Modeling early Parkinson’s disease pathology with chronic low dose MPTP treatment

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Abstract

Purpose: Parkinson's disease (PD) is a movement disorder mainly characterized by progressive neurodegeneration of dopaminergic (DAergic) neurons in the substantia nigra (SN). As yet, unknown molecular changes contribute to the development of PD leading to a great need for in vivo models that herald this disorder. Here we characterize an animal model presenting early PD pathology. Methods: Young, adult C57/BL6 mice were treated for five weeks twice a week with 15mg/kg 1-methyl-4-phenyl1,2,3,6-tetrahydropyridine (MPTP) in combination with 250mg/kg probenecid. During the treatment mice were tested on their dopamine dependent movement skills. The integrity of their nigrostriatal system was examined through immunohistochemical studies. Results: During the treatment, mice developed dopamine-dependent movement deficits induced by loss of tyrosine hydroxylase (TH) positive nigrostriatal axon terminals. Immunohistochemical study identified astrogliosis and microgliosis in the SN but no decrease of TH immunostaining, demonstrating lack of DAergic neuron degeneration. We also observed formation of α-synuclein inclusion bodies in the SN. Conclusions: The combined features of this MPTP model appear to represent an early neurotoxic cellular stress to the SN neurons bearing a striking resemblance to the early stages of PD neuropathology. This model might prove very useful to investigate early neurodegenerative events in the nigrostriatal DAergic system and to study the effects of potential treatment strategies counteracting the early PD cellular changes.
1. Introduction

Parkinson’s disease (PD), the second most prevalent age-related neurodegenerative disease, is characterized by a progressive loss of nigrostriatal dopaminergic (DAergic) terminals and a selective loss of DAergic neurons in the substantia nigra pars compacta (SN), thereby inducing a movement disorder (Dauer & Przedborski, 2003). Additionally, there is a progressive formation of ubiquitin and α-synuclein positive cytoplasmic inclusions during the progression of the disease, starting in the lower brainstem areas and ascending to the higher cortical levels of the brain (Braak et al., 2003). The etiology of PD appears to be multifactorial, involving both genetic and environmental components (Gorell et al., 2004). So far several cellular mechanisms, such as aberrant protein folding, oxidative stress and mitochondrial dysfunction have been linked to the development and progression of this disease, but these cannot fully explain the underlying neuropathology (Greenamyre & Hastings, 2004). Therefore, there is a great need in PD research for animal models to further explore the role of specific molecular players in PD pathology and to test novel, potential treatments. So far, multiple PD mouse models have been developed by either genetic manipulation (such as mice overexpressing human α-synuclein protein) or acute treatment with neurotoxic compounds that selectively damage DAergic neurons in the SN (rotenone, 6-Hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl1,2,3,6-tetrahydropyridine (MPTP) (Betarbet et al., 2002; Bove et al., 2005; Schober, 2004).

The neurotoxin MPTP was discovered in 1982, after drug addicts have been found to self-administrate this meperidine analog synthesis by-product. These addicts rapidly developed PD symptoms and proved responsive to treatment with L-DOPA. Later neuropathological examination of these patients’ brains revealed moderate to severe loss of DAergic neurons in SN (Langston et al., 1999). After crossing the blood brain barrier, MPTP is taken up by astrocytes and converted by monoamine oxidase B to its active toxic component 1-methyl-4-phenyl-pyridium ion (MPP(+)). MPP(+) is then released and taken up specifically by DAergic neurons via the dopamine transporter (DAT). In neurons, MPP(+) specifically inhibits the mitochondrial complex 1 of the electron transport chain, resulting in ATP depletion, loss of mitochondrial membrane potential and the formation of reactive oxygen species (ROS). These stress events eventually lead to the selective loss of DAergic neurons, similar to neuronal degeneration in the PD SN (Nicotra & Parvez, 2002; Beal, 2003).

