

# Spontaneous Partial Recovery of Striatal Dopaminergic Uptake Despite Nigral Cell Loss in Asymptomatic MPTP-Lesioned Minipigs

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## Research Article

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# Abstract

The gold standard animal model of Parkinson's disease is the non-human primate rendered parkinsonian with the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin. Low availability, ethical issues, and primate-specific biohazards make alternative large animal models necessary. Here, we investigate the temporal evolution of presynaptic dopaminergic function after MPTP in another large animal model, the Göttingen minipig. We subcutaneously injected seven sedated minipigs with 1–2 mg/kg of MPTP, and two minipigs with saline, three times a week over 4 weeks. We monitored behavioral deficits using a validated motor scale and a Gait4Dog® walking mat. Minipig brains were imaged with (+)-[<sup>11</sup>C]-dihydrotrabenzazine ([<sup>11</sup>C]-DTBZ) and [<sup>18</sup>F]-fluorodopa ([<sup>18</sup>F]-FDOPA) PET at baseline and 1, 3, 9 and 12 months after the final MPTP injection. Immunohistochemical tyrosine hydroxylase (TH) staining was used to assay nigral TH+ area loss post-mortem. The minipigs showed only mild bradykinesia and impaired coordination at early timepoints after MPTP. PET revealed decreases of striatal [<sup>11</sup>C]-DTBZ and [<sup>18</sup>F]-FDOPA uptake post-MPTP with a partial spontaneous recovery of [<sup>18</sup>F]-FDOPA after 9 months. Postmortem histological analysis showed a loss of 71% TH-immunopositive area in the substantia nigra. When testing the efficacy of putative neuroprotective agents, partial spontaneous recovery of dopamine terminal function must be taken into account in the MPTP minipig model of parkinsonism.

## Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by degeneration of nigro-striatal dopaminergic neurons associated with motor symptoms including bradykinesia, rigidity and tremor. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD in non-human primates is a well-established animal lesion model of PD<sup>1</sup>. MPTP is metabolised to a toxic by-product, MPP+, which enters dopaminergic neurons and induces neuronal degeneration by blocking complex 1 activity of the mitochondrial electron transport chain<sup>2</sup>. The model presents with parkinsonian motor symptoms, shows loss of nigral dopamine neurons and striatal levels of dopamine, and responds to levodopa therapy, making it an ideal model system to evaluate the efficacy of neuroprotective agents<sup>3–6</sup>. MPTP also induces dopamine deficiency in mice<sup>7–9</sup> and a number of other small species, including the goldfish<sup>10</sup>. Although it is regarded as the 'gold standard' model of PD, there are major limitations inherent to the model, including variable dose response, poorly understood compensatory mechanisms, and spontaneous recovery from motor symptoms.

Non-human primates are often the preferred species for the MPTP model, but low availability, ethical, and cost issues limit their use. The Göttingen minipig is a strain specifically designed for research as their limited growth permits long-term longitudinal follow-up with non-invasive neuroimaging while their brain size allows identification of the sub-regional striatal distribution of pre- and post-synaptic dopaminergic tracers<sup>11–14</sup>. Our team has previously developed and validated MPTP-lesion pig models and studied the efficacy of stem cell transplantation and deep brain stimulation of the subthalamic nucleus<sup>15–20</sup>.

In the current report, we aimed to study the longitudinal effects of MPTP administration to minipigs on behavior and uptake of presynaptic markers of the dopamine system using serial positron emission tomography (PET) over one year. Despite large doses of MPTP, the minipigs became only mildly symptomatic at early stages after MPTP injections. Exogenous dopa decarboxylation in dopaminergic terminals was serially studied with fluoro-3,4-dihydroxyphenylalanine ( $[^{18}\text{F}]$ -FDOPA) PET<sup>21</sup> while vesicular monoamine transporter 2 (VMAT2) availability was measured with (+)- $[^{11}\text{C}]$ -dihydrotetrabenazine ( $[^{11}\text{C}]$ -DTBZ) PET<sup>22</sup>. The longitudinal PET data are discussed in light of the mild behavioral changes and neuropathology of the minipigs after MPTP and compared to reported findings in non-human primates.

