% at ages above 65 [66, 174]. The male to female ratio varies among studies between 1.4-3.7, with males being more prone to develop PD [174]. PD was first described by James Parkinson in "An essay of the shaking palsy" in 1817 (reprint: [129]). Nowadays, roughly 200 years later, the observations made by Dr. Parkinson are still of clinical relevance for the diagnosis of the disease. PD is initially characterized by motor symptoms such as resting tremor, postural instabilities, bradykinesia (slow movement), disturbances in initiating and terminating movements, rigidity and difficulty with swallowing and speech [40, 66, 111, 191]. However, non-motor symptoms associated with PD can often reduce the patients' quality of life even more than motor aspects [122]. Non-motor symptoms associated with early PD and generally presenting in a prodromal phase are REM-sleep behaviour disorder (RBD), constipation and hyposmia (loss of smell) [23, 24, 111, 122]. Later on in the course of disease, patients may also experience cognitive impairments, depression, other sleep abnormalities, dementia and autonomic dysfunction [40, 82, 158]. Early non-motor symptoms are often too general for diagnosis of PD, however, can be used as risk indicators as individuals showing these non-motor symptoms are frequently diagnosed with PD at a later stage [122].

Risk factors

It is important to distinguish between early-onset and late-onset PD when addressing the issue of risk factors. Early-onset PD is often familial and caused by genetic predisposition resulting in dopaminergic cell loss [111]. A variety of processes involved in PD are linked to gene mutations, such as those in genes encoding PINK1, PARKIN, DJ-1, LRRK2, MAPT, SNCA [31, 35, 40, 162]. Early-onset PD is observed in patients younger than 40 and reflects 5% of the total PD-population [122]. The largest population of patients is diagnosed with late-onset PD also known as idiopathic or the sporadic form of PD. For these patients age is the largest risk factor [40, 144], although history of anxiety or depression and pesticide exposure are also associated with PD [122]. More recently also head trauma was proposed as a risk factor [79].

Proposed protective mechanisms are found to correlate negatively with PD, such as use of non-steroidal anti-inflammatory drugs (NSAID) or calcium channel blockers, coffee drinking, hypertension and use of alcohol and nicotine [122]. Moreover, oestrogen has been proposed as a possible neuroprotective mechanism, maybe explaining why males are more prone to develop PD [174]. In line with this, early menopause, hysterectomy, or ovariectomy increases female risk relative to male risk, possibly due to the loss of oestrogen [139, 143].

Sleep disorders (in particular RBD) and olfactory dysfunction are non-motor symptoms that are not exclusively linked to PD, and arise in the early phase of the disease often before diagnosis [60, 141, 145]. In 38-75% of the RBD patients PD develops at a later stage, and 30-65% of PD patients suffer from RBD [78, 141, 145].

Neuropathology

PD is hallmarked by dopaminergic (DA) cell loss in nigro-striatal regions. DA cell loss can be observed in the substantia nigra, but also as a loss of dopaminergic terminals in the caudate nucleus and putamen [31, 33, 49, 89]. Dopamine finds its origin in the substantia nigra and ventral tegmental area, which project to the basal ganglia and frontal cortical regions [17, 39, 64, 93, 164]. The basal ganglia are functionally involved in motor execution, motivation and reward related behaviour [20, 42], whereas the frontal cortex controls executive planning, decision-making and higher cognitive processes [131, 199]. Cell loss in the substantia nigra causes loss of dopamine-innervation to the striatal regions, the so-called direct pathway. The indirect pathway involves the globus pallidus, thalamus and frontal cortex, and is affected through feedback and feedforward loops by glutamatergic and GABA-ergic projections. Dysfunction of both pathways result in the above-mentioned symptoms [17].