In an attempt to reproduce the chronic and slowly progressive nature of PD associated neurodegeneration, a rat model was developed based on multiple increasing intrastriatal dosage of 6-OHDA (Fleming et al., 2005), and a mouse model based on multiple low dose injections of MPTP (Petroske et al., 2001). This mouse model was based on a chronic 5 week 25mg/kg MPTP treatment scheme in combination with probenecid. Probenecid inhibits MPTP clearance from the periphery and increases its efficiency in crossing the blood brain bar-
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rier to the CNS. This chronic treatment regimen, apart from inducing progressive
daergic neuron degeneration in the SN, decreased the number of daergic nerve
terminals in the striatum, the DA levels in the striatum (Petroske et al., 2001),
and induced glial reactivity in the midbrain (Schintu et al., 2009a; Schintu et al.,
2009b). Also, this treatment is the only toxicological treatment so far that can
induce the formation of α-synuclein and ubiquitin positive inclusions in the cyto-
plasm of daergic and cortical neurons (Meredith et al., 2002). Although chronic
and progressive, after a 5-week treatment period this model still represents an
end stage PD-like phenotype as most of the daergic neurons have already degener-
ated by this time.

In this study, we describe the early neurodegenerative characteristics of a
PD mouse model, based on a 5-week chronic 15mg/kg MPTP treatment scheme.
This treatment leads to movement deficits, the loss of nigrostriatal terminal
projections, gliosis in the SN and the formation of α-synuclein positive inclusion
bodies. Importantly, we did not observe daergic neuronal cell loss in this model.
These combined features bear a striking resemblance to the dying-back hypoth-
thesis in PD, where striatal denervation is the initiating event in the loss of the SN
daergic neurons (Dauer & Przedborski, 2003). Thus, we are the first to report
that this early PD model provides an interesting window of opportunity to study
the mechanisms that underlie the early neurodegenerative events that initiate
the cellular death of daergic neurons, and can be a useful tool to explore ap-
proaches to rescue the PD-phenotype during the earliest stages of development.

2. Materials and Methods

2.1. Reagents

MPTP hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO,
USA) and dissolved in 0.9% NaCl (10mg aliquots, prepared fresh before each in-
jection session). Probenecid (Sigma-Aldrich) was dissolved first in 1N NaOH and
adjusted to pH of 7.4 with 0.1M Tris-HCl (pH 6.8) prior to injection. The final con-
centration of probenecid was 12.5mg/ml. Fresh aliquots were used for each in-
jection session.

2.2. Animal care and MPTP chronic model PD generation

21 male C57/BL6 mice 12 weeks of age (weight ~25g) were used (Harlan,
Zeist, The Netherlands). Animals were housed socially (5 animals per cage) in a
standard plastic housing with food and water ad libitum, 12 hour light and dark
cycles in 20°C controlled housing facility. All the experimental procedures and
post treatment care were carried out in accordance with the Animal Experimen-
tal Committee of the Royal Netherlands Academy of Arts and Sciences.
Mice were randomly divided into three groups prior to the behavioral testing. The first group consisted of a control group (n=8) receiving subcutaneous (s.c.) saline injections along with intraperitoneal (i.p.) 250mg/kg probenecid, a MPTP clearance inhibitor having no effects on the DAergic system (Barber-Singh et al., 2009). The second group (n=6) received 15mg/kg MPTP s.c. and 250mg/kg i.p. probenecid injections and the third group (n=7) received 25mg/kg MPTP s.c and 250mg/kg i.p. probenecid in order to recreate the model previously described in Petroske et al (Petroske et al., 2001). All animals received a total of 10 injections, every 3.5 days for 5 weeks. Following the treatment, all cages were placed on heating pads overnight, and throughout the experiment animals received sucrose gel packs. Safety measures were followed according to previously published guidelines (Przedborski et al., 2001; Jackson-Lewis & Przedborski, 2007).

2.3. Behavioral testing

All animals were behaviorally tested using the grid test where movement coordination and balance were assessed. This test assesses forepaw use, in particular the use of distal musculature and digit manipulation sensitive to dopaminergic input in the striatum (Tillerson & Miller, 2003). Briefly, mice were suspended upside down on a metal grid and allowed to move freely throughout the grid. A successful trial meant that an animal had taken at least 10 steps in at least 10 seconds. Each trial lasted a maximum of 30 seconds. The number of steps with both forepaws was counted as well as each unsuccessful step with either forepaw. For each animal, the three runs were averaged and the ratio between the total forepaw foot faults/total forepaw steps was calculated (Meredith & Kang, 2006). Tests were performed once a week, always before the MPTP injections to avoid the reduction of movement bias that may occur directly after the treatment. The first test took place on the day of the first MPTP treatment and the last test was performed 3 days after the last MPTP treatment (total of 6 testing sessions).

