

Continuous MPTP intoxication in the Göttingen minipig results in chronic parkinsonian deficits

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Parkinson's disease (PD) is a common neurodegenerative disorder, resulting from progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Neuroprotective therapies in PD are still not available, perhaps because animal models do not imitate the chronic and progressive nature of the clinical state of PD. To address this, we performed a feasibility study aimed at establishing a chronic non-primate large animal PD model in Göttingen minipigs based on continuous infusion of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Twelve female Göttingen minipigs were divided into groups of 2–4 animals and implanted with infusion pumps for continuous intramuscular MPTP delivery of 4–24 mg MPTP/day for 11 weeks. The animals showed parkinsonian symptoms with bradykinesia, rigidity, coordination and chewing difficulties. Symptoms were stable in the 12 and 18 mg MPTP/day groups, whereas the remaining groups showed partial or full behavioral recovery. Digital gait analysis, high performance liquid chromatography (HPLC) measurements and stereological counts of tyrosine hydroxylase-positive (TH+) neurons in the SNc revealed a dose-related decrease in gait velocity, striatal metabolite levels and neuron numbers with increasing doses of MPTP. No neuronal inclusions were observed, but alpha-synuclein staining intensified with increased cumulative MPTP dosages. We conclude that this large-animal model of chronic MPTP administration in Göttingen minipigs shows trends of stable parkinsonian deficits at 18 mg MPTP/day in all modalities examined. This PD model shares many of the characteristics seen in patients and, although preliminary, holds considerable promise for future pre-clinical trials of neuroprotective therapies.

Key words: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), animal model, behavior, minipig, neuropathology, Parkinson's disease

INTRODUCTION

Parkinson disease (PD) is a progressive neurodegenerative disorder resulting from an excessive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), leading to a subsequent loss of striatal dopamine (DA). Great advances in the treatment of PD and the understanding of the biochemical pathology of the disease have been achieved using animal models exhibiting nigro-striatal damage in a variety of species such as rodents and primates (Bergman et al. 1990, Carlsson 2002, Jankovic 2006). However, most existing animal models of PD exhibit acute neuronal damage and not the progressive neuronal loss as seen in PD patients and many do not develop the characteristic protein aggregates known as Lewy bodies (Braak et al. 2003, Jenner 2008, Giráldez-Pérez et al. 2014). The lack of slow

onset and progressive course of the existing PD models may be an important contributing factor to the fact that substances displaying promising neuroprotective effects in animal studies of PD have failed in human clinical trials (Kalia et al. 2015, Suchowersky et al. 2006, Yacoubian and Standaert 2009). Therefore, better, more representative animal models are needed in order to adequately test neuroprotective strategies in PD (Carvey et al. 2006, Jenner 2008). Continuous MPTP or rotenone infusion in mice or rats seemed promising, with animals displaying progressive behavioral changes and even alpha-synuclein (α -syn) positive neuronal inclusion bodies (Betarbet et al. 2000, Fornai et al. 2005). Nevertheless, alternatives to rodent models are greatly needed to approach neuroprotection research to PD patients. In this pursuit we found the Göttingen minipig advantageous as a non-primate large experimental

animal. The animal weighs 20–40 kg and its large gyrated brain (6×5×4 cm, weight approximately 70 g) has a great subcortical anatomical resemblance to primates (Bjarkam et al. 2001, Ettrup et al. 2010, Félix et al. 1999, Larsen et al. 2004, Nielsen et al. 2009, Rosendal et al. 2009a). PET and MRI scanning procedures are possible in the minipig brain as well as conventional neurosurgery, stereotaxic neurosurgery and surgical implantation of stem cells and human-sized devices (Andersen et al. 2005, Bjarkam et al. 2001, 2004, 2008a, 2008b, 2009, 2010, Dall et al. 2002, Dalmose et al. 2004, 2005, Danielsen et al. 2000, Ettrup et al. 2011, 2012, Fjord-Larsen et al. 2010, Glud et al. 2011, Jensen et al. 2009, Lind et al. 2007, Norgaard Glud et al. 2010, Rosendal et al. 2009b, 2010, Sørensen et al. 2000, Watanabe et al. 2001). The pig genome resembles that of primates (Wernersson et al. 2005), and as opposed to primates, pigs are not a biohazard, making handling and housing easier and more affordable. Furthermore, many of the ethical considerations associated with the use of primates as experimental animals may be avoided, enabling inclusion of more animals in each study and thereby potentially obtaining more valid results (Aziz 2006, Goodman and Check 2002, Dolezalova et al. 2014).

To address the need of a non-primate large animal model for neuroprotective studies in PD we set out to develop, thoroughly describe and validate a modified model of parkinsonism in the Göttingen minipig based on continuous MPTP-infusion. In this feasibility study we included few animals in each group for the purpose of providing preliminary evidence of optimal MPTP doses and methods for evaluating parkinsonian deficits and degrees of nigro-striatal damage. This study represents the first of its kind in a large animal and its results are of importance to future large-scale validation studies, aimed at developing an animal model of PD more closely imitating the clinical state and course of PD seen in patients.

MATERIALS AND METHODS

Animals

A total of 16 female Göttingen minipigs (Ellegård Göttingen Minipigs A/S, 4261 Dalmose, Denmark) approximately 1-year-old were used for this study approved by the Danish National Council for Animal Research Ethics (DANCARE). Twelve animals were chronically MPTP intoxicated as described below, and the remaining 4 were kept as normal controls. The animals were kept in pairs in a stable with an air temperature of 21 ±1°C, a relative humidity of 50 ±2% and an air change of 12 times/hour. The stable was artificially illuminated with 12 h light and 12 h darkness. The animals were allowed to acclimate for 3–4 weeks before the surgical pump implantation.

MPTP stability.

Prior to the experiment the stability of the MPTP HCl (M-0896, Sigma-Aldrich, St. Louis, Missouri) was tested with high performance liquid chromatography (HPLC), confirming its stability at 39°C (the body temperature of the minipig) for 5 months. Briefly, HPLC was performed employing a reverse-phase column Suplex pKb-100 (4.6×150 mm, 5 µm; Supelco Inc., Bellefonte, Pennsylvania). The mobile phase was a mixture of 25% acetonitrile and 75% 25 mM potassium phosphate (pH 7.4) at a flow rate of 1 ml/min and monitoring was performed at 265 nm. See Yoshihara and others (2000) for details.

MPTP intoxication

On the day of surgery, the animals were deeply anesthetized with a combination of ketamine (125 mg im+62.5 mg iv), midazolam (25 mg im+12.5 mg iv) and isoflurane (inhalation, 5% for 2 minutes, then reduced to 1.5%). Under sterile conditions, a Medtronic Syncromed II 8637-40 (40 mL) programmable pump (Medtronic, Minneapolis, Minnesota) filled with MPTP HCl 10 mg/ml was implanted subcutaneously on the right side of the back of each pig (Figs 1A and 1B). A Medtronic catheter 8709SC was connected to the pump, cut to an appropriate length and its tip placed intramuscularly in the back of the animal. The skin was sutured tightly in three layers. Antibiotics (benzylpenicillin 300,000 IE im) were administered 1 day pre-operatively, on the day of the surgery and 5 days postoperatively. Analgetics (flunixin 50 mg im) were administered 3 days postoperatively. Each pump was programmed with an external Medtronic En'Vision unit for continuous MPTP delivery. The twelve animals in the MPTP group were divided into 5 groups, receiving 4 mg MPTP/day (n=2), 6 mg MPTP/day (n=2), 12 mg MPTP/day (n=2), 18 mg MPTP/day (n=4) or 24 mg MPTP/day (n=2). When required, the pumps were refilled transcutaneously under sterile conditions in animals anesthetized with azaperone (200 mg) and midazolam (25 mg+25 mg pn) im. The ten pigs receiving 4–18 mg MPTP/day had the infusion protocol last 11 weeks. The two animals receiving 24 mg MPTP/day had the infusion stopped after 11 days as the animals expressed severe parkinsonian deficits compromising food and water intake, and levodopa (L-dopa) (levodopa 50 mg/carbidopa 12.5 mg p.o., 1–3 times daily) was administered to these animals as supportive measures for the remainder of the study. These two minipigs were sacrificed at the end of the 11 weeks' protocol i.e. after 11 days of intoxication followed by 9 weeks of recovery. Two animals in the 18 mg MPTP/day group also received L-dopa substitution therapy due to severe parkinsonism, but without cessation of the MPTP infusion.

Two animals developed a skin infection over the pump area, and this was managed with antibiotics (benzylpenicillin 300,000 IE im) and transcutaneous drainage under general anesthesia with azaperone (200 mg) and midazolam (25 mg) im. The pumps were not removed and the infusions continued throughout the treatment.

The remaining four control animals were anesthetized as described above and received a skin incision and were sutured in a location similar to the location of the pump in the MPTP group. However, due to the high cost of the pumps, the control animals did not have pumps implanted.