Besides pathological features observed in the nigro-striatal systems, PD is also hallmarked by the presence of Lewy bodies in cells of the brain [2, 82, 114], named after Frederic Lewy (1885-1950). Lewy bodies are aggregated clusters of the protein α-synuclein [46, 80, 109]. Whereas the link between dopamine and PD was already established in the early 1950's, α-synuclein deposits were first identified as the main component of Lewy bodies in the late 1990's [162]. These protein aggregates can increase with age and are classified according to Braak-staging [12]. Appearance of Lewy bodies starts in the brainstem and spreads to more frontal regions of the brain over time [80]. Lewy bodies are formed from monomers of α-synuclein, which coagulate to oligomers and finally evolve into highly insoluble fibrillar aggregates [46]. Debate is still ongoing which if these appearances of α-synuclein is most toxic to the cell [155]. Lewy bodies are not exclusively involved in PD, but are also found in Lewy body dementia (LBD) in which patients suffer from dementia, but lack the dopaminergic cell loss and accompanying motor symptoms [61, 114]. A causal role of Lewy bodies in non-motor related symptoms is evident [155, 173].

Mechanisms of neurodegeneration

It is deemed unlikely that neuronal cell death in PD emerges from one single factor, and instead likely results from a multifactorial cascade of deleterious events. The different processes involved besides protein aggregation are oxidative stress, proteasomal dysfunction, mitochondrial dysfunction, inflammatory responses and excitotoxicity [19, 31, 40, 111, 133, 191]. All are intertwined, and dysfunction in one process may lead to disturbances in the others (Figure 1).

Excitoxicity occurs when neurons are exposed to high levels of glutamate. The ensuing activation of multiple cascades via AMPA receptors, NMDA receptors, and voltage-gated calcium channels results in an increased influx of extracellular calcium, leading to cell death [19, 92].

Mitochondria are the main source of energy supply in a cell, generating ATP by oxidative phosphorylation of glucose [57]. When the cell does not die as a result of the energy depletion, dysfunctional mitochondria will produce increased amounts of reactive oxygen species (ROS) [1]. Oxidative stress can be characterized as a state in which a cell is insufficiently capable of keeping levels of ROS below a toxic threshold [1, 162]. ROS levels can increase due to a variety of processes. Several neurotoxins, among others, 1-methyl-4-fenyl-1,2,3,6-

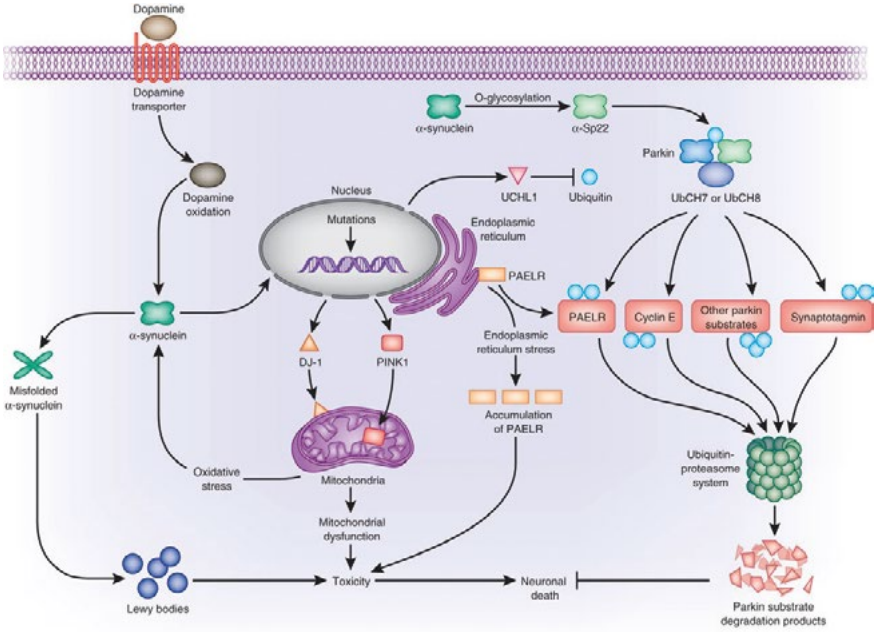

Figure 1: Schematic overview of proposed mechanisms and interactions in the dopaminergic cells in PD (adapted from [123]). Pathology seen in PD patients is most likely a result of disturbances of multiple processes in the dopaminergic cells in the substantia nigra.