2.4. Tissue processing

3.5 days after the last MPTP treatment all mice were sacrificed by an i.p. overdose with Pentobarbital (50mg/µl) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA, Sigma-Aldrich) in sodium phosphate buffer (PBS) pH 7.4. The brains were further post-fixed overnight and 4 series of 30µm thick coronal sections of the entire SN and the striatum were cut on a vibratome. The anatomical level of the sections was identified using Mouse Brain Atlas (Paxinos.G. & Franklin.K.B.J., 2001) and sections were collected starting at the posterior end of the SN and ending at the anterior side of the striatum. The sections were stored free-floating at 4°C in 1% PFA in PBS pH 7.6.
2.5. Immunohistochemistry

Prior to the free-floating immunostaining, sections were washed twice times for 10 minutes with 1x tris buffered saline (TBS) (Sigma-Aldrich) with 0.2% Triton X-100 (Sigma) (TBS-T) and then blocked in blocking buffer (2.5% fetal calf serum (FCS) (DAKO A/S, Glostrup, Denmark) in TBS-T) for 1 hour at room temperature. Sections were then incubated for 1 hour at room temperature, followed by overnight incubation at 4°C in TBS-T 2.5% FCS containing 1:1000 anti-tyrosine hydroxylase (TH) rabbit polyclonal antibody (Institute Jacques Boy SA, Reims, France for SN sections and Pel-Freez Biologicals P40101, AR, USA for striatal sections) or 1:1000 anti-TH mouse monoclonal antibody, clone LNC1 (Millipore MAB318, Temecula, CA, USA) in combination with either anti-glial fibrillary acidic protein (GFAP)-Cy3 conjugated mouse monoclonal antibody (Sigma) at 1:1500 dilution, anti-Iba1 rabbit polyclonal antibody (activated microglia marker, Wako, Osaka, Japan) at 1:2000 dilution, anti-VAMT2 polyclonal antibody (Chemicon International, CA, USA) at 1:400 dilution, or anti-α-synuclein monoclonal antibody (BD Biosciences, Breda, The Netherlands) at 1:500 dilution. Subsequently, sections were washed 3 times with TBS-T and incubated with anti-rabbit or anti-mouse Alexa 488 antibody at 1:800 (Invitrogen, Carlsbad, CA, USA), anti-rabbit Cy3 1:800 (Jackson Laboratories, PA, USA) and Hoechst 33258 1:10000 (BioRad, Hercules, CA, USA) for 1 hour at room temperature in blocking buffer. Sections were then mounted on chrome-aluin and gelatin coated glass slides and embedded in Mowiol (0.1 M Tris pH 8.5, 25% glycerol, 10% w/v Mowiol 4-88 (Sigma)).

2.6. Image acquisition and analysis

Images were obtained on an AxioPlan 2 microscope (Zeiss, Sliedrecht, The Netherlands) with Planapochromat objectives, using Evolution QEi black and white camera (MediaCybernetics) and ImagePro software. 10x magnification pictures were taken for SN sections and 2.5x magnification pictures were taken for the striatum sections with fixed exposure times for each channel. The exposure time was selected so the fluorescence signal was not overexposed. High resolution confocal z stack images were acquired using the Leica confocal microscope (Wetzlar, Germany).

Masking and quantification were processed blinded to the treatment groups. In SN sections, based on the TH staining and anatomical borders, an area of interest was outlined in the ImagePro Plus Measure Threshold macro representing the SN pars compacta (SNpc) structure and an area just outside of the SN to measure the background fluorescence levels (Figure 1A, B and C). Total intensity of TH, GFAP and Iba1 was measured in both areas and the background value was subtracted from the SN measure. The total intensity value was then corrected for the size of the outline area, resulting in the measure of average intensity in that area. Striatal sections were outlined based on the Hoechst staining and the anatomical borders (Figure 1E and F). Here also TH and GFAP fluorescence in-
Figure 1. Masking procedure for fluorescence intensity quantification. A. Image of control SN stained for both TH (green) and GFAP (red). Immunefluorescent intensity was measured in the SN (red outline) and the background (blue outline). B. Gray image of TH immunefluorescent signal in the SN outline. C. Gray image of GFAP immunefluorescent signal in the SN outline. D. For TH positive area measurement TH signal higher than 2x background was marked in each image (red mask). The area of this signal within the outlined SN (red outline) and background outline (blue outline) was quantified. Scale bar represents 0.25mm. E. Gray image of Hoechst immunofluorescent signal in the mouse striatum section. Based on this signal a striatal area (red outline) and background area (blue outline) in the cortex was outlined. F. Image of control striatum stained for TH (green), GFAP (red) and Hoechst (blue). Immunofluorescent intensity was measured in the SN (red outline) and the background outlines (blue outline). Scale bar represents 1mm.
tensity was measured and corrected for background and size of the area. Finally all sections from each animal were averaged out, resulting in a readout of average fluorescence intensity in the SN or the striatum for each animal.