Behavioral analysis

The pigs were observed for the next 11 weeks at 0, 1 week, 2, 2.5, 4, 5, 7 and 10–11 weeks. Evaluations were performed in the stable with at least 10 minutes of observation by authors MSN and AM according to a behavioral score scheme modified from Mikkelsen and others (1999) to include motility (0–5), coordination (0–5), rigidity (0–2), chewing (0–2) and vocalization (0–2), with 0 denoting the normal state and 5/2 the most severe expression. Motility was evaluated in degree of bradykinesia; coordination in crossing of hind or front limbs or both; rigidity by flexion/extension of the hind limb; chewing by time spent to eat chocolate treats; and vocalization by the ability to make normal sounds, a pathological high pitch sound or by loss of ability to vocalize. A total score of 0 was normal, 1–5 was regarded mild, 6–11 moderate and 12–16 indicated severe parkinsonism. L-dopa substitution therapy was paused for at least 18 hrs prior to behavioral evaluation.

Gait analysis

Digital gait analysis was modified from previous clinical studies (Johnsen et al. 2009, Mogensen and Jakobsen 2001) and performed for each pig pre-operatively and after 4 and

11 weeks of MPTP intoxication. Each pig served as its own internal control, comparing its pre- and post-operative gait velocities. Prior to the first analysis the animals were trained to walk in a flat, fenced area with author MSN using chocolate (e.g. M&M's®) as treats (Fig. 1C). Animals in need of L-dopa therapy did not receive the drug for at least 24 hrs prior to the gait analysis. Six infrared Vicon cameras were placed on tripods around the fenced area. Reflective ball-shaped markers were placed with double-stick tape distally on both hind limbs and the right fore limb, on the right thigh and between the highest point of the shoulders. The cameras recorded the positions of the markers in space. The 2D coordinates from each camera were digitalized and transformed to one set of 3D coordinates and from this, gait velocity was calculated.

Euthanasia

At the end of the 11 weeks of infusion or observation, the animals were anesthetized with ketamine and midazolam as described above and through a burr hole in the skull biopsies for HPLC were taken from the left striatum and snap frozen in liquid nitrogen. Each animal was then euthanized with intracardial pentobarbital injection (2 g) before transcatheter perfusion with 5 L 4% paraformaldehyde (PFA). The brains were removed and the brain stem containing SNc dissected according to the method described in Nielsen and others (2009). The right striatum was cut coronally and divided in a rostral and caudal part. The pumps were removed and their function verified.

Histology

The brain stems and right striatae were paraffin embedded and serial 30- μ m-thick sections mounted on glass slides before immunohistochemical staining for tyrosine hydroxylase (TH) or α -syn. Immunohistochemistry was



Fig. 1. (A) Implantation of pump for continuous MPTP delivery. The pump was placed subcutaneously behind the scapula of the minipig, and a catheter connected to the pump placed in the deep muscles of the back for im drug delivery. (B) The 40 ml pump was programmable and refillable transcutaneously. (C) For the digital gait analysis, each pig was trained with chocolate treats to walk in a fenced area. Reflective markers were placed on the limbs and between the shoulders of the animal, and 6 infrared Vicon cameras registered the location of the markers in space. From this, gait velocity was objectively measured.

performed directly on the slides as described previously (Nielsen et al. 2009), using monoclonal rabbit anti-tyrosine hydroxylase (P 40101-0, Pel Freez, Rogers, Arkansas) diluted 1:600 or polyclonal sheep anti-alpha-synuclein (ab6162, Abcam, Cambridge, UK) diluted 1:100 as primary antibodies. Visualization was performed by use of a biotinylated secondary antibody (anti-rabbit Ig, Amersham, Buckinghamshire, UK diluted 1:200, or anti-sheep Ig, diluted 1:400) followed by streptavidin horseradish peroxidase (Sigma) and final exposure to diaminobenzidine (DAB). α -syn stained sections were counter-stained with toluidine blue. Omission of both primary antibodies resulted in lack of staining. In addition, separate nigral sections were stained with hematoxylin-eosin (HE).

Metabolite measurements

To determine the concentration of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) the left striatal samples were thawed and homogenized with a

Teflon pistil, adding 0.5 ml ice-cold 0.5 M/l perchloric acid containing 0.05% w/v sodium thiosulfate (Pharmacy, Aarhus University Hospital, Aarhus, Denmark). The homogenate was centrifuged for 5 min at 13000 rpm. The supernatant was analyzed by HPLC using Perkin-Elmer Series 200 HPLC pump (Titan) connected in series with a Rheodyne 7025 injector (80 μ l loop), a Phenomenex Spherclone 5 μ ODS (2) (250 \times 4.6 mm, Phenomenex, Torrance, California), a Dionex PED II electrochemical detector in amperometry mode (sensitivity 1 μ A). For data acquisition Dionex Chromeleon Software 6.8 was used. The mobile phase was a mixture of 80% buffer containing 0.1 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (13.8 g/l), 2.6 mM octanesulphonic acid sodium salt (0.6 g/l), 0.1mM EDTA (38 mg/l), pH 3.3 adjusted with acetic acid and 20% methanol. At a flow rate of 1 ml/min, DOPAC eluted around 9 min, dopamine around 15 min, HVA around 21 min and 3-MT around 36 min. Further details can be seen in Gamache and others (1993) and Mikkelsen and colleagues (1999). Metabolite concentrations were determined by comparison to the peaks produced by injection of the respective standards at the fixed concentration of 2 μ g/ml each.

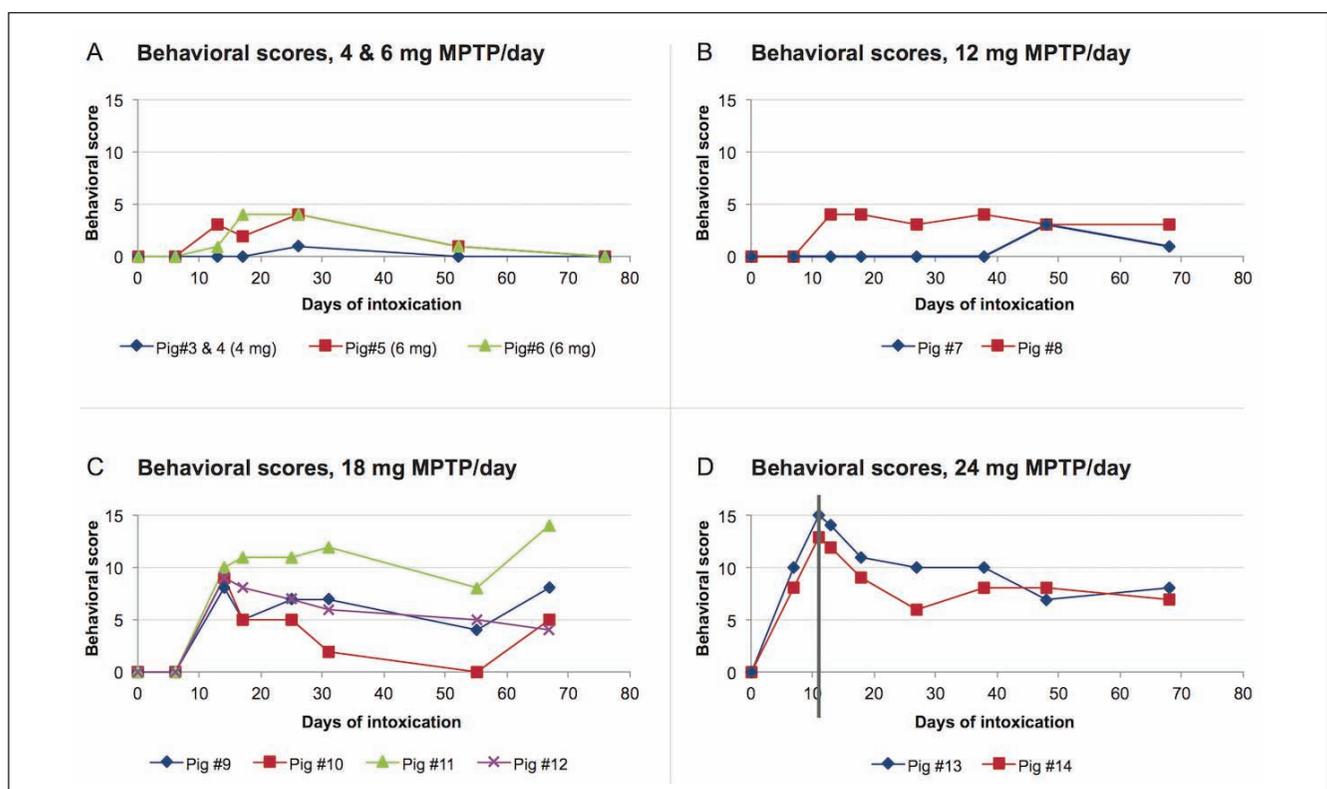


Fig. 2. Behavioral scores during the observation period. (A) The 4 mg MPTP pigs (n=2) displayed the same mild and remitting scores and are therefore depicted in one line. The 6 mg MPTP animals (n=2) showed mild symptoms, also with remission to the normal state. (B) The 12 mg MPTP group (n=2) showed mild, if any, parkinsonian symptoms. (C) In the 18 mg MPTP group (n=4) three animals (#9, 11, 12) showed moderate and stable symptoms. Pig #10 showed signs of remission following the initial effect. However, parkinsonian symptoms increased towards the end of the observation period in all but #12. (D) The 24 mg MPTP animals (n=2) showed severe parkinsonian deficits and had their pumps turned off after 11 days of intoxication (gray vertical line). These animals were regarded acutely and not chronically intoxicated, and remitted from severe to moderate PD, remaining stable throughout the rest of the observation period.