## Results

### Behavior

During the first weeks after MPTP injections, all seven pigs had mildly reduced motility, three had abnormal standing/leg positions, one displayed freezing of gait, one had head tremor, and three had abnormal swallowing. All of these symptoms, however, were mild and the total score for each animal ranged from 0–2, with only one animal with a score of 3 out of 12 in the first weeks after the MPTP treatment. The animals in this study were, therefore, classified as asymptomatic or mildly symptomatic during the most affected period, and did not need treatment of symptoms. No score above 0 was observed in the two saline-injected animals at any time during the study.

Using the GAIT4Dog® walking mat, we measured gait symmetry of the minipigs by assessing the Total Pressure Index (TPI) for all limbs, which is expected to be 30% for each front limb (LF and RF) and 20% for each hind limb (LH and RH) under healthy conditions. Both saline (n = 2) and MPTP injected minipigs (n = 7) had TPIs close to the expected 30% for front limbs (range: 28.0-32.5%) and 20% for hind limbs (range: 17.5–20.9%) at all measured timepoints accounting for small individual variances (Table 1).

Table 1

Total Pressure Index (TPI, %) for saline and MPTP-injected minipigs at baseline, 1 week (MPTP only), 4 months and 15 months after the last injection. LF = Left front limb, RF = Right front limb, LH = Left hind limb, RH = Right hind limb

	Saline (n = 2)			MPTP (n = 7)			
	Baseline	4 mo	15 mo	Baseline	1 week	4 mo	15 mo
<b>TPI% LF</b>	32.3 ± 0.4	31.5 ± 1.6	31.8 ± 1.3	30.6 ± 1.6	29.9 ± 1.0	29.2 ± 1.6	28.0 ± 1.0
<b>TPI% RF</b>	32.5 ± 0.5	32.3 ± 2.6	31.6 ± 1.1	30.3 ± 1.2	30.1 ± 1.2	30.9 ± 1.1	30.2 ± 1.9
<b>TPI% LH</b>	17.9 ± 1.3	18.3 ± 2.1	19.0 ± 2.5	19.7 ± 1.6	20.0 ± 1.2	20.4 ± 1.1	20.9 ± 1.5
<b>TPI% RH</b>	17.5 ± 0.3	17.8 ± 1.8	17.5 ± 0.1	19.4 ± 0.4	20.0 ± 0.7	19.5 ± 0.7	20.8 ± 1.3

We also used the GAIT4Dog® walking mat to study gait velocity and found a small trend towards a decrease (12%,  $p = 0.23$ ) in the 7 pigs injected with MPTP one month after the final MPTP injection. By the next testing on the gait mat at 4 months after the final MPTP injection, the gait velocity in all pigs had returned to baseline values, and by the final timepoint 15 months after the last MPTP injection, the values had significantly increased by 29% from baseline values ( $p < 0.016$ ) (Fig. 1).

## Longitudinal effects of MPTP neurotransmission

Average parametric  $K_i$  and  $BP_{ND}$  maps at baseline and 1, 3, 9 and 12 months after MPTP are shown in Fig. 2a and b.

MPTP significantly reduced striatal [ $^{18}F$ ]-FDOPA  $K_i$  values at each of the 4 timepoints by an average of 65.7%, 63.7%, 52.4% and 50.4%, respectively, after MPTP injections compared to baseline (one-way ANOVA, Bonferroni correction,  $p < 0.0001$ ), while no significant changes were observed in response to saline injections (Fig. 3a). Similarly, MPTP significantly reduced striatal [ $^{11}C$ ]-DTBZ  $BP_{ND}$  values at each of the four timepoints by 58.9%, 59.0%, 54.9% and 55.4%, respectively, after the MPTP injections compared to baseline (one-way ANOVA, Bonferroni correction,  $p < 0.0001$ ) while saline-injected animals showed no significant changes from baseline (Fig. 3b).

