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GENERAL DISCUSSION

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The primary aim of the studies reported in this thesis was to gain insight into the behavioural, molecular and cellular processes during clinical progression of Parkinson's disease (PD) and molecular and cellular processes during recovery from PD symptoms. The chronic MPTP model in the common marmoset was used as a clinically relevant model of the human disease. The novelty of this model is that by repetitive injections of low doses of the neurotoxin MPTP the toxic dose for dopaminergic neurons is built up gradually while a-specific toxicity is avoided. This leads to interesting modifications of the model compared one high dose injection: 1) In the early phase of the disease recovery of symptoms can be observed; 2) A disconnection between clinical and pathological expression (clinic-pathological paradox) was found; 3) Familiar traits of PD sensitivity were noticed.

Recovery of PD symptoms after discontinuation of MPTP was examined to investigate underlying clinically relevant compensatory mechanisms. Proteomics analysis was performed on post-mortem tissue from marmosets displaying different severities of disease manifestation or recovery thereof.

The experiments described in this thesis provided a unique opportunity to investigate and compare different stages of parkinsonism induced by MPTP administration in the common marmoset (*Callithrix jacchus*). Figure 1 shows typical expression curves of clinical signs. Also the time points of sacrifice for post-mortem tissue collection of the five experimental groups, described in Chapters 2 and 3, are indicated by arrows in the figure. The behavioural differences, measured by a clinical score, of intermediate-, high- and low- responders to MPTP, are depicted. The intermediate responders were continuously treaded with MPTP until a disease score similar to patients diagnosed with PD in de clinic was reached. Subsequently, recovery was observed until animals reached a disease score similar to the maximum disease score of the high responders.

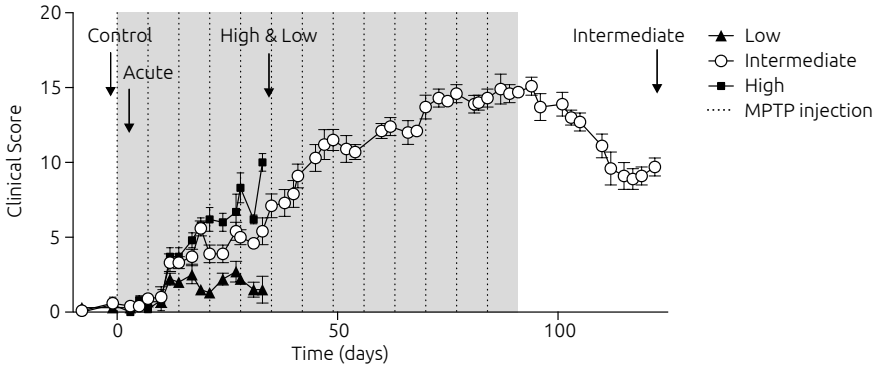

Figure 1: Clinical score observed in the five experimental groups of marmosets used in this thesis. Grey shaded area indicates the period of MPTP exposure. Dotted lines designate weekly MPTP injections. The curves represent three groups that were separated based on their clinical scores, i.e. intermediate responders (open circles), high responders (closed squares) and low responders (closed triangles). Arrows indicate the moment of euthanization of each of he treatment groups.

Clinical relevance of the MPTP Model

PD is currently diagnosed when the first symptoms are identified. At this early stage PD is already accompanied by substantial damage to several brain regions [33, 170, 191]. Unfortunately, current treatment of patients even at this early stage of disease only alleviates symptoms whereas the process of neurodegeneration cannot be slowed down or stopped [40]. The MPTP animal model has been used for many years and is a relevant preclinical model for translational research into the pathogenesis and treatment of PD [81]. MPTP was discovered as a neurotoxin and a side product of synthetic heroin in the early 1980's [164]. It selectively destroys dopaminergic neurons in the substantia nigra, mimicking the loss of DA neurons in the human PD brain as described in Chapter 1. In the marmoset model, MPTP administration leads to clinical manifestation of Parkinson symptoms, including pathological features and neurochemical alterations [44]. Although other types of models exist, such as the 6-OHDA animal model or the PC12 cell line, the MPTP animal model is preferred for the study of PD [31]. It has been widely used to unravel pathogenic mechanisms underlying the neurodegenerative component of the disease or to screen potential PD drug candidates for their ability to modify neurodegeneration. In many studies, the focus has been on acute manifestation of severe parkinsonian symptoms after a relatively high dose of MPTP [54, 189]. The studies in this thesis are an example of a refinement of this model and use a chronic low dose of MPTP to achieve an early PD-like stage and to investigate possible compensatory mechanisms that may occur before the onset of neurodegeneration. Modelling early PD might lead to the discovery of novel biomarkers, which in turn would allow earlier diagnosis of PD patients. It has been shown that early treatment is more beneficial to patients than treating later diagnosed patients [152]. In addition, a low dose protocol would potentially allow the investigation of pathogenic processes operating prior to neurodegeneration in the brain and may subsequently lead to the discovery of new disease-modifying strategies.