Stereology

The total number of TH-positive (TH+) neurons in the SNc was estimated on both the right and left sides on TH-stained sections according to our previous study (Nielsen et al. 2009), utilizing the optical fractionator technique (West 1999), using a Zeiss AxioPlan microscope interfaced with MicroBrightField StereoInvestigator software. A 40× air lens was used. The final section thickness was measured to 28 μm . $\sum Q^-$ represents the total counts from each SNc, and the total number of neurons, N, was calculated as follows: Every 10th section of the SNc was used, making the section sampling fraction, $\text{ssf}=1/10$. The counting frame had an area of 7,000 μm^2 , and the area associated with each step made by the computer over the section surface was 22,500 μm^2 , 90,000 μm^2 or 160,000 μm^2 , depending on the MPTP dose of the animal (the larger the dose, the smaller the step due to assumed fewer neurons), making the area sampling fraction, $\text{asf}=0.311$, $\text{asf}=0.078$, or $\text{asf}=0.044$, respectively. The relation between the section thickness ($t=28 \mu\text{m}$) and the height of the optical dissector ($h=15 \mu\text{m}$), t/h , was 28/15. Thus $N=\sum Q^- \times (1/\text{ssf}) \times (1/\text{asf}) \times (t/h)$.

RESULTS

Behavior

Abnormal behavior was observed in all groups of MPTP-intoxicated animals. Initially, the animal would lean its thigh against the wall. Then followed lack of coordination, primarily of the hind limbs and in severe cases the front limbs. General bradykinesia and freezing episodes could progress to akinesia in the most extreme cases. We also observed prolonged chewing and disturbances in normal vocalization (normal sounds progressing to abnormal sounds and finally to inability to vocalize). Only severely affected animals displayed reliably assessable rigidity of the limbs. There was no tremor, except in the acute stage of intoxication in the 24 mg MPTP animals. For the 4–12 mg MPTP pigs, mild symptoms were observed after 10–21 days of intoxication, with most of the animals showing partial or full behavioral recovery after 7 weeks of intoxication, despite the on-going active infusion (Figs 2A and 2B). The four 18 mg MPTP animals developed symptoms of moderate to severe PD after approximately 2 weeks and all but one (pig #10) showed moderate and stable

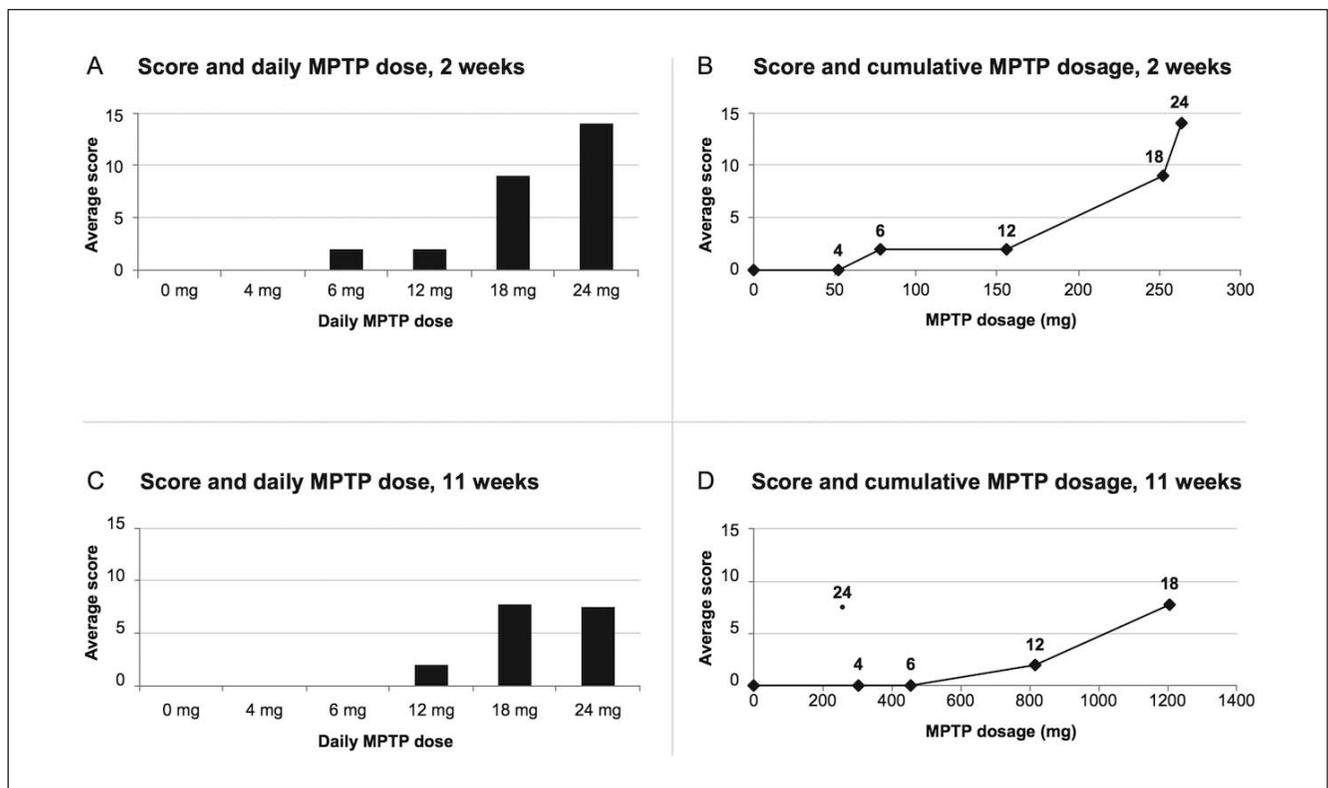


Fig. 3. Average behavioral scores compared to the daily MPTP dose and the cumulative MPTP dosage at 4 and 11 weeks of observation. Note the recovery of the 6 mg MPTP animals, despite high cumulative dosage compared with the 24 mg animals, where infusion was stopped after just 11 days. Also note that the 12 and 18 mg groups as the only groups show stable behavioral scores from the 4- to the 11-week observations (A and C). The numbers 4–24 in B and D refer to mg MPTP/day.

behavioral deficits. In three animals, progressive impairments and behavioral scores was seen towards the end of the observation period (Fig. 2C). The two most severely affected animals in this group received L-dopa to ensure proper food intake. The 24 mg MPTP animals employed severe parkinsonism after just 11 days of intoxication. Their pumps were turned off to prevent further progression of the symptoms and the animals were administered L-dopa therapy. They quickly recovered and stabilized into moderate PD without any signs of progression (Fig. 2D). Comparing the average behavioral scores at 2 and 11 weeks with the daily MPTP doses confirmed full recovery in the 4 and 6 mg groups overall and partial recovery of the 24 mg group (Figs 3A and 3C). The 12 and 18 mg groups showed stable scores. When comparing the average behavioral scores with the total cumulative MPTP dosage, stable scores were again seen in the 12 and 18 mg MPTP groups from 2 to 11 weeks (Figs 3B and 3D). The 24 mg animals demonstrated the classical behavioral feature of acute toxicity: initial severe scores with partial recovery following interruption of MPTP administration. These animals were rated moderately parkinsonian at 11 weeks despite the much lower cumulative dose compared with the continuously infused animals.

Digital gait analysis

The digital gait analysis pointed to decreased gait velocity in the 12 and 18 mg MPTP groups after 4 and 11 weeks of intoxication (Fig. 4), most distinctly demonstrated in the 18 mg MPTP group. Results from a representative 24 mg animal showed an initial decrease in gait velocity at 4 weeks with recovery to normal values at the 11-week test, consistent with the observed behavioral recovery. The 4–6 mg MPTP animals displayed normal gait velocity throughout the observation period and are not shown in Fig. 4. We did not observe any effect of training, learning or exercise i.e. normal animals did not increase or decrease their gait velocity significantly during the course of the observation period.