In order to test the impression that the binding values recovered between the time of initial intoxication and the end of the study, we performed a two-tailed, paired t-test on the data from the 6 minipigs injected with MPTP that were scanned at both the 1- and 9-month timepoints. We found that striatal [ $^{18}F$ ]-FDOPA  $K_i$  values recovered significantly (38.6%,  $p < 0.05$ ) (Fig. 3c) from 1 to 9 months but with no significant recovery in striatal [ $^{11}C$ ]-DTBZ  $BP_{ND}$  that only increased by 9.7% between the 1- and 9-month timepoints (Fig. 3d). The recovery in striatal [ $^{18}F$ ]-FDOPA  $K_i$  values further progressed to 44.4% at 12 months vs 1 month after MPTP.

# Histology

The SN anti-TH staining was semi-quantified by measuring the nigral TH-immunopositive area on both hemispheres on five corresponding sections per animal. In Fig. 4 the mean immunopositive area ( $\mu\text{m}^2$ ) is shown for each animal (top) and each group (bottom). The TH-immunopositive area was significantly reduced by 71% (95% CI: 1,353,966 to 546,152,  $p < 0.001$ ) in the MPTP-injected pigs ( $n = 7$ ) compared to the saline-injected pigs ( $n = 2$ ), confirming the presence of cell death in the model.

## Correlations

We interrogated whether [ $^{18}\text{F}$ ]-FDOPA uptake and [ $^{11}\text{C}$ ]-DTBZ binding were correlated using measures at each time-point after MPTP (Fig. 5). We found a significant, positive, linear correlation between the binding of the two radioligands at 9 months after MPTP ( $r = 0.9131$ ,  $n = 6$ ,  $p < 0.05$ ) but not at any other timepoint (1 month:  $r = 0.697$ ,  $n = 7$ ,  $p > 0.05$ ; 3 months:  $r = 0.5093$ ,  $n = 6$ ,  $p > 0.05$ ; 12 months:  $r = 0.7772$ ,  $n = 6$ ,  $p > 0.05$ ).

We then tested potential correlations between binding of each PET ligand at 12 months after MPTP and our semi-quantitative measure of TH positive SN area (Fig. 6) and found a significant, positive, linear correlation for [ $^{11}\text{C}$ ]-DTBZ ( $r = 0.888$ ,  $n = 6$ ,  $p < 0.05$ ) with TH+ area, but not for [ $^{18}\text{F}$ ]-FDOPA ( $r = 0.6092$ ,  $n = 6$ ,  $p > 0.05$ ).

## Discussion

We followed longitudinally the behavior and dopaminergic function of Göttingen minipigs exposed repetitively to MPTP with multi-tracer PET imaging. The MPTP paradigm was similar to that previously used in a large animal model which successfully induced parkinsonism<sup>15-20</sup>. Our data show that minipigs dosed with MPTP over a one month period demonstrate significant reductions in dopaminergic presynaptic function, observed up to one year later, along with cell death, even in the absence of sustained behavioral symptoms. Despite the significant reductions in tracer binding at all timepoints post-MPTP, we found spontaneous partial recovery of striatal [ $^{18}\text{F}$ ]-FDOPA Ki uptake when comparing data 1 month vs 9 months after the final MPTP injection. Even though the minipigs showed only mild and reversible behavioral changes after MPTP in the most aggressive phase, the administration protocol was effective and sufficient to induce *in vivo* dopamine storage and VMAT2 binding changes along with pathological nigral dopamine cell loss.