Furthermore, the total area positive for TH immunofluorescence was measured in the SN and background outlines (outlines are indicated in Figure 1D). Only TH signal higher than 2x average background value of all sections was measured. In case any signal was detected in the background outline, these values were subtracted from the SN area. For each animal an average of TH positive area per section was calculated.

2.7. Statistical Analysis

Statistical analysis was performed using SPSS PASW Statistics software version 18. The Mann Whitney U test was used to estimate the differences between all treatment conditions. A p-value < 0.05 was considered significant.

3. Results

3.1. Both low and high dose chronic MPTP-probenecid treatments induce movement deficits in mice

All animals were weighed and subjected to a weekly grid test to assess their movement skills starting before the first MPTP treatment (week 0) and ending 3.5 days after the last treatment (week 5, Figure 2). MPTP and probenecid injections had no significant effect on an animal’s weight throughout the treatment period (Figure 2A). The neurotoxic treatment in both concentrations induced behavioral deficits already after two injections (week 2B, p=0.016 and 0.017). For the high dose regimen (25mg/kg) this effect continued until the end of the experiment (week 5, p=0.005). A low dose regimen of 15mg/kg MPTP caused an increase in the number of forepaw faults made by the animals until the 3rd week after the start of the experiment (p=0.003). Further treatments did not show this behavior deficit either at week 4 and 5 (p=0.108, and p=0.368 respectively). No significant difference was seen between the high and low dose treatment groups in any of the time points. Control animals did not show any effect of probenecid treatment on their movement skills.

3.2. Only high dose chronic MPTP treatment induced DAergic neuron degeneration

Immunohistochemical analysis of the SN revealed that a high dose MPTP regimen induces DAergic neuron cell death (Figure 3). In these animals, TH fluorescence levels, marking cell bodies and axons of DAergic neurons, significantly decreased in comparison to the control situation (65% fluorescence loss
p<0.001) and to 15mg/kg MPTP treated mice (p=0.001, arrows in Figure 3A and C, quantification Figure 3E). In contrast to the observations in the high dose MPTP regimen, the 15mg/kg MPTP treatment did not significantly decrease TH immunoreactivity in the SN (18% decline in fluorescence compared to control, p=0.142).

Similarly, average TH positive area in the SNpc also showed a significant decrease in 25mg/kg MPTP treated animals compared to both control (decrease of 75%, p=0.002, Figure 3E) and 15mg/kg MPTP treated animals (p=0.014). 15mg/kg MPTP treatment showed a slight trend towards decreasing the average TH positive area in the SNpc compared to the probenecid only treated animals (36% decrease, p=0.181).