tetrahydropyridine (MPTP) and paraquat, lead to deficiencies in the complex I of the mitochondria [27, 43, 47]. Additionally, mutations in genes encoding SNCA, Parkin, DJ-1, and LRRK2, have been linked to malfunction of several processes, including mitochondrial dysfunction [33, 40], which consequently may result in elevated ROS levels [62]. Furthermore, oxidative stress can be due to lack of homeostasis in antioxidants in the brain, exposure to environmental toxins, high consumption of oxygen in the brain, or low levels of radical scavenging enzymes [62]. Likewise, increased iron and copper concentrations cause an increase in ROS levels in PD [63, 112]. Dopamine itself is not a source for oxidative stress, but toxic intermediates derived from its catabolism are [31]. On the other hand, homovanillic acid (HVA), a metabolite of dopamine, is a potential antioxidant [134]. It can thus be envisaged that depletion of dopamine may reduce the antioxidant protection of the neurons. Furthermore, problems with calcium influx by overstimulation of glutamate receptors and the selective vulnerability of the L-type calcium channels may lead to oxidative stress in dopaminergic neurons [62].

Healthy protein homeostasis of a cell warrants degradation of improperly folded or otherwise dysfunctional proteins [84]. Normally, ubiquitin forms a complex with these proteins and uses activating, conjugating and ligating enzymes to complete protein degradation [75]. Malfunction of this system leads to protein aggregates and high levels of misfolded proteins, which in the end lead to cell death. Errors in the ubiquitin-proteasome system (UPS) arise mainly as a consequence of malfunctioning of UPS-involved enzymes by gene mutations, i.e. PARKIN and UCH-L1 [27, 75, 84].

Inflammatory processes in the brain involve activated microglia and astrocytes, which in turn secrete substances causing neuronal cell death [6, 9, 37]. Post-mortem studies revealed elevated levels of inflammatory markers in the brain of PD-patients, such as TNF-alpha, IL1-beta, IFN-gamma [40]. These inflammatory markers are found in the nigro-striatal circuitry, and also throughout the brain in structures such as hippocampus and frontal cortex [37]. In addition to the central nervous system (CNS), there is also a peripheral immune response observed in the serum of PD-patients mediated by several cytokines [167], with recently proposed the involvement of the gut [118]. It is still debated whether the peripheral and CNS immune responses are a cause or a consequence of the disease, however, the involvement of the immune system is evident [37, 167]. Separately or synergistically, these different pathological events all play a role in the neurodegeneration of brain structures involved in PD.

Treatment

Treatment is only available for alleviating symptoms for the patient and this does not stop or slow down disease progression. The majority of patients is treated with dopaminergic medication such as L-dihydroxy-phenylalanine (L-dopa) and DUO-dopa [157]. Treatment can also be supported by anticholinergic drugs or enzyme inhibitors, e.g. catechol-O-methyl transferase (COMT) inhibitors, and monoamine oxidase-B (MAO-B) inhibitors [40, 190]. As with many drug treatments, adverse effects and tolerance to the drug may occur. Tolerance often necessitates the increase of drug dosage and thereby increasing the number, duration or severity of side effects [186]. In addition, also physical therapy can improve the quality of life for PD patients [41, 152], which obviously can be offered in parallel to pharmacological treatment. There is also a part of the PD patients that benefits from deep brain stimulation (DBS). With this invasive method, an intracranial electrode is surgically placed and pulses of electricity are administered in a coherent matter to improve PD symptoms [40, 210]. Although patients can benefit greatly from this type of treatment, it is also associated with the adverse effects and risk of complications during the surgery [67, 99]. In addition, selection criteria for these surgeries are strict and not every patient qualifies due to age, medication history or poor general health [106].