The MPTP model in non-human primates is still the gold-standard in PD animal lesion models, especially for trialing the efficacy of novel symptomatic medications<sup>1</sup>. However, there are well known limitations to MPTP administration, including spontaneous recovery from motor symptoms<sup>23,24</sup> which is not observed in idiopathic PD patients. This limits its potential to investigate long-term efficacy of putative neuroprotective medications on motor symptoms. Studies have found that cell loss, however, is not

statistically different between non-human primates that recover from MPTP exposure compared to those with stable motor deficits<sup>25</sup> suggesting variable degrees of dopamine terminal adaptation to the nigral lesions are occurring. Indeed, it has been found that remaining TH+ fibers can sprout and increase branching after partial lesion with MPTP in non-human primates<sup>26</sup> and our data suggest an increase in aromatic L-amino acid decarboxylase (AADC) activity important for the intracellular retention of [<sup>18</sup>F]-FDOPA in remaining terminals at both 9 and 12 months post-MPTP. It is also known that the same MPTP dose can lead to mild to severe motor deficits in the same species. Previous studies of Göttingen minipigs administered MPTP have shown behavioral sensitivity and moderate parkinsonism, though they reported a non-significant tendency towards improved scores throughout the 6-month study period<sup>17,18</sup>. In contrast to their low 0.7-1 mg/kg daily SC injection (cumulative dose of 6.6 ± 1.4 mg/kg over a 22-day injection period), our initial dose of 1 mg/kg three times a week, was doubled in the final 2 weeks of injections due to the apparent lack of motor symptoms, resulting in a cumulative dose of 18 mg/kg. Previous studies in non-human primates administered cumulative doses of 1.6 mg/kg<sup>27</sup> and 1.8 mg/kg as a progressive intoxication dose, and 3.2 mg/kg as an acute intoxication dose<sup>25</sup>. It is unclear why the MPTP dose in the current cohort of minipigs, which far exceeded the dose administered in previous large animal studies, was not sufficient to elicit moderate parkinsonian symptoms, or any stable behavioral impairment.

However, we speculate that our use of midazolam and (S)-ketamine anesthesia prior to MPTP-injections to increase safety of the staff handling MPTP might have protected the minipigs against extensive fiber loss. Midazolam and MPTP are both competitive inhibitors of various cytochrome P450 isozymes, that are involved in the neurotoxicity of MPTP<sup>28,29</sup>. Furthermore, a recent study found that (R)-ketamine attenuated MPTP-induced reduction in striatal dopamine transporter (DAT) and TH, while (S)-ketamine only had a small effect on DAT<sup>30</sup>. Therefore, the combination of midazolam (S)-ketamine may have reduced the dopamine terminal loss. Nevertheless, the dose was sufficient to induce 71% TH+ area loss in the SN and significant reductions in PET markers of striatal presynaptic dopamine neurotransmission.

[<sup>18</sup>F]-FDOPA PET has previously been used to assess decline in striatal dopamine terminal function in MPTP-induced parkinsonism and its response to fetal mesencephalic dopaminergic allografts in pigs<sup>17</sup> and humans<sup>31</sup>. The previous transplant study reported motor symptoms and a stable 60% loss of dopa decarboxylase activity 3- and 6-months after MPTP in the non-grafted minipigs. We show here that asymptomatic minipigs demonstrate a clear and persistent reduction in [<sup>18</sup>F]-FDOPA uptake even when behaviorally intact but, for the first time, we also demonstrate a significant recovery at 9 months after MPTP intoxication, which was also sustained at 12 months post-MPTP. The observed decline in striatal [<sup>18</sup>F]-FDOPA uptake of MPTP-lesioned pigs is consistent with changes reported in MPTP-exposed non-human primates<sup>32</sup> and both idiopathic and MPTP-exposed PD patients<sup>33</sup>. Franke *et al.* also suggested that mild pathological changes were reversible after cessation of MPTP administration in marmosets<sup>34</sup>.