### 3.3. Both low and high dose MPTP treatments result in astrogliosis and microgliosis in the SN

Interestingly, animals treated with 15mg/kg and 25mg/kg MPTP both displayed significantly higher levels of GFAP immunoreactivity in the SN in comparison to the control group (p=0.003 and p< 0.001 respectively, arrow heads in Figure 3A and B, quantification Figure 3F). Additionally, Iba1 immunoreactivity
Figure 3. Tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP) and Iba1 immunological detection in mouse SN after low and high dose MPTP treatment. A and B. Photographs represent SN of mice treated with saline, 15mg/kg MPTP and 25mg/kg MPTP stained for TH (green) and GFAP (red). Panel B represents a high magnification Z stack confocal image of panel A. Arrows point to the loss of TH staining in the SN, whereas arrowheads point to the increase of GFAP staining. C and D. Photographs represent SN of mice treated with saline, 15mg/kg MPTP and 25mg/kg MPTP stained for TH (green) and Iba1 (red). Panel D represents a high magnification Z stack confocal image of panel C. Arrows point to the loss of TH staining in the SN, whereas arrowheads point to the activated microglia stained for Iba1. The scale bars in A and C represent 0.25 mm, and in B and D 20µm. E. Effect of MPTP treatment on TH expression. TH average fluorescence intensity levels (gray level/mm²) and TH positive area (mm²) in the SN were quantified in saline (black bars), 15mg/kg (light gray bars) and 25mg/kg (dark gray bars) treated animals. TH immunoreactivity levels and TH positive area were significantly reduced in 25mg/kg MPTP treated mice compared to the saline and the 15mg/kg MPTP treated mice (* p <0.05, ** p < 0.01, *** p <0.001). F. MPTP treatment induces gliosis in the SN. GFAP and Iba1 fluorescent intensities were significantly increased in the SN in both MPTP treatment groups compared to the saline injected mice (* p <0.05, ** p < 0.01, *** p <0.001). Error bars represent standard error of mean.
indicated higher levels of activated microglia in the SN in both of the MPTP treatment groups compared to the control situation (15mg/kg group p=0.002, 25mg/kg group p=0.021, arrowheads Figure 3C and D, quantification Figure 3F). Morphology of microglia confirmed their activated state (Figure 3D).

3.4. Chronic low and high dose MPTP treatments result in a loss of nigrostriatal endings in the striatum

We also investigated the effect of MPTP treatment on the integrity of axonal projections of SN DAergic neurons to the whole mouse striatum. Interestingly, in both MPTP treatment groups a decrease in TH immunoreactivity in the striatum was observed, suggesting a loss of SN DAergic nerve endings in this structure (Figure 4). This amount of denervation was dose dependent (significant difference between two MPTP treatments, p value=0.022), with a 53% decrease of TH immunoreactivity in the 15mg/kg MPTP group (p=0.02) and an 85% decrease in the 25mg/kg group (p< 0.001, Figure 4D). Similarly, VMAT2 fluorescence levels decreased in MPTP treated animals by 50% compared to the saline treated mice when measured in three randomly selected animals for each group (p=0.039 and p=0.044, Figure 4C and E). GFAP levels were not significantly different between the treatment groups (p=0.95 and p=0.78, Figure 4D).

3.5. Chronic MPTP treatment induces formation of α-synuclein positive inclusion bodies in SNpc

Immunohistochemical detection of α-synuclein revealed a large presence of large α-synuclein positive puncta in the SN of the MPTP treated animals (Figure 5). We also observed an increased amount of α-synuclein positive inclusions in the SN with higher dosage of MPTP treatment (Figure 5A and 5B). High magnification revealed α-synuclein positive diffuse staining and large granular puncta in the TH positive and other SN neurons (Figure 5C). There were a few α-synuclein positive small puncta present in the saline treated animals, but much less when compared to the MPTP treated animals.

4. Discussion

In this study, we present a chronic PD mouse model based on a 5-week low dose (15mg/kg) MPTP treatment in combination with probenecid. This model is characterized by movement deficits and loss of DAergic nigrostriatal projections. We are the first to report that this model is also associated with astrogliosis and microgliosis in the SN and formation of α-synuclein positive inclusions. Interestingly, we did not observe a significant loss of TH-positive DAergic neurons in the SN in this model, in contrast to DAergic neuron death in the well characterized high dose (25mg/kg) MPTP model. These interesting features of the 15mg/kg model resemble early pathogenic events in PD, and this could therefore be a use-
Figure 4. Tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), Hoechst and VMAT2 immunofluorescence levels in the mouse striatum after MPTP treatment. A. Immunofluorescent detection of TH (green), GFAP (red) and Hoechst (blue) in the striatum of mice treated with saline, 15mg/kg MPTP and 25mg/kg MPTP. B. High magnification Z stack confocal image of panel A. C. Immunofluorescent detection of VMAT2 (red), another marker of dopaminergic terminals.
ful model to study the early neurodegenerative events that initiate the cellular death of DAergic neurons.