HPLC

HPLC analysis of striatal DA and its metabolites at the end of the 11-week study presented in Figs 5A–5D suggested decreased concentrations of DA and its metabolites in response to exposure to increasing doses of MPTP, with occasional outliers. The relative increase in metabolites in the 4 animals receiving 18 mg MPTP/day in comparison with the further decrease in DA at that dose suggested an increase in turnover. This is supported by Fig. 5E graph in which

a routinely used index of DA turnover, DA metabolites/DA, suggested an increase in the 18 mg daily dose animals as well as the animals with the acute moderate to severe lesion.

Histopathology

A trend of dose-dependent neuron loss in the SNc was demonstrated on TH-stained sections (Figs 6A–6D). The neuron loss was most prominent in the rostral and lateral 2/3 of the SNc in the lower daily dose groups, but the 18 and 24 mg MPTP animals displayed a neuronal loss in all parts of the SNc. Surviving neurons appeared smaller and more heavily stained than in normal animals. In the striatum, the 12 and 18 mg animals showed swollen TH+ terminals with fewer fibers and terminals in the 18 mg group (Figs 6F–6G). Some sense of direction of the fibers remained in the 18 mg animals, but was lost in the 24 mg animals, which also displayed smaller, fewer terminals (Fig. 6H). These changes were most prominent in the rostral part of the striatum corresponding to the rostral nigral projection.

The SNc and striatae of MPTP intoxicated animals stained positive for α -syn (Fig. 7). α -syn was mostly visible in the neuropil, but occasional neuronal staining

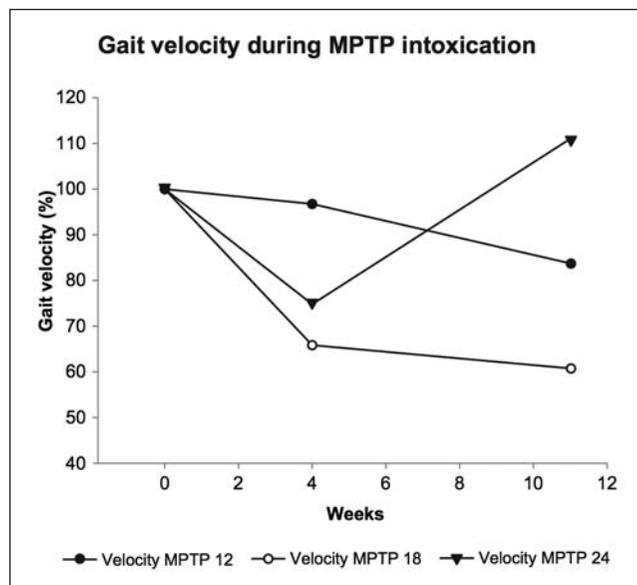


Fig. 4. Mean gait velocity during the 11-week observation period in the 12, 18 and 24 mg MPTP groups. The 4–6 mg animals showed normal gait velocities throughout the study. Each animal served as its own normal control due to inter-individual variability of normal gait velocity. The pre-operative gait velocity was set at 100% and changes are depicted in percent points. The 12 mg MPTP group, comprising two animals, showed decreased gait velocity throughout the observation period. The 18 mg MPTP animals, comprising four animals, showed stable and decreased gait velocity at both 4 and 11 weeks of intoxication. The 24 mg MPTP group is presented here with one representative animal, showed decreased gait velocity at 4 weeks and increased velocity at 11 weeks. The MPTP intoxication of this group was stopped after 11 days and corresponded to acute intoxication.

in the SNc was also observed, primarily confined to the cytoplasm and the cell membrane (Fig. 7B). There were no neuronal inclusions. α -syn stained most intense in 12 and 18 mg animals receiving continuous MPTP infusion compared with the acutely intoxicated 24 mg animals. This finding was most prominent in the striatum (Figs 7E and 7F). Control animals were devoid of specific α -syn reactivity.

Stereology

The stereological cell counts also suggested a dose-dependant loss of TH+ neurons (Fig. 6 graph). For reference, normal Göttingen minipigs have an estimated 80,700 neurons in their SNc unilaterally (Nielsen et al. 2009) and this number including SD's was included in Fig. 6. The TH and the HE stainings were compared, and the specific

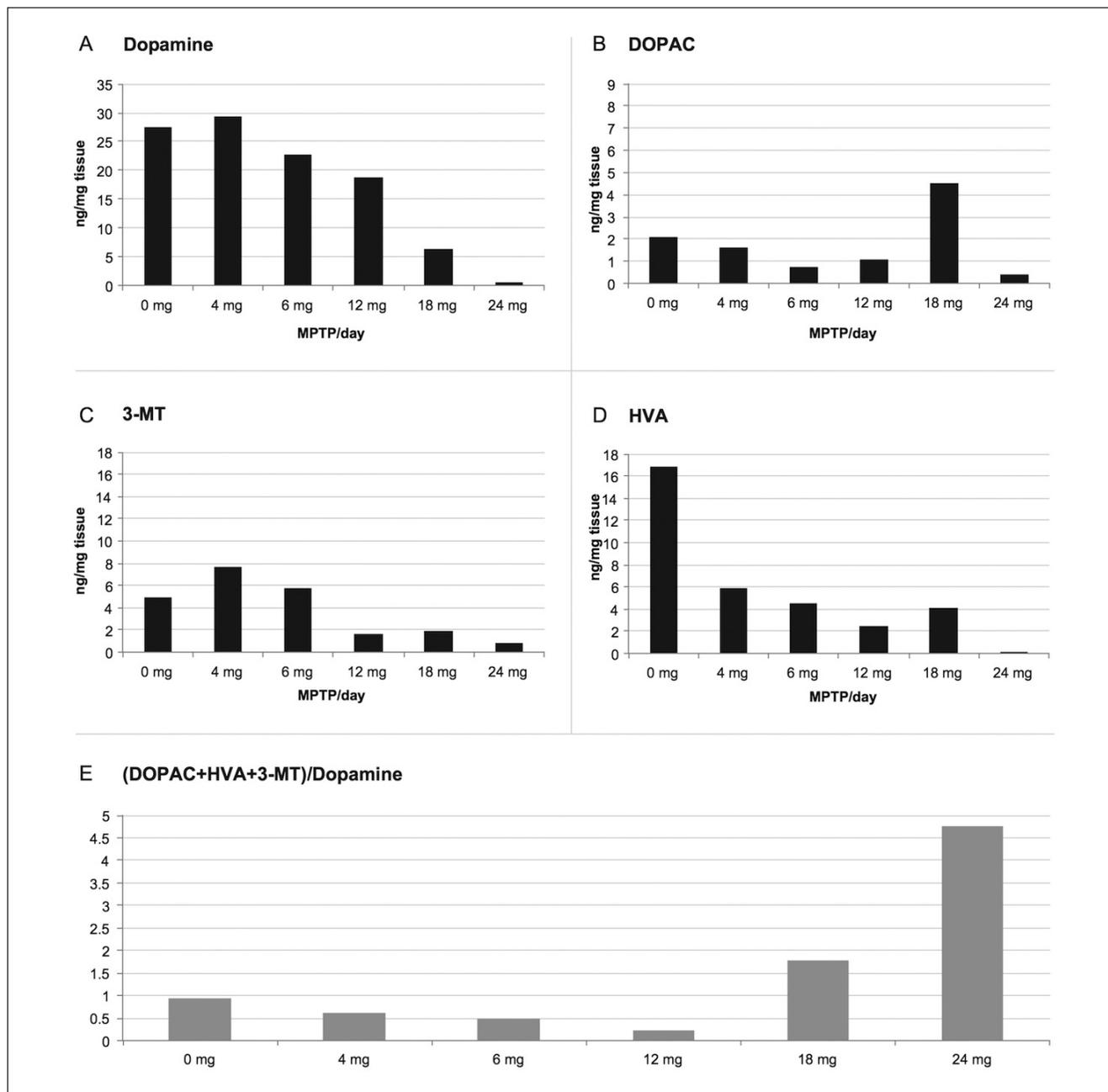


Fig. 5. HPLC for DA, DOPAC, 3-MT and HVA in the different MPTP groups and normal animals. The number of observations was limited (1 in the 6 and 24 mg MPTP groups; 2 in the 0, 4 and 12 mg MPTP groups; and 4 in the 18 mg MPTP group). However, the overall trend was that of a dose-dependent decrease in dopamine and related metabolites. The (DOPAC+HVA+3-MT)/Dopamine ratio shown in E is a sensitive marker of dopamine turnover, increasing in the 18 and 24 mg groups.

dopaminergic neuronal loss seen on the TH stained sections corresponded well with the neuronal loss observed in the HE stained sections (data not shown).