More research is necessary to improve treatment opportunities for patients. Thereby, it is crucial to make use of clinical relevant research models.

ANIMAL MODELS OF PD

Types of PD models

Different animal models are available that mimic PD symptoms and dopaminergic cell loss. As mentioned, PD has not one single cause, and most likely different systems are involved in PD pathology. Consequently, multiple animal models are available reflecting different aspects of PD. As quoted by George Box: “All models are wrong, but some are useful” [11]. None of the available models replicates the complexity of PD, but some can be used to gain insight in certain aspects of the disease.

Genetic models include different transgenic, knockout and knock-in animals as well as overexpression models of particular PD genes. In most cases mice are used as the model organism, because of the relatively low costs and the relative ease of genetic modification. Using these models, different genes were found to play a role in the pathology, onset, mechanism of action or affected pathway in PD [31, 40]. Most commonly used genetic variants are DJ-1 and PINK1 mutated animals, however also other PD genes have been genetically manipulated [159, 162].

Besides the genetic models, chemically induced models are available. Several neurotoxins can be administered to induce PD-like symptoms via different pathways. 6-Hydroxydopamine (6-OHDA) and paraquat cause direct oxidative stress, whereas intracerebral injections of lipopolysachariden (LPS) cause nitrative stress (caused by reactive nitrogen species) and an innate inflammatory response [40, 47]. Proteosomal dysfunction can be induced by Z-Ile-Glu(OBut)-Ala-Leu-al (PSI), epoxymycin and lactacystin [205], while quinolinic and ibotinic acid cause immediate excitotoxicity [40]. Rotenone and MPTP are involved in mitochondrial dysfunction of dopaminergic neuronal cells [31, 47, 97, 126]. MPTP is most frequently used in animal models to induce PD [142] and is also the neurotoxin of choice in the studies reported in this thesis.

The MPTP model

MPTP was discovered as a side-product formed during the chemical synthesis of meperidine, a synthetic form of heroin [96, 97, 164]. Unfortunately, drug users developed PD-like symptoms after the use of MPTP-polluted synthetic heroin. PET scans confirmed similar damage to striatal regions as seen in PD patients, already after mild exposure [96, 164]. Quickly thereafter, researchers started to investigate MPTP and its relevance to Parkinson, leading to the now preferred protocols for modelling PD in animals [31].

MPTP readily crosses the brain-blood barrier and is metabolized to MPDP by MOA-B, and subsequently converted to MPP+ by glial cells [153] (Figure 2, next page). MPP+ is then taken up via the dopamine transporter (DAT) into DA neurons where it binds to complex 1 of the mitochondrial electron transport chain [96]. As mentioned, mitochondria are the energy suppliers of the cell, and mitochondrial dysfunction leads to a multitude of problems inside the cell that ultimately result in cell death. Thus MPTP intoxication resembles both the dopaminergic cell loss and the behavioural and neurochemical deficits seen in PD patients [47, 164]. Although MPP+ is the metabolite that actually causes these mitochondrial deficits, it is common to refer to MPTP as the toxic agent in this model, as the authors will also do in this thesis.

Different dosing regimes of MPTP lead to induction of different mechanisms of cell death. Acute administration of high doses of MPTP leads to rapid degeneration through necrotic cell death [121, 126, 189], whereas subchronic regimes using lower MPTP doses administered over a longer period of time achieve delayed degeneration with apoptotic cell death [83, 94, 148]. However, a progressive chronic model, in which even lower doses of MPTP are administered over a longer period of time, often with more time between injections, is generally regarded the preferred animal model of PD [70, 90, 150]. It best resembles PD and allows investigation of early disease symptoms that closely mimic disease stages at which