It is intriguing to observe that the chronic low dose MPTP treatment results in clinical symptoms in the absence of DA cell death, and this potentially offers unique opportunities to study early pathogenic cellular mechanisms underlying PD, which cannot be studied in acute MPTP models, 6-OHDA models or alpha-synuclein models which are primarily mimicking the neuropathological and neurodegenerative components of the disease. On the other hand we do have to keep in mind that it is not clear yet why and how clinical symptoms develop in the absence of DA cell death. Interestingly, this clinical-pathological paradox in humans is the reverse, i.e., clinical symptoms start to present only when a substantial degree of DA cell death has already occurred. Apparently, humans can either tolerate substantial DA cell loss or compensate early clinical signs better than monkeys. It is also possible that early symptoms in humans are not easily recognized in the course of PD, as they are not exclusively linked to PD but rather to the general processes of aging. Finally, some early linked symptoms observed in monkeys after chronic low dose MPTP exposure may only be seen in marmosets and do not manifest in a similar way in humans suffering from PD. None of these explanations invalidates our hypothesis that our chronic low dose MPTP model is relevant for early PD, however, a further characterization of the effects of low dose MPTP treatment on DA neuron function in various brain areas should be conducted in order to be conclusive on this issue.

Compared with MPTP models in rodents the chronic slow progressive marmoset MPTP model presented here also has another important advantage. By selecting animals from multiple breeding families it allowed us to consider genetic heterogeneity comparable to the human population. For this reason our model is superior to for instance MPTP mouse models, since these are often genetically identical to each other.

As a potential disadvantage it could be argued that MPTP itself does not result in the formation of Lewy bodies, as seen in PD patients. However, alpha-synuclein aggregates have recently been identified in the olfactory bulb of the aged common marmoset [87a], which offers opportunities to start investigating the link between MPTP toxicity and PD-like neuropathology more closely in the future.

Behavioural analysis

The marmoset MPTP model is clinically characterized by the expression of Parkinson-like behavioural features. We used multiple types of behaviour tests to investigate both motor and non-motor related PD symptoms. The non-motor measurements included homecage measurements. The assignment of clinical scores was to assess disease manifestation, the general well-being of the animals and to monitor the derived activity patterns to examine sleep and resting periods.

Motor-related behaviour was measured using several tasks that capture subtle differences in motor execution. Locomotor activity was scored as measurement of general activity, jumping behaviour was measured to investigate the ability to perform large movements [182], whereas the hand-eye coordination task [198] provided information on fine motor skills. Righting reflexes were tested as a measure analogous to human axial reflexes [182].

Using the slow induction protocol a gradual increase of impairment of both motor and non-motor related behaviour was observed. This slow induction protocol describes more detail the PD course in patients than the acute models, in which animals become seriously disabled within one week. The multilevel analysis in combination with slow progression of parkinsonian symptoms in the marmoset thus provides a clinically relevant model to investigate the early phase of PD.

The behavioural improvements observed after MPTP discontinuation described in Chapter 2 provided an additional opportunity to investigate the mechanism underlying clinical recovery in more detail, and might potentially lead to treatment that prevents, delays or even reverses neurodegeneration. Unfortunately, the experimental design did not allow post-mortem analysis of animals at the maximum disease score, although the recovery group was compared with the high responders that had the same clinical score. Behavioural recovery after MPTP discontinuation was probably due to the plasticity of cells and the ability to cope with mild (mitochondrial) stress. This can be concluded from the absence of dopaminergic cell loss in these animals, and is also supported by normal neurotransmitter levels compared to the high responders, suggesting a reversal of disturbed DA turnover. An intriguing question not resolved in this thesis is what causes the clinical symptoms in the absence of detectable DA damage after MPTP discontinuation.

Proteome analysis

In the absence of detectable DA cell damage, proteomics analysis might provide a way to investigate in more detail the molecular disturbances that underlie the induction and development of PD-like symptoms in the marmoset MPTP model, as well as the recovery from these symptoms after MPTP discontinuation. In Chapter 4 we attempted to investigate proteome changes in the SN and in the striatum in all five experimental groups of marmosets. Unfortunately, these experiments did not lead to reproducible and significant differences in protein expression. When the significance threshold was lowered, it became apparent that most differential regulation/expression of proteins was observed in putamen compared to the SN, which might well be in line with the dying back hypothesis described by others [156]. According to this theory, dopaminergic cell loss seen in the SN is initiated in the neurites in the target area (striatal regions) rather than at the cell body of the neuron, due to disturbed processes at the synapses preceding cell death [40]. However, we have not investigated this possibility. Of note, this interpretation should be considered with great care, because the major reason for not detecting significantly regulated proteins in the first place turned out to be variability in the samples and the measurements. Based on findings described in Chapter 5, we therefore propose to improve proteomics measurements to overcome the problem of technical variation on top of the inherently high biological variation observed in marmosets. In addition, improvements to sample dissection or protein isolation need to be considered, as alternatively, these may have hampered the analysis.