We observed a significant loss of TH immunoreactivity in the whole striatum of both the low and high dose MPTP mice. This is in accordance with earlier reports where both these doses induced loss of DA and DA uptake in the striatum resulting in movement impairments (Xu et al., 2010; Schintu et al., 2009a; Schintu et al., 2009b; Lau et al., 1990; Petroske et al., 2001; Novikova et al., 2006). Indeed, our observation of impaired performance in both the low and high dose MPTP groups on the grid test, a test that is sensitive to DA levels and DA uptake in the striatum (Tillerson & Miller, 2003), suggests that the loss of striatal TH expression induced these movement deficits. Two MPTP/probenecid injections were needed for the mice to show a significant increase in errors during the grid test, suggesting that at early stages of this model there is already depletion of DA in the striatum. This DA depletion in the striatum has been previously shown already 1 day after a single 15mg/kg MPTP/probenecid injection and lasting up to 7 days post injection (Lau et al., 1990). The only other report testing behavioural impariments during the MPTP treatment showed no significant increase in error rate at beam transveral test after the 1st 25mg/kg MPTP/probenecid injection, but only after the 10th injection, suggesting the progressive nature of this model (Schintu et al., 2009a). As no testing was performed between the first and tenth injection, we can not directly compare their data to our results. However, a significant delay in pellet retrieval during an olfactory test was reported already after the 1st MPTP/probenecid injection (Schintu et al., 2009a). Although a non-motor symptom, olfactory dysfunction is one of the earliest deficits in PD (Haehner et al., 2007).

In the high dose MPTP/probenecid treatment motor deficit is seen to last until 6 months after the treatment (Petroske et al., 2001; Schintu et al., 2009b). In our study, the low dose group shows recovery directly at the end of the treatment. This recovery phenomenon has been reported by others after many dif-
fertent MPTP treatment regimens, where mice performed as good or even better (referred to as so called ‘overshooting’) as their control groups, despite deficiencies in the DAergic system (Meredith & Kang, 2006; Sedelis et al., 2001). A similar recovery may have also taken place in our low dose MPTP treated mice. There are several possible explanations for this effect. Since we do not see DAergic neuronal death in the SN of the low dose group, these neurons may have increased the production of DA during the recovery phase to compensate for the possible

Figure 5. MPTP treatment induces formation of α-synuclein inclusion in the SN. All panels represent the same image. A. Identification of mouse SN by TH immunoreactivity (green, arrows identify the decrease of TH staining in 25mg/kg MPTP treated animals). The images here are representative of three animals per each treatment group. B. Presence of α-synuclein (red) inclusion bodies is detected in the SN of 15mg/kg and 25mg/kg MPTP treated mice, but not in the saline injected mice (arrow heads). C. High magnification z stack confocal images of panels A and B illustrate in both MPTP dosage treated animals large α-synuclein immunoreactive puncta (arrow heads), large puncta present in the TH positive neurons (white arrows) and diffused α-synuclein staining in TH positive neurons (red arrows). A few of the α-synuclein positive puncta can also be identified in the saline treated animals, but to a much lesser extent then in the MPTP treated animals. The scale bar in A represents 0.25 mm, and in C 20µm.
loss of this neurotransmitter in the striatum. Such a compensatory mechanism has been previously reported and resulted in an increased transmitter-metabolite ratio in the striatum of MPTP treated mice (Schwarting et al., 1999; Yurek et al., 1989). Also, there may be an increase of DA receptors in the surviving striatal terminals, increasing the efficiency of DA uptake. One study illustrated that this effect indeed takes place three weeks after the MPTP treatment, which is in line with the onset of recovery in our study (Weihmuller et al., 1990). Lastly, the effect of neurotransmitter 'volume transmission' may play a role here, where the released DA diffuses over longer distances in the striatum and affects multiple synapses throughout the structure. This effect has been described in MPTP treated cats (Schneider et al., 1994).

One of the hallmarks of PD is gradual and progressive gliosis in the affected SN (Langston et al., 1999; McGeer & McGeer, 2008). Both microgliosis and astrogliosis have been reported in the human PD SN and in the monkey PD-MPTP model (Langston et al., 1999; Hirsch et al., 2003; McGeer & McGeer, 2008; Barcia et al., 2003). We observed a large increase in the number of reactive astrocytes and activated microglia in the SN of both the low and high dose MPTP treated animals. This has been previously corroborated for the 25mg/kg MPTP treatment (Alvarez-Fischer et al., 2008; Novikova et al., 2006; Schintu et al., 2009a; Schintu et al., 2009b). We are however the first to report that glial activation also occurs in the low dose MPTP treatment. Gliosis can either be a result of neuronal stress or can itself induce neuronal damage, for instance by secretion of pro-inflammatory cytotoxins by microglia (Hanisch, 2002). Schintu et al showed that the glial response preceded neuronal loss in the high dose MPTP-probenecid model, suggesting a causal role of the gliosis in the process of neurodegeneration (2009b). Indeed, the anti-inflammatory reagent rosiglitazone prevented neuronal loss in the SN, fully inhibiting microglial activation and partially reducing astroglial activation in these mice (Schintu et al., 2009a). Based on these reports, our data on the low dose MPTP group suggests that gliosis in the SN plays a prominent initiating role in the introduction of DAergic deficits after MPTP treatment, and may be sufficient to significantly reduce TH levels in the striatum.