DISCUSSION

For the first time, this study suggests that continuous im delivery of MPTP for 11 weeks results in chronic and stable PD deficits in the Göttingen minipig. We found 18 mg MPTP/day to be the optimal dose, as the animals in this group displayed moderate to severe and stable parkinsonian behavior, evidence of decreased gait velocity, loss of TH⁺ neurons evaluated histopathologically and stereologically, and a HPLC-verified decrease in striatal dopamine and metabolites. As this was the first study of its kind in a large animal, we included just 2 animals in each group to target the optimal MPTP dose and doubled the number of animals to 4 once this dose was established. The small n precluded detailed statistical analysis, but provided valuable information for future studies of its kind in animals of this size and weight.

The past 10 years' of research aimed at making the pig an attractive animal model for neuroscience research has led to detailed descriptions of this animal's genome, subcortical anatomy, neurosurgical, PET and MRI scanning procedures and actual disease modeling such as PD, Alzheimer's disease, Huntington's disease, and autonomic and micturition disturbances (Andersen et al. 2005, Baxa et al. 2013, Bjarkam et al. 2001, 2004, 2008a, 2008b, 2009, 2010, Dall et al. 2002, Dalmose et al. 2004, 2005, Danielsen et al. 2000, Ettrup et al. 2010, 2011, 2012, Félix et al. 1999, Fjord-Larsen et al. 2010, Glud et al. 2011, Jensen et al. 2009, Kragh et al. 2009, Larsen et al. 2004, Lind et al. 2007, Lindvall and Wahlberg 2008, Mikkelsen et al. 1999, Nielsen et al. 2009, Norgaard Glud et al. 2010, Ostergaard et al. 1992, Rosendal et al. 2009a, 2009b, 2010, Sørensen et al. 2000, Watanabe et al. 2001, Wernersson et al. 2005). These studies showed that the pig has commonalities with non-human primates and humans and may serve as a large animal model, filling the gap between neuroscience on rodents and clinical trials on patients, as use of non-human primates is becoming more difficult.

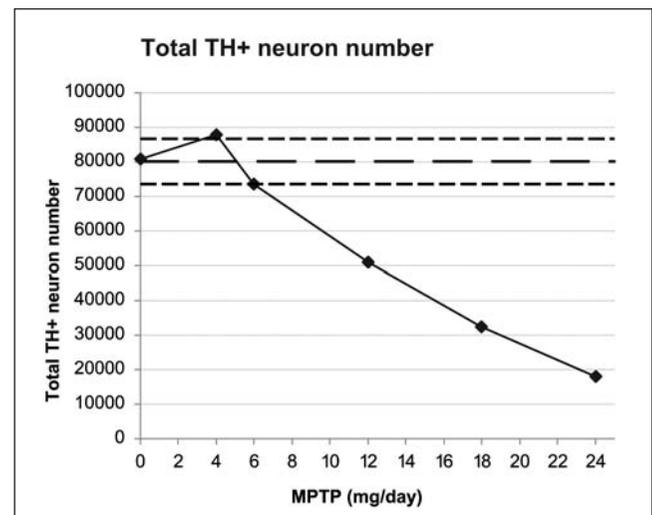
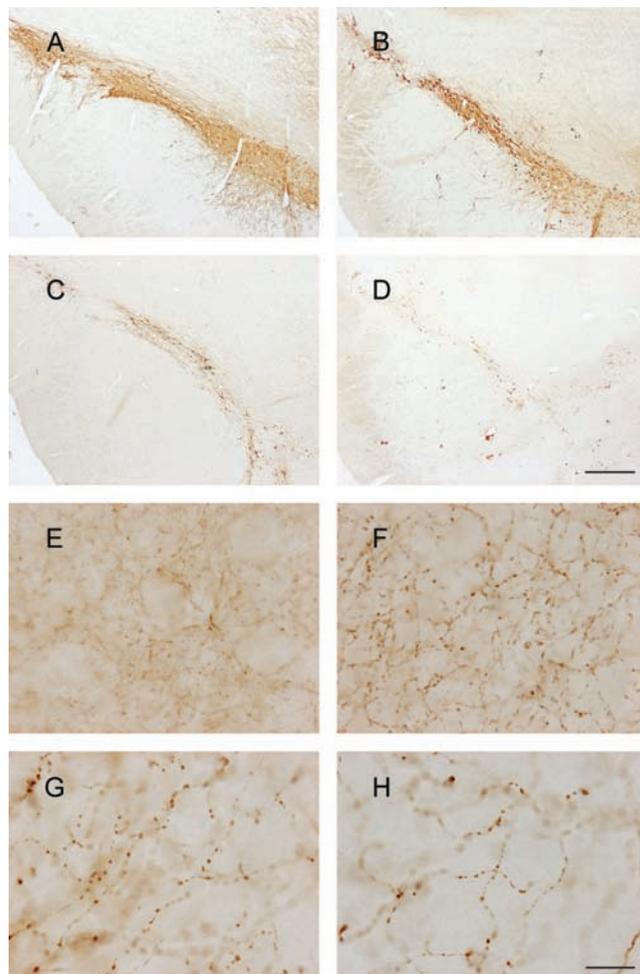


Fig. 6. (A–D) TH-staining of the SNc in A – normal; B – 12 mg; C – 18 mg; and D – 24 mg MPTP animals at corresponding levels. A dose-dependent decrease in the number of TH⁺ neurons was visible. Scale bar: 1 mm. (E–H) TH-staining in the striatum in E – normal; F – 12 mg; G – 18 mg; and H – 24 mg MPTP animals. TH⁺ terminals were swollen in the 12 mg animals. Likewise, the 18 mg animals showed swollen terminals, reduced in number and with fewer fibers. The 24 mg animals displayed only few terminals and fibers, and the fibers appeared completely disorganized. Terminals were not as swollen as in the 12 and 18 mg groups. Scale bar: 20 μ m. Graph: the stereological results. n=4 in the 18 mg group, n=2 in all other groups. Both SNc's in each animal were counted. The results show the average number of neurons unilaterally. The dose-dependent decrease in TH⁺ neurons seen in Figs 6B–6D was confirmed, pointing towards fewer neurons in the 12, 18 and 24 mg MPTP groups. The dashed horizontal lines indicate the mean and SD's in normal Göttingen minipigs based on Nielsen and others (2009).

The MPTP model of selective DA neuron damage has been used to investigate the pathogenesis in PD, the pharmacological consequences of the DA loss, the compensatory adaptations, efficacy testing of new symptomatic treatments and the potential of gene therapies and alternate neuroprotective approaches. Promising results of neuroprotection in rodent and primate models with a variety of agents have failed to show convincing effects in subsequent clinical trials. A plausible explanation could be the lack of current animal models for preclinical screening to accurately mimic the slow progressive course of the idiopathic disease (Kiebertz and Ravina 2007, Olanow et al. 2008). Conventionally, acute nigral DA neuron loss has been performed by injecting high MPTP doses hours or days apart but usually within a short time frame, until the animal displayed persistent PD symptoms (Emborg 2004, Przedborski et al. 2001). Attempts to mimic progressive neuron loss by subacute/chronic MPTP intoxication using lower doses over longer periods of time have been performed (Bezard et al. 1997, Gibrat et al. 2009, Meredith et al. 2008). However, it has been argued that this also in essence represents acute

intoxication (Jenner 2008). Continuous administration of MPTP exposes the SNc neurons to chronic toxic stress and therefore supposedly models the progressive pathology seen in PD patients. This was performed in mice by osmotic minipumps (Fornai et al. 2005, Gibrat et al. 2009) and may be the closest yet to a true chronic PD toxin model. To date, we have not found evidence of continuous administration of MPTP or other PD-related neurotoxins in a large animal model as performed here. Additionally, our study is the first to administer MPTP continuously for as long as 11 weeks.

The behavioral analysis performed in the minipig resembles those described for primates with obvious exceptions such as evaluation of facial expression, fine hand movements etc. (Bezard et al. 1997, Doudet et al. 2004, Emborg 2004). Behavioral evaluation in an animal model of neuroprotection is very important, and require a standardized scoring system, which would optimally correspond to the Unified Parkinson Disease Rating Scale (UPDRS) assessment in PD patients, as this scale is often used in clinical trials for evaluation of treatment efficacy. Although blind assessment would be preferred, it was impossible in this feasibility study due to the obvious presence of the large implanted pumps. Nevertheless, the subjective behavioral assessment by two investigators with experience in pig behavior was remarkably similar to the data obtained from the objective gait analysis, measuring movement velocity (see below).

Continuous delivery of 18 mg MPTP/day led to stable PD symptoms in 3 out of 4 animals (Fig. 2C). Comparison of the average motor scores and daily or cumulative MPTP doses suggested stability of the behavioral scores in the 12 and 18 mg MPTP/day groups (Fig. 3). These observations also suggested that the 24 mg animals could be regarded as acutely and not chronically intoxicated, as severe symptoms occurred quickly and persisted after slight recovery despite cessation of the MPTP infusion. This behavioral time course was similar to that of acute MPTP intoxication described previously in pigs and non-human primates (Emborg 2007, Mikkelsen et al. 1999). Clinical recovery after acute exposure to MPTP intoxication is thoroughly described in the literature (Boulet et al. 2008, Mikkelsen et al. 1999, Mounayar et al. 2007) in both bilaterally and unilaterally lesioned animals. Additionally, examination of the animals' individual clinical responses shown in Fig. 2 clearly demonstrated significant variability in the sensitivity of the different animals exposed to the same dose of MPTP. Similar variability is commonly described with toxin exposure in primates, minipigs and MPTP-exposed addicts (Ballard et al. 1985, Emborg 2007, Mikkelsen et al. 1999).