We have previously shown reductions in striatal [ $^{11}\text{C}$ ]-DTBZ binding in minipig models of parkinsonism induced by acute and chronic proteasome inhibition<sup>11,14</sup>, and preformed  $\alpha$ -synuclein fibrils<sup>35</sup> or adeno-associated viral (AAV) vector induced  $\alpha$ -syn overexpression in rodents<sup>36</sup>. However, this is the first study of VMAT2 binding in MPTP-injected minipigs. Radio-labeled DTBZ binds with nanomolar-affinity to the VMAT2 transporter protein of synaptic vesicles in monoaminergic neurons<sup>37</sup> and can non-invasively track presynaptic dopamine terminal density in the striatum<sup>38</sup>. It was once thought that DTBZ binding was insensitive to pharmacological challenges<sup>39</sup>, however, it is now accepted that binding may be altered by changes in intravesicular concentrations of stored dopamine<sup>40–42</sup>. Selective loss of VMAT2 is one of the first measurable effects of aging and active PD in human, and aging and MPTP exposure in non-human primates<sup>22,43</sup> and precedes the appearance of clinical features. VMAT2 reductions in striatum and SN correlate with disease severity of PD patients<sup>44</sup>. Here we show 55–60% decreases in VMAT2 availability in MPTP-treated minipigs in the absence of overt motor features of parkinsonism. Unlike [ $^{18}\text{F}$ ]-FDOPA, at the time of the final imaging sessions there was no significant recovery in [ $^{11}\text{C}$ ]-DTBZ binding. Previous studies have found 35–46% loss of VMAT2 in striatal regions of asymptomatic non-human primates 2 months after onset of MPTP exposure which progressed to a 69–78% loss by 10 months<sup>43</sup>. The different results are likely attributable to the fact that the investigators continued to administer MPTP throughout the entire non-human primate study, whereas the scans conducted in minipig were done at specific times after the final MPTP administration in our subchronic protocol.

As [ $^{18}\text{F}$ ]-FDOPA  $K_i$  reflects blood-brain barrier transport, AADC activity and vesicular storage, whereas [ $^{11}\text{C}$ ]-DTBZ  $\text{BP}_{\text{ND}}$  only reflects synaptic monoamine vesicle availability, the main differences between the uptake and binding should be reflected by the decarboxylation. Using [ $^{18}\text{F}$ ]-FDOPA, [ $^{11}\text{C}$ ]-DTBZ and [ $^{11}\text{C}$ ]-methylphenidate (labeling DAT) PET, Lee et al. have found that [ $^{18}\text{F}$ ]-FDOPA uptake was reduced to a lesser extent than [ $^{11}\text{C}$ ]-DTBZ and [ $^{11}\text{C}$ ]-methylphenidate in PD basal ganglia<sup>45</sup>. This difference in uptake and binding was argued to reflect up-regulation of AADC activity in the parkinsonian striatum as a plastic mechanism to increase availability of dopamine to postsynaptic dopamine receptors<sup>45</sup>. Our functional recovery in [ $^{18}\text{F}$ ]-FDOPA uptake at 9 and 12 months post-MPTP might therefore be explained by AADC activity being more prone to up-regulation following dopamine denervation than VMAT2 due to dynamic regulation and compensatory mechanisms<sup>46,47</sup>. Furthermore, the significant correlation of striatal [ $^{18}\text{F}$ ]-FDOPA uptake and [ $^{11}\text{C}$ ]-DTBZ binding only at the 9 month timepoint supports the differential behavior of the two PET tracers across time after MPTP lesioning in minipigs. We show the temporal stability of [ $^{11}\text{C}$ ]-DTBZ as a marker of terminal damage that correlates with TH+ area. This makes the case for using [ $^{11}\text{C}$ ]-DTBZ when planning trials of neuroprotective agents in MPTP-lesioned animals.

In conclusion, we show here for the first time the temporal evolution of two markers of presynaptic dopamine function in a large animal model, the Göttingen minipig, as an alternate to the non-human primate, in response to MPTP. Our asymptomatic to mildly symptomatic animals showed similar radioligand changes observed in behaviorally impaired animal models as well as in human PD. The

recovery of [<sup>18</sup>F]-FDOPA binding 9 and 12 months after the final dose of MPTP suggests that caution should be taken when planning investigations of therapeutic interventions, which may be confounded by spontaneous recovery of the model.