The most intriguing finding of our study was the lack of a significant decrease of TH levels in the SN of the low dose MPTP treated mice, suggesting that this treatment does not induce a loss of SN DAergic neurons. This is in contrast to the high dose regimen, where the loss of TH immunoreactivity is consistent with the reports of DAergic neuron loss after the application of MPTP (Meredith et al., 2009; Novikova et al., 2006). The phenotype of the low dose MPTP model (similar TH immunoreactivity in the SN compared to controls combined with gliosis in the SN, loss of the TH levels in the striatum and movement deficits) appear to support the 'dying back' hypothesis of PD (reviewed in Cheng et al., 2010; Dauer & Przedborski, 2003; Burke & O’Malley, 2012). In this hypothesis, the TH-positive terminal loss in the striatum is the first neurodegenerative event in PD, which later induces neuronal degeneration in the SN. Evidence for this can be observed in different PD animals models such as in rats overexpressing human
α-synuclein in the SN (Chung et al., 2009), in the α-synuclein (Tofaris et al., 2006) and LRRK2 transgenic mouse models (Li et al., 2009b), and in MPTP treated mice (Li et al., 2009a). The present low dose MPTP model might therefore represent an early PD-like phenotype expressing early PD pathology. The current observations suggest that the SN DAergic neurons are initially stressed by the combined toxic effects of MPTP and gliosis and as consequence lose their terminal connections in the striatum. Additionally, the presence of α-synuclein inclusion bodies, previously reported to be present in the 25mg/kg MPTP model (Meredith et al., 2002; Meredith et al., 2008) further supports early neuronal disfunction in this low dose model. It would be interesting to follow these animals for a longer period of time to assess whether these initial neurodegenerative events will eventually lead to a more advanced neurodegenerative state in the SN and further induce neuronal loss in the ventral tegmental area (VTA). Advanced SN damage is indeed reported in two recent studies, which used similar 5 week MPTP treatment strategies, where severe degeneration of SN DAergic neurons was observed either 4 weeks post-treatment with 15mg/kg MPTP (Xu et al., 2010) or 12 weeks post-treatment with 12.5mg/kg toxin concentration(Ahmad et al., 2009). In the latter, a significant decrease of TH positive neurons in the VTA was also observed (Ahmad et al., 2009). These late stage data suggest that the combined effects of first time reported gliosis in the SN and observed loss of TH-positive terminals in the striatum directly after the low dose MPTP treatment period are sufficient to induce the main hallmark of PD- the loss of DAergic neurons in the SN. Interestingly, in contrast to our findings, the same low dose chronic MPTP treatment regimen in older, more sensitive mice (6-12 months old) resulted in a loss of TH positive neurons in the SN directly after the MPTP treatment (Lau et al., 2011; Barber-Singh et al., 2009). As age has been reported to play a dramatic role in the sensitivity to MPTP, with older animals being more vulnerable to this treatment (Jossan et al., 1989; Ricaurte et al., 1987; Przedborski et al., 2001), we hypothesize that the age difference between these two experiments underlies this apparent discrepancy.

In conclusion, we have extensively characterized the immediate effects on neuropathology, gliosis and behavior in a PD mouse model induced by a 5-week 15mg/kg chronic MPTP-probenecid treatment. Immediately after the treatment period, this model is characterized by movement deficits, loss of TH-positive fibers in the striatum, and astro-gliosis and micro-gliosis in the SN without loss of DAergic neurons. Together, these neuropathological features closely resemble events associated with the ‘dying-back’ hypothesis in PD. This model may therefore prove useful for the investigation of the early neurodegenerative events in the nigrostriatal DAergic system and shed more light on the molecular processes involved in the development of PD. Moreover, it can be an excellent tool to study the effects of potential treatment strategies that counteract the early cellular changes observed in Parkinson’s disease.