As described previously by our group (Glud et al. 2011), we confirmed that digital gait analysis is possible on properly trained minipigs and offers a sensitive method of motor performance assessment. The trends of decreased

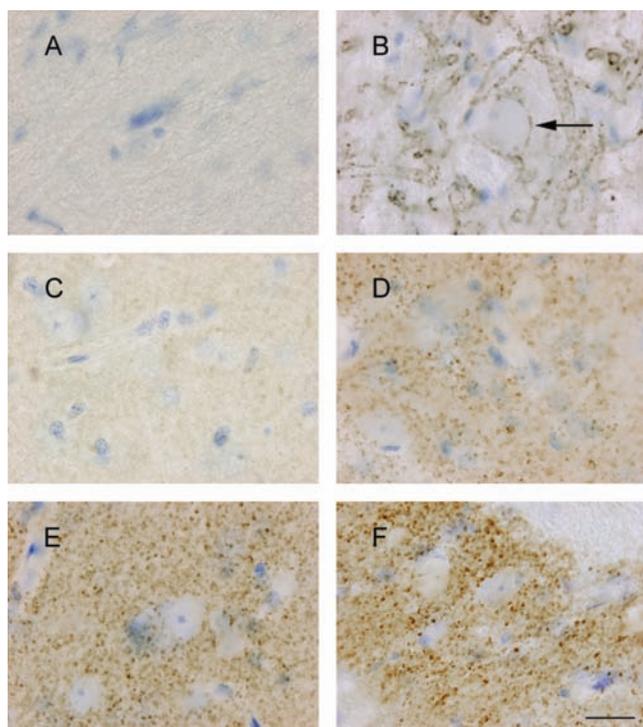


Fig. 7. (A-B) α -syn staining of the SNc in A – control animal and B – 18 mg animal. Staining was ubiquitous with a predilection to fibers and membranes. No neuronal inclusions were observed. Arrow: staining of neuronal membrane. (C-F) α -syn staining in the striatum in C – control animal, D – 24 mg, E – 12 mg and F – 18 mg animal, showing increased staining intensity in the two chronically intoxicated animals (E and F), compared with the acutely intoxicated 24 mg animals and intensified staining in the 18 mg animal compared with the 12 mg animal. Scale bar: 20 μ m.

gait velocity in the 18 mg MPTP group confirmed that this was the optimal dose for animals of this age and weight. The decreasing trend of the gait velocity from 4 to 11 weeks in the 12 and 18 mg groups corresponded with the behavioral signs of stability to possibly slight progression, which however the subjective behavioral analysis was not sensitive enough to register. In addition, digital gait analysis confirmed the behavioral recovery observed in the 24 mg group. The gait analysis equipment was previously used to assess gait parameters in PD patients (Johnsen et al. 2009), emphasizing that the pig may help bridge laboratory and clinical neuroscience. The minipigs were easily trained for the procedure. However, training must be started well before the first data analysis acquisition (baseline) to avoid the effects of learning and habituation (i.e. the animals walking faster and more confidently following the investigator) during the actual experimental part of the study. Such effects could otherwise easily confound the velocity measurements in the preclinical or early phase of the intoxication. Decreases in gait velocity after MPTP can be reliably measured and used as an objective estimate of motor disability. Due to inter-individual variations in the normal gait velocity among animals, although the set-up is time consuming, it is necessary to obtain sufficient baseline, pre-MPTP gait velocity measurements for each individual animal for post-operative gait velocity data comparison, instead of using a population average gait velocity.

The histopathology suggested a dose-dependent loss of TH+ neurons in the SNc, confirmed by stereological cell counting. The number of neurons on HE stains correlated well with the TH stains, confirming that neurons were permanently lost and not just devoid of TH (data not shown). This was also reported previously in acute MPTP intoxication (Seniuk et al. 1990). The 12 mg MPTP group, which showed mild and stable PD symptoms and slightly decreased gait velocity, displayed a neuron loss in the SNc of about 37%. This observation was consistent with previous reports in animal models and in PD patients suggesting the existence of a significant amount of internal compensation early in the disease: indeed, it is widely accepted that at least 50% DA neuronal loss in the SNc is necessary for clearly recognizable clinical symptoms. The 18 mg MPTP group showed greater DA neuron loss (about 60%), accompanied with significantly more severe motor symptoms and decreased DA and metabolites. Interestingly, Fig. 5E showed an increased metabolite/DA ratio in this group, suggesting increased DA turnover. Increased DA turnover has been reported both postmortem and in vivo by PET in non-human primates (Doudet et al. 1998) as well as in early PD patients (Sossi et al. 2002) and is believed to represent one of the early compensatory mechanisms to neuronal loss. Our 4–12 mg animals showed variable loss of DA and metabolites, relatively stable turnovers but little significant motor impairments or decreases

in TH+ neurons, confirming the very early existence of internal compensatory biochemical changes. The 24 mg group, however, also showed increased DA turnover, similarly consistent with the behavioral and pathological observations of serious motor impairments and 78% loss of TH+ neurons in the SNc.

The striatal TH staining showed pronounced loss of TH+ fibers rostrally, loss becoming more severe and eventually more widespread with increasing dose and exposure to the toxin. In PD, the dorsolateral striatal quadrant is typically the most affected (Bové et al. 2005). This observation supports the validity of the proposed model of continuous infusion. We did not see evidence of sprouting in any of the infused animals as has otherwise been reported previously (Song and Haber 2000).

In contrast to Fornai and others (2005) and Gibrat and colleagues (2009) we did not detect α -syn positive intraneuronal inclusions, which is in accordance with others studies in chronic MPTP models and monkeys examined after 10 years of parkinsonism (Alvarez-Fischer et al. 2008, Halliday et al. 2009, Shimoji et al. 2005). However, the neuronal inclusions described by Fornai and Gibrat were restricted to the mice receiving the highest daily doses. It is likely that the doses used in our study were not sufficient to produce similar effect. We did, however, observe a tendency to a more intense α -syn stain with increased continued MPTP doses in contrast to the acutely intoxicated 24 mg animals. This could suggest different pathological mechanisms of the two different administration regimens. Thus, it has been previously shown that acute MPTP intoxication causes necrotic cell death whereas chronic intoxication causes apoptotic neuron loss (Schmidt and Ferger 2001). Double staining of TH and α -syn to confirm the α -syn pathology in dopaminergic neurons under chronic toxic stress as seen in mice is worth performing in future studies in the minipig (Gibrat et al. 2009).

The continuous MPTP intoxication used in this study required large amounts of toxin. Each 18 mg animal received approximately 1200 mg MPTP to produce moderate and stable PD. In comparison, the 24 mg acutely intoxicated animals received 264 mg MPTP, whereas the acutely intoxicated minipigs described by Mikkelsen and others (1999) just received approximately 120 mg. Thus the chronic administration of MPTP appeared to induce a substantial tolerance to the toxin, perhaps as a result of hepatic detoxification (Markey and Schmuft 1986, Yoshihara et al. 2000). Chronic MPTP intoxication seemed to require 10 times as much MPTP as acute intoxication, which may raise economical issues. However, if further studies of this administration route confirm greater similarities to the clinical state of PD than the acute model or even show progression when carried out for longer than 11 weeks, the possibility of valid translational results may by far exceed the economical drawback of the model.

CONCLUSION

The Göttingen minipig model of the chronic PD based on continuous MPTP infusion presented here has been preliminarily characterized behaviorally, chemically, histopathologically and stereologically. Although based on few animals, this model already suggests many resemblances to the clinical state of PD and provides hope for future demonstration of actual disease progression.

The continued lack of accordance between preclinical data of neuroprotective agents and clinical efficacy in PD patients keep emphasizing the need for improved PD animal models. The pig with its large gyrated brain, low cost and easy handling compared with primates along with its genetic and subcortical anatomical resemblance to primates and possibilities of implantation of stem cells, human-sized devices and gene transfer offers a valuable platform for preclinical efficacy and safety experiments. We thus propose that this Göttingen minipig model of PD based on continuous MPTP intoxication may prove valuable in future preclinical neuroprotective studies in PD.