## Material And Methods

### Animals

We studied nine 1-year old female Göttingen minipigs (Ellegaard Minipigs ApS, Dalmose, Denmark) each weighing approximately 25 kg at the beginning of the experiment. This study was approved and regulated by The Danish Council for Animal Experiments (License: 2016 - 15 - 0201 - 00878) and all experiments were carried out in accordance with Danish law and regulations for the Protection of Animals used for Scientific Purposes, the ARRIVE guidelines and the 2010/63/EU directive. An animal technician and veterinarian monitored animals daily and weekly, respectively. The pigs were acclimatized for several weeks prior to the experiment. We fed minipigs a restricted pellet diet (SDS Diet, Witham, UK) and fasted them overnight, with free access to tap water, prior to the day of the experiment. We group-housed pigs in a 4.6 m<sup>2</sup> enclosure with fence-line visual contact to one another under environmental conditions of 20°C and 50–55 % relative humidity, with air changed 8 times every hour and 12 hour light/dark cycles. We sedated minipigs during subcutaneous MPTP- or saline-injections and anesthetized them for the scanning procedures. We followed humane endpoints of weight loss of over 10% body weight in combination with other observations suggestive of poor well-being, bleeding complications or signs of hypoxia under anaesthesia. All minipigs were clinically healthy prior to the study, and they were randomized to either MPTP or saline treatment groups.

### Study overview

Minipigs were imaged at baseline with [<sup>18</sup>F]-FDOPA and [<sup>11</sup>C]-DTBZ PET and then injected 3 times per week with 1–2 mg/kg MPTP over a 4-week period. Pigs were scanned four additional times with the same two radioligands at 1, 3, 9 and 12 months after their last MPTP injection. Two animals were injected with saline 3 times per week over a 4-week period and were scanned at baseline and 1, 6 and 9 months after the final saline injection. Due to the radiochemistry schedule, it was not possible to conduct PET with both radioligands in each animal at each timepoint and one [<sup>18</sup>F]-FDOPA scan was excluded due to an acquisition error. This led to a varying number of scans across the animals. The MPTP group PET data was obtained with group sizes of at least 5 minipigs (5–6 for baseline and 6–7 for post MPTP scans). Neurological scoring and gait were assessed throughout the study. Histology for tyrosine hydroxylase (TH) immunopositive neurons was performed to assess nigral cell death.

The MPTP-injected minipigs were part of a larger study of the effects of human engineered stem cells as therapeutic agents in the MPTP pig model of PD. Due to lack of behavioral symptoms, spontaneous partial recovery of striatal dopaminergic neurotransmission, and insufficient immunosuppression (> 2 µg/l tacrolimus blood level), there were no differences between the minipigs that received stem cells and

sham treatment in any of the studied parameters. We, therefore, pooled the data for the purpose of this study to investigate the long-term effects of MPTP on [<sup>18</sup>F]-FDOPA and [<sup>11</sup>C]-DTBZ binding, and potential recovery of PET measures in near asymptomatic MPTP-injected minipigs.

## MPTP treatments

Three times a week over a 4-week period, we sedated the minipigs with approximately 0.8 mg/kg of midazolam (Hameln) and 5 mg/kg of S-ketamine (Pfizer) or 1.2–2.8 mg/kg midazolam alone. We injected 1 mg/kg of MPTP (BioNordika Denmark A/S, Herlev) for the first 2 weeks and then 2 mg/kg for the last 2 weeks resulting in a cumulative dose of 18 mg/kg subcutaneously into the hind leg groin (SC) designed to induce a stable syndrome of parkinsonism. MPTP intoxication using this dose range previously resulted in a moderate parkinsonian syndrome, characterized by reduced spontaneous movement, and by rigidity and limb incoordination, especially of the hind limbs<sup>17,18</sup>. Control pigs had an equivalent volume of saline injected.