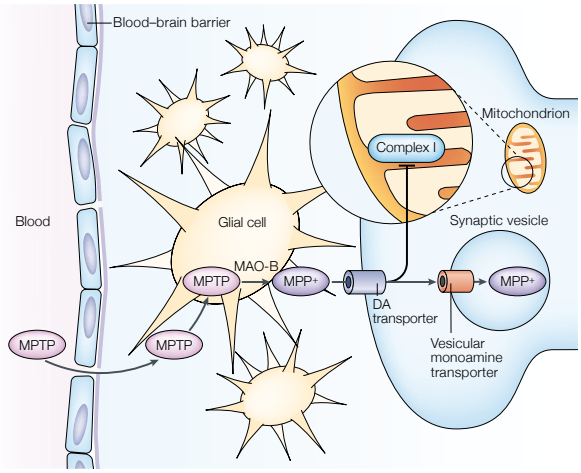

Figure 2: MPTP metabolism in the brain (adapted from [185]). MPTP crosses the blood-brain barrier and is metabolized to MPDP by MAO-B in glia cells and then converted to MPP+, the latter of which is then taken up via the dopamine transporter into DA neurons. MPP+ binds subsequently to complex 1 of the mitochondrial electron transport chain.

patients are often diagnosed. Of note, animals treated with anti-PD drugs after MPTP admission, react in a similar fashion as patients do [83].

In summary, MPTP causes dopaminergic cell loss and induces similar clinical and pathological features as observed in PD, which makes the MPTP model a very important and clinically relevant model for studies of PD. In addition, the MPTP model matches all four criteria of validity according to van der Staay et al.; reliability and replicability (internal validity), predictive validity, construct validity, and external validity (i.e. generalizability) [175].

Non-human primate models

Many experiments have been performed with MPTP on different species to test the efficacy of different treatment regimes. Mice and monkeys have been the preferred choice [31], whereas rats have a different metabolism of MPTP, leading to virtually no MPP+ in the brain after MPTP administration [154]. Intracerebral injections with MPP+ in the rats however led to similar dopaminergic cell loss as seen in mice and non-human primates [200].

Non-human primate studies are primarily performed in macaques or common marmosets. Because non-human primates are more similar to humans at all levels of analysis, primate research has a high validity with respect to many human diseases [180]. For PD research specifically, non-human primates share with humans a very similar functional organization of the striatal regions [70, 137]. As a consequence, monkeys have better developed fine motor skills than rodents, which is important for testing of motor-related dysfunctions in PD. In addition, non-human primates have a vertical body position, which allows better analysis of postural imbalances characteristic of PD.

In particular common marmosets are frequently used in the MPTP model [56, 69, 70, 83, 148, 206]. These New World monkeys are genetically approximately 96% identical to humans [88, 192], are small in size, easy to handle and survive well in captivity. In addition, research with marmoset has proven to reduce costs and safety hazards in comparison with Old World monkeys [47].

PD RESEARCH IN MARMOSETS

Monitoring parkinsonian behaviours in marmosets

PD patients experience motor and non-motor dysfunction throughout the disease course. Therefore, different behavioural tasks have been developed to monitor several PD-related symptoms, both motor and non-motor symptoms, in the marmoset (Figure 3). Non-motor symptoms measured in this study are clinical observations, home-cage activity and observations of fear- and arousal-related behaviours. Motor behaviour is examined by investigating jumping behaviour, locomotor activity, axial movement and fine motor skills, such as hand-eye coordination.

Key tests to assess PD-related features in marmosets are clinical observations, such as tremor, rigidity, mobility and general well being [181, 182]. In addition, evoked fear- and arousal-related behaviours can be observed in a human interaction test, in which the observer is positioned in front of the cage [177]. Moreover, an activity meter connected to the collar of the animals allows 24-hour measurements of activity in the home cage [134]. With this non-invasive method animals are followed during day and night in order to monitor their circadian rhythms and resting behaviour.