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REFERENCES

- Alvarez-Fischer D, Guerreiro S, Hunot S, Saurini F, Marien M, Sokoloff P, Hirsch EC, Hartmann A, Michelet PP (2008) Modelling Parkinson-like neurodegeneration via osmotic minipump delivery of MPTP and probenecid. *J Neurochem* 107: 701–711.
- Andersen F, Watanabe H, Bjarkam C, Danielsen EH, Cumming P, DaNeX Study Group (2005) Pig brain stereotaxic standard space: mapping of cerebral blood flow normative values and effect of MPTP-lesioning. *Brain Res Bull* 66: 17–29.
- Aziz T (2006) Essential animals. *New Sci* 191: 20.
- Ballard PA, Tetrad 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.
- Baxa M, Hruska-Plochan M, Juhas S, Vodicka P, Pavlok A, Juhasova J, Miyanochara A, Nejime T, Klima J, Macakova M, Marsala S, Weiss A, Kubickova S, Musilova P, Vrtel R, Sontag EM, Thompson LM, Schier J, Hansikova H, Howland DS, Cattaneo E, DiFiglia M, Marsala M, Motlik J (2013) A transgenic minipig model of Huntington's Disease. *J Huntingtons Dis* 2(1): 47–68.
- Bergman H, Wichmann T, DeLong MR (1990) Reversal of experimental parkinsonism by lesions of the subthalamic nucleus. *Science* 249: 1436–1438.
- 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, Deloire X, Bioulac B, Gross CE (1997) A chronic MPTP model reproducing the slow evolution of Parkinson's disease: evolution of motor symptoms in the monkey. *Brain Res* 766: 107–112.
- Bjarkam CR, Cancian G, Glud AN, Ettrup KS, Jorgensen RL, Sorensen JC (2009) MRI-guided stereotaxic targeting in pigs based on a stereotaxic localizer box fitted with an isocentric frame and use of SurgiPlan computer planning software. *J Neurosci Methods* 183: 119–126.
- Bjarkam CR, Cancian G, Larsen M, Rosendahl F, Ettrup KS, Zeidler D, Blankholm AD, Østergaard L, Sunde N, Sørensen JC (2004) A MRI-compatible stereotaxic localizer box enables high-precision stereotaxic procedures in pigs. *J Neurosci Methods* 139: 293–298.
- Bjarkam CR, Glud AN, Margolin L, Reinhart K, Franklin R, Deding D, Ettrup KS, Fitting LM, Nielsen MS, Sørensen JC, Cunningham MG (2010) Safety and function of a new clinical intracerebral microinjection instrument for stem cells and therapeutics examined in the Göttingen minipig. *Stereotact Funct Neurosurg* 88: 56–63.
- Bjarkam CR, Jorgensen R, Jensen KN, Sunde NA, Sørensen JCH (2008a) Deep brain stimulation electrode anchoring using BioGlue®, a protective electrode covering, and a titanium microplate. *J Neurosci Methods* 168: 151–155.
- Bjarkam CR, Nielsen, MS, Glud AN, Rosendahl F, Mogensen P, Bender D, Doudet D, Møller A, Sørensen JC (2008b) Neuromodulation in a minipig MPTP model of Parkinson disease. *Br J Neurosurg* 22 Suppl 1: S9–S12.
- Bjarkam CR, Sørensen JC, Sunde NA, Geneser FA, Ostergaard K (2001) New strategies for the treatment of Parkinson's disease hold considerable promise for the future management of neurodegenerative disorders. *Biogerontology* 2: 193–207.
- Boulet S, Mounayar S, Poupard A, Bertrand A, Jan C, Pessiglione M, Hirsch EC, Feuerstein C, François C, Féger J, Savasta M, Tremblay L (2008) Behavioral recovery in MPTP-treated monkeys: neurochemical mechanisms studied by intrastriatal microdialysis. *J Neurosci* 28: 9575–9584.
- Bové J, Prou D, Perier C, Przedborski S (2005) Toxin-induced models of Parkinson's disease. *NeuroRx* 2: 484–494.
- Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E (2003) Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 24: 197–211.
- Carlsson A (2002) Treatment of Parkinson's with L-DOPA. The early discovery phase, and a comment on current problems. *J Neural Transm (Vienna)* 109: 777–787.
- Carvey PM, Punati A, Newman MB (2006) Progressive dopamine neuron loss in Parkinson's disease: the multiple hit hypothesis. *Cell Transplant* 15: 239–250.
- Dall AM, Danielsen EH, Sørensen JC, Andersen F, Møller A, Zimmer J, Gjedde AH, Cumming P, Danish Neuronal Xenografting Group (2002) Quantitative [18F]fluorodopa/PET and histology of fetal mesencephalic dopaminergic grafts to the striatum of MPTP-poisoned minipigs. *Cell Transplant* 11: 733–746.
- Dalmoose AL, Bjarkam CR, Djurhuus JC (2005) Stereotactic electrical stimulation of the pontine micturition centre in the pig. *BJU Int* 95: 886–889.
- Dalmoose AL, Bjarkam CR, Sørensen JC, Djurhuus JC, Jørgensen TM (2004) Effects of high frequency deep brain stimulation on urine storage and voiding function in conscious minipigs. *NeuroUrol Urodyn* 23: 265–272.
- Danielsen EH, Cumming P, Andersen F, Bender D, Brevig T, Falborg L, Gee A, Gillings NM, Hansen SB, Hermansen F, Johansen J, Johansen TE, Dahl-Jørgensen A, Jørgensen HA, Meyer M, Munk O, Pedersen EB, Poulsen PH, Rodell AB, Sakoh M, Simonsen CZ, Smith DF, Sørensen JC, Ostergård L, Zimmer J, Gjedde A, Møller A (2000) The DaNeX study of