Typically, our proteomic experiments were carried out using in-gel digestion (IGD) in combination with MS analysis in a data dependant acquisition mode (DDA). A newer and promising method of isolation-MS detection, is FASP/SWATH, as examined in Chapter 5. With this method the number of steps of sample preparation are drastically reduced using a filter aided separation method (FASP) and measurement on MS is optimised using SWATH. FASP/SWATH analysis seems to introduce less variability compared to IGD/DDA and therefore should be able to identify smaller effect sizes. In Chapter 5 we used FASP/SWATH analysis only on specific cellular compartments, i.e., pre- and postsynaptic compartments of hippocampal neurons. This proof of principle experiment showed that technical variation can be reduced using FASP/SWATH. Future experiments will be needed to develop a method for also measuring total lysate using the FASP/SWATH methods.

Overall we might conclude that the technique of in-gel digestion and DDA for proteomic analysis is not the prime tool to investigate subtle differences among samples from a genetically heterogeneous group of marmosets. Secondly, as immunoblotting shows, much care has to go into sample dissection and/or protein isolation as these introduced variation as well. As stated in Chapter 4 and 5, increasing the group sizes is not an option due to ethical reasons and high costs. Increasing the effect size by treatment with MPTP for a longer period of time, in particular for high vs. low responders, might exacerbate the behavioural phenotype and probably also the differences in cellular and molecular players involved in these processes, but may not be the favoured option.

Variation and biodiversity of marmosets

Chapter 3 and 4 show the opportunities and limitations of using an outbred group of marmosets, with a biodiversity presumably comparable to human individuals. Proteomics analysis in Chapter 5 shows that marmosets are, in terms of protein expression, close to humans, and genome projects tell us that the protein sequence identity is close to 96%. Thus, given that the molecular make-up of marmosets is similar to humans, this makes them a good translational model. In addition, much variation comes from the genetic diversity of these animals, which resembles the human population. However, this is also a caveat of the current marmoset research, as effect sizes in variables must be large to achieve significant differences between groups.

Future directions

Previous Chapters presented a marmoset MPTP model that addresses the early stage of PD and also recovery from PD-like symptoms. Future experiments should examine these phenomena in more depth aiming at the discovery of new targets and development of new disease modifying strategies, taking along the recommendations from the studies described in this thesis.

In Chapter 2 we postulated that parkinsonian symptoms might be reversible when diagnosed and treated early. The slow progressive MPTP model is in principle adequate to investigate underlying mechanisms, and can be used in the search for biomarkers and screening of drugs for early staged PD. Closer examination of the recovery phase might provide additional strategies for novel therapies.

Our experiments show behavioural resemblance with PD symptoms at many different levels. Interestingly, whereas we clearly observed behavioural changes indicative of PD symptoms, no dopaminergic cell damage could be observed in these experiments. This led us to conclude that dopaminergic neurons might have been only stressed and not damaged enough to die. Taking into account that the primary neurotoxic effect of MPTP is on the mitochondria [142], we recommend to focus future analysis on the metabolism of DA neurons. Although differences in protein expression were observed in the putamen, we cannot link these non-significant observations to severity of clinical symptoms.

Techniques for integrated genomics, transcriptomics or metabolomics are already used on human tissue to characterize sickness and medication in PD. Transfer of these techniques to the chronic progressive marmoset MPTP model could provide valuable information [18, 68, 73, 77]. However, still the considerable inter-individual variation observed in the proteomics analysis needs attention first.

The differences observed between high and low responders in the current experiments could be further explored by examination of still unused post mortem tissue available of these animals. The observation that the responsiveness to MPTP-induced disease segregated into high and low responders on the basis of non-motor symptoms, mainly expressed as disturbed sleep patterns during the day, is a good starting point for further investigation whether this behavioural observation is accompanied by changes in the brain areas classically involved in the regulation of sleep, such as the brainstem [59, 74]. Also areas of the NA

pathways, such as the locus coeruleus, which are known to be involved in sleep and other non-motor symptoms [48, 146] would be of interest. Irregularities in the gastro- intestinal circuit were reported to occur already in the early phase of PD [118]. Faeces specimens of the tested animals are still available to investigate whether the non-motor symptoms that differentiated these two groups, also may include irregularities in gastro- intestinal circuit. Experimental techniques to study gene expression might reveal the cause of the different susceptibility to MPTP in high and low responders. Another aspect worthwhile examining is the ability of the animals to metabolize MPTP and to verify possible tolerance to the neurotoxin in low responders.

Since more marmosets from the families studied here are still present in the pedigreed marmoset breeding colony at BPRC, it is possible to investigate familial genetic differences in more detail. Basal neurotransmitter levels were not measured in this study, since these values were obtained from post-mortem tissue. Samples of untreated family members might shed light on basal differences between families. Microdialysis could provide more information on baseline levels of monoamines accompanied by their metabolites during MPTP administration and recovery phase. In particular, information on the NA-circuitry could provide more information on the possible neuroprotective properties of NA to MPTP administration.