## Neurological Score and behavioral impairment

During the intoxication period and every week after MPTP or saline injections, we scored the neurological status of the pigs. We used previously published protocols<sup>15,17,18</sup> with a few modifications. Ratings included **(A)** Motility – 0: normal, 1: slightly compromised, 2: moderately compromised, 3: severely compromised, 4: lean against wall, 5: freezing of muscles, **(B)** Position abnormality/Coordination – 0: normal, 1: abnormal leg positioning (i.e. crossing of hindlimbs), 2: lowered head, 3: clear coordination issues, **(C)** Muscle rigidity – 0: normal, 1: slight rigidity, 2: severe rigidity, and **(D)** Feeding function – 0: normal, 1: slightly compromised feeding function, 2: rigid jaws and reduced feeding function (total maximum score = 12).

We also assessed walking speed and total pressure index (TPI) of the gait using a GAIT4Dog® walking mat prior to any treatments and at 1 week, 4 months and 15 months after the last MPTP or saline treatment. Minipigs walked at their preferred velocity on the pressure sensing walkway system. Three consecutive gait cycles (12 steps) or more accounted for one quality reading and the final measures were an average of at least 3 quality readings per animal.

## PET Imaging

As previously described<sup>12</sup>, minipigs were anesthetized with a mixture of 1.25 mg/kg midazolam (Hameln) and 6.25 mg/kg S-ketamine (Pfizer) IM, intubated for mechanical ventilation and prepared for PET imaging. We monitored physiological parameters which all remained at normal porcine values in line with Alstrup (2010)<sup>48</sup>.

We placed minipigs supine in a human PET/CT scanner (Siemens Biograph 64 Truepoint PET) and immobilised their head and body. We positioned the animal within the field of view of the PET camera and performed a low-dose CT scan prior to each PET recording for anatomical definition and attenuation correction of PET emission data.

We intravenously administered an average of  $369 \pm 26$  MBq (range 315–415 MBq) (+)-a-[ $^{11}\text{C}$ ] labeled DTBZ (saline with 10% ethanol) via an ear vein catheter in 10 mL saline, during the initial 60 seconds of a 90-minute scan. After the DTBZ scan, we intravenously administered carbidopa (50 mg/10 mL, 42 mM  $\text{NaH}_2\text{PO}_4$ , pH 3) in each animal to reduce their peripheral DOPA decarboxylase activity and then after 30 minutes scanned for 90 minutes with an average of  $199 \pm 22$  MBq (range 107–222 MBq) [ $^{18}\text{F}$ ] labeled FDOPA (70 mM  $\text{NaH}_2\text{PO}_4$ , pH 4.5). [ $^{11}\text{C}$ ]-DTBZ data was collected first due to the shorter 20-minute half-life of [ $^{11}\text{C}$ ]. We reconstructed PET data using TrueX 3D OSEM (3 iterations), a 256 x 256 x 109 matrix, and a 2-mm Gauss filter, using a time-frame structure of 5 x 60, 3 x 300, 4 x 600, 2 x 900 seconds (total 14 frames, 90 minutes).

## Quantitative PET analyses

PET studies were analysed using routine existing procedures for pigs developed in Aarhus<sup>11,14,49</sup>. We performed all preprocessing steps using PMOD™ 3.7 (PMOD Technologies Ltd, Zurich, Switzerland), a commercially available software package for biomedical image quantification. To define the stereotaxic transformation parameters from time-averaged PET images of [ $^{11}\text{C}$ ]-DTBZ, we used a ligand-specific template. We applied the generated transformation matrices and warping fields onto the corresponding dynamic PET time series. We generated parametric  $\text{BP}_{\text{ND}}$  images of [ $^{11}\text{C}$ ]-DTBZ binding using the Simplified Reference Tissue Model 2 (SRTM2). [ $^