Non-motor symptoms can be complemented by several tasks testing motor behaviour. Axial movement is tested in the so-called Hourglass setup, in which animals are placed in a narrow cylinder that is turned vertically 180 degrees [182]. The time that the monkey needs to return to its upward position is a measure for axial movement capacity. Natural jumping behaviour is tested in the so-called Tower test, where animals can move freely in a vertical direction by jumping on platforms placed at different heights [182]. Spontaneous locomotor activity is measured in the so-called Bungalow-setup [132]. Here animals can freely move horizontally between four compartments that are interconnected with tubes. Finally, animals

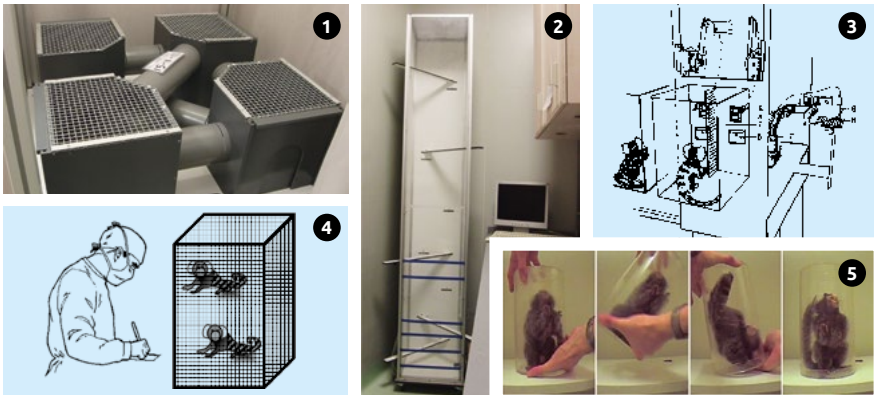

Figure 3: Behavioural assessments in the common marmoset. Bungalow (1), Tower (2), Hourglass (3) and hand-eye coordination test (5) assess motor related behaviour and clinical homecage observations (4) are concerned with non-motor related behaviour. Illustrations from the hand-eye coordination and hourglass are adapted from references [182, 198].

are subjected to a test of hand-eye-coordination (HEC). Marmosets are encouraged to display fine motor skill by reward-related grabbing of a moving or static object [197]. Together these tests capture multiple features of PD-related motor and non-motor dysfunction.

Recovery from MPTP treatment

In the studies described in this thesis, the PD-related features are well characterized by the use of different motor and non-motor related tests in the induction phase with MPTP and during the progression of the disease. Interestingly, whereas patients show an increase of neurodegeneration and aggravation of symptoms during the course of the disease [40, 191], non-human primates show an improvement of PD-related behaviour after discontinuation of MPTP treatment [69, 70, 183, 184]. This very interesting finding allows to study compensatory or recovery mechanisms that are activated after discontinuation of the MPTP challenge. These mechanisms could possibly contribute to novel strategies to delay or eventually stop neurodegeneration.

Post-mortem analyses

To find targets for a disease modifying treatment, to prevent or stop neurodegeneration or to learn about the mechanisms behind recovery, brain tissue can be collected and analysed at the final stage of the study. Pathology of brain tissue in the current study included histological, neurochemical and proteomics analyses. Cells are structurally examined by their size, shape and dendritic arbors using immunohistochemistry, and neurotransmitter levels of dopamine and its metabolites are measured using high-pressure liquid chromatography (HPLC). In addition, different brain areas were measured for protein expression using quantitative mass spectrometry. Importantly, the progressive chronic MPTP model used in this study allows us to investigate possible underlying cellular and molecular mechanism at different stages of disease progression and recovery, in the absence of dopaminergic loss. Early PD cannot yet be diagnosed with biomarkers, and patients are diagnosed when major dopaminergic cell loss is already apparent. Therefore it is of great importance to unravel the mechanisms that determine disease onset and progression prior to dopaminergic cell loss, preferably in species that resemble human PD patients as close as possible.

Proteomics

Proteins are key functional molecules that form cellular structures, act as messengers of cellular processes, and serve as the overall link between genotype and phenotype [188]. Proteins are encoded by the genome, but in contrast to genes they are the direct bio-functional mediators of all cellular functions in living organisms [207]. The term proteomics refers to the large-scale studies of proteins, including information on abundances, functions, structures (variations and modifications), along with their interacting partners and networks [18, 104, 127, 188]. The core analytical tool in proteomics technology is the mass spectrometer (MS) [18].

Typical proteomics experiments first involve separation of proteins based on their molecular weight. Subsequently, proteins are digested into smaller peptides, which are then subjected to MS analysis. In the MS, peptides are fragmented and characterized based on the

mass of the fragments. Peptide identification can be achieved by matching the spectra derived from peptide fragmentation with theoretical spectra generated from a protein database [207]. In addition, validation of candidate proteins revealed by proteomics analyses by an independent method, such as immunoblotting, is often used to confirm differences in protein levels between experimental groups [18].

In the past decade, there has been a rapid advance in the resolution, mass accuracy, sensitivity and scan rate of mass spectrometers, as well as significant improvements in bioinformatics tools used to analyse proteins and protein complexes [207]. Proteome analysis can be performed on different species, types of tissues and even different compartments of the cell, and thus provides many opportunities for research. Proteomics in PD-research has focused mainly on the differential expression of proteins in the substantia nigra between patients and healthy controls [4, 38, 104, 105]. In addition, different brain areas and subcellular structures obtained from animal models have been analysed with proteomics [34, 201, 209]. Our understanding of the molecular pathology of PD has significantly increased by the use of proteomics [18], and will continue to do so in the future, e.g. in the quest for a reliable biomarkers for PD. Possible caveats in proteomics research in the field of neurodegeneration concern that measurements may be biased due to neuronal loss [18], depending on the study design. Therefore, proteomics analysis of brain tissues as a final step towards understanding disease-causing and -modifying mechanisms in our model is promising and could potentially uncover novel therapeutic targets.

AIMS AND OUTLINE

The primary aim of the studies reported in this thesis was to gain insight into the molecular and cellular pathology underlying the clinical expression of PD. The chronic MPTP model in de common marmoset was used as a clinically relevant model of the human disease. In addition, the recovery of behaviour after discontinuation of MPTP was examined in order to investigate underlying compensatory or recovery mechanisms.

Chapter 2 describes the dynamic properties of various clinical symptoms during disease progression and recovery in the MPTP model in the common marmoset. Marmosets exposed to low doses of MPTP are behaviourally examined prior to, during and after MPTP exposure. Post-mortem morphological and molecular analyses confirmed the absence of neuronal damage or alterations in protein expression in different regions of the brain after recovery.

Variation of susceptibility to the neurotoxin MPTP is explored in Chapter 3. Two groups of animals were selected based on a difference in their response to MPTP. Behavioural and post-mortem analyses were studied in these high- and low-responders to gain insight into the mechanism involved in the difference in sensitivity to the neurotoxin, which may also underlie differences in susceptibility to PD in humans.

Chapter 4 describes the proteomics analyses of two brain areas, substantia nigra and putamen, of animals that received acute, chronic or no MPTP treatment. The data is discussed in the light of quality control and general data processing. Different angles of data exploration will be examined to loosen statistical boundaries, capture possible subtle effects and identify

protein networks. Finally, the limitations of a proteomics approach when working with a relatively small number of outbred animals is discussed.

In Chapter 5, a novel method of proteomics measurement and data analysis is presented which might solve some of the limitations observed in Chapter 4. The FASP/SWATH method was used on synaptosomal fractions of the hippocampus of the marmoset, and the data was used to investigate technical and biological variation. The data demonstrate that with these recent advances in proteomics technology it is in principle possible to obtain high-quality quantitative proteomics measurements based on a limited number of marmoset tissue samples.

The relevance of behavioural, cellular and molecular research of the marmoset in a clinical setting is discussed in Chapter 6. Implications and future perspectives are presented, including the importance of current research in the broader scope of clinical research.