- embryonic mesencephalic, dopaminergic tissue grafted to a minipig model of Parkinson's disease: preliminary findings of effect of MPTP poisoning on striatal dopaminergic markers. *Cell Transplant* 9: 247–259.
- Dolezalova D, Hruska-Plochan M, Bjarkam CR, Sørensen JCH, Cunningham M, Weingarten D, Ciacci JD, Juhas S, Juhasova J, Motlik J, Hefferan MP, Hazel T, Johe K, Carromeu C, Muotri A, Bui J, Strnadel J, Marsala M (2014) Pig models of neurodegenerative disorders: Utilization in cell replacement-based preclinical and efficacy studies. *J Comp Neurol* 522: 2784–2801.
- Doudet DJ, Chan GL, Holden JE, McGeer EG, Aigner TA, Wyatt RJ, Ruth TJ (1998) 6-[18F]Fluoro-L-DOPA PET studies of the turnover of dopamine in MPTP-induced parkinsonism in monkeys. *Synapse* 29: 225–232.
- Doudet DJ, Cornfeldt ML, Honey CR, Schweikert AW, Allen RC (2004) PET imaging of implanted human retinal pigment epithelial cells in the MPTP-induced primate model of Parkinson's disease. *Exp Neurol* 189: 361–368.
- Emborg M (2004) Evaluation of animal models of Parkinson's disease for neuroprotective strategies. *J Neurosci Methods* 139: 121–143.
- Emborg ME (2007) Nonhuman primate models of Parkinson's disease. *ILAR J* 48: 339–355.
- Ettrup KS, Sørensen JC, Bjarkam CR (2010) The anatomy of the Göttingen minipig hypothalamus. *J Chem Neuroanat* 39: 151–165.
- Ettrup KS, Sørensen JC, Rodella A, Alstrup AK, Bjarkam CR (2012) Hypothalamic deep brain stimulation influences autonomic and limbic circuitry involved in the regulation of aggression and cardiocerebrovascular control in the Göttingen minipig. *Stereotact Funct Neurosurg* 90(5): 281–291.
- Ettrup KS, Tornøe J, Sørensen JC, Bjarkam CR (2011) A surgical device for minimally invasive implantation of experimental deep brain stimulation leads in large research animals. *J Neurosci Methods* 200(1): 41–46.
- Félix B, Léger ME, Albe-Fessard D, Marcilloux JC, Rampin O, Laplace JP (1999) Stereotaxic atlas of the pig brain. *Brain Res Bull* 49: 1–137.
- Fjord-Larsen L, Kusk P, Tornøe J, Juliusson B, Torp M, Bjarkam CR, Nielsen MS, Handberg A, Sørensen JC, Wahlberg LU (2010) Long-term delivery of nerve growth factor by encapsulated cell biodelivery in the Göttingen minipig basal forebrain. *Mol Ther* 18: 2164–2172.
- Fornai F, Schlüter 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, Südhof 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.
- Gamache P, Ryan E, Svendsen C, Murayama K, Acworth IN (1993) Simultaneous Measurement of Monoamines, Metabolites and Amino Acids in Brain Tissue and Microdialysis Perfusates. *J Chromatogr* 614: 213–220.
- Gibrat C, Saint-Pierre M, Bousquet M, Lévesque D, Rouillard C, Cicchetti F (2009) Differences between subacute and chronic MPTP mice models: investigation of dopaminergic neuronal degeneration and alpha-synuclein inclusions. *J Neurochem* 109: 1469–1482.
- Giráldez-Pérez RM, Antolín-Vallespín M, Muñoz MD, Sánchez-Capelo A (2014) Models of α -synuclein aggregation in Parkinson's disease. *Acta Neuropathol Commun* 2: 176.
- Glud AN, Hedegaard C, Nielsen MS, Sørensen JC, Bendixen C, Jensen PH, Mogensen PH, Larsen K, Bjarkam CR (2011) Direct MRI-guided stereotaxic viral mediated gene transfer of alpha-synuclein in the Göttingen minipig CNS. *Acta Neurobiol Exp (Wars)* 71(4): 508–518.
- Goodman S, Check E (2002) The great primate debate. *Nature* 417: 684–687.
- Halliday G, Herrero MT, Murphy K, McCann H, Ros-Bernal F, Barcia C, Mori H, Blesa FJ, Obeso JA (2009) No Lewy pathology in monkeys with over 10 years of severe MPTP parkinsonism. *Mov Disord* 24: 1519–1523.
- Jankovic J (2006) An update on the treatment of Parkinson's disease. *Mt Sinai J Med* 73: 682–689.
- Jenner P (2008) Functional models of Parkinson's disease: a valuable tool in the development of novel therapies. *Ann Neurol* 64 Suppl 2: S16–S29.
- Jensen KN, Deding D, Sørensen JC, Bjarkam CR (2009) Long-term implantation of deep brain stimulation electrodes in the pontine micturition centre of the Göttingen minipig. *Acta Neurochir (Wien)* 151: 785–794.
- Johnsen EL, Mogensen PH, Sunde NA, Østergaard K (2009) Improved asymmetry of gait in Parkinson's disease with DBS: gait and postural instability in Parkinson's disease treated with bilateral deep brain stimulation in the subthalamic nucleus. *Mov Disord* 24: 590–597.
- Kalia LV, Kalia SK, Lang AE (2015) Disease-modifying strategies for Parkinson's disease. *Mov Disord* 30(11): 1442–1450.
- Kiebertz K, Ravina B (2007) Why hasn't neuroprotection worked in Parkinson's disease?. *Nat Clin Pract Neurol* 3: 240–241.
- Kragh PM, Nielsen AL, Li J, Du Y, Lin L, Schmidt M, Bøgh IB, Holm IE, Jakobsen JE, Johansen MG, Purup S, Bolund L, Vajta G, Jørgensen AL (2009) Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. *Transgenic Res* 2009;18: 545–558.
- Larsen M, Bjarkam CR, Østergaard K, West MJ, Sørensen JC (2004) The anatomy of the porcine subthalamic nucleus evaluated with immunohistochemistry and design-based stereology. *Anat Embryol (Berl)* 208: 239–247.
- Lind NM, Moustgaard A, Jelsing J, Vajta G, Cumming P, Hansen AK (2007) The use of pigs in neuroscience: modeling brain disorders. *Neurosci Biobehav Rev* 31: 728–751.
- Lindvall O, Wahlberg LU (2008) Encapsulated cell biodelivery of GDNF: a novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease?. *Exp Neurol* 209: 82–88.
- Markey S, Schmuft N (1986) The pharmacology of the parkinsonian syndrome producing neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and structurally related compounds. *Med Res Rev* 6: 389–429.
- Meredith GE, Totterdell S, Potashkin JA, Surmeier DJ (2008) Modeling PD pathogenesis in mice: advantages of a chronic MPTP protocol. *Parkinsonism Relat Disord* 14 Suppl 2: S112–S115.
- Mikkelsen M, Møller A, Jensen LH, Pedersen A, Harajehi JB, Pakkenberg H (1999) MPTP-induced Parkinsonism in minipigs: A behavioral, biochemical, and histological study. *Neurotoxicol Teratol* 21: 169–175.
- Mogensen PH, Jakobsen J (2001) Cued and non-cued repetitive ballistic movements. A kinematic study in healthy subjects. *Acta Neurol Scand* 103: 12–19.
- Mounayar S, Boulet S, Tandé D, Jan C, Pessiglione M, Hirsch EC, Féger J, Savasta M, François C, Tremblay L (2007) A new model to study compensatory mechanisms in MPTP-treated monkeys exhibiting recovery. *Brain* 130: 2898–2914.
- Nielsen MS, Sørensen JC, Bjarkam CR (2009) The substantia nigra pars compacta of the Göttingen minipig: an anatomical and stereological study. *Brain Struct Funct* 213: 481–488.
- Norgaard Glud A, Hedegaard C, Nielsen MS, Sørensen JC, Bendixen C, Jensen PH, Larsen K, Bjarkam CR (2010) Direct gene transfer in the Göttingen minipig CNS using stereotaxic lentiviral microinjections. *Acta Neurobiol Exp (Wars)* 70(3): 308–315.
- Olanow CW, Kiebertz K, Schapira AHV (2008) Why have we failed to achieve neuroprotection in Parkinson's disease?. *Ann Neurol* 64 Suppl 2: S101–S110.
- Østergaard K, Holm IE, Zimmer J (1992) Tyrosine hydroxylase and acetylcholinesterase in the domestic pig mesencephalon: an immunocytochemical and histochemical study. *J Comp Neurol* 322: 149–166.
- 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.
- Rosendal F, Chakravarty MM, Sunde N, Rodell A, Jonsdottir KY, Pedersen M, Bjarkam C, Sørensen JC (2010) Defining the intercommissural plane and stereotactic coordinates for the Basal Ganglia in the Göttingen minipig brain. *Stereotact Funct Neurosurg* 88: 138–146.

- Rosendal F, Frandsen J, Chakravarty MM, Bjarkam CR, Pedersen M, Sangill R, Sorensen JC (2009a) New surgical technique reduces the susceptibility artefact at air-tissue interfaces on in vivo cerebral MRI in the Gottingen minipig. *Brain Res Bull* 80: 403–407.
- Rosendal F, Pedersen M, Sangill R, Stødkilde-Jørgensen H, Nielsen MS, Bjarkam CR, Sunde N, Sorensen JC (2009b) MRI protocol for in vivo visualization of the Gottingen minipig brain improves targeting in experimental functional neurosurgery. *Brain Res Bull* 79: 41–45.
- Schmidt N, Ferger B (2001) Neurochemical findings in the MPTP model of Parkinson's disease. *J Neural Transm (Vienna)* 108: 1263–1282.
- Seniuk NA, Tatton WG, Greenwood CE (1990) Dose-dependent destruction of the coeruleus-cortical and nigral-striatal projections by MPTP. *Brain Res* 527: 7–20.
- Shimoji M, Zhang L, Mandir AS, Dawson VL, Dawson TM (2005) Absence of inclusion body formation in the MPTP mouse model of Parkinson's disease. *Brain Res Mol Brain Res* 134: 103–108.
- Song DD, Haber SN (2000) Striatal responses to partial dopaminergic lesion: evidence for compensatory sprouting. *J Neurosci* 20: 5102–5114.
- Sossi V, de La Fuente-Fernandez R, Holden JE, Doudet DJ, McKenzie J, Stoessl AJ, Ruth TJ (2002) Increase in dopamine turnover occurs early in Parkinson's disease: evidence from a new modeling approach to PET 18 F-fluorodopa data. *J Cereb Blood Flow Metab* 22: 232–239.
- Sørensen JC, Bjarkam CR, Danielsen EH, Simonsen CZ, Geneser FA (2000) Oriented sectioning of irregular tissue blocks in relation to computerized scanning modalities: results from the domestic pig brain. *J Neurosci Methods* 104: 93–98.
- Suchowersky O, Gronseth G, Perlmutter J, Reich S, Zesiewicz T, Weiner WJ (2006) Practice Parameter: neuroprotective strategies and alternative therapies for Parkinson disease (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 66: 976–982.
- Watanabe H, Andersen F, Simonsen CZ, Evans SM, Gjedde A, Cumming P, DaNex Study Group (2001) MR-based statistical atlas of the Gottingen minipig brain. *Neuroimage* 14: 1089–1096.
- Wernersson R, Schierup MH, Jørgensen FG, Gorodkin J, Panitz F, Staerfeldt H-H, Christensen OF, Mailund T, Hornshøj H, Klein A, Wang J, Liu B, Hu S, Dong W, Li W, Wong GKS, Yu J, Wang J, Bendixen C, Fredholm M, Brunak S, Yang H, Bolund L (2005) Pigs in sequence space: a 0.66X coverage pig genome survey based on shotgun sequencing. *BMC Genomics* 6: 70.
- West MJ (1999) Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. *Trends Neurosci* 22: 51–61.
- Yacoubian TA, Standaert DG (2009) Targets for neuroprotection in Parkinson's disease. *Biochim Biophys Acta* 1792: 676–687.
- Yoshihara S, Harada K, Ohta S (2000) Metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in perfused rat liver: involvement of hepatic aldehyde oxidase as a detoxification enzyme. *Drug Metab Dispos* 28: 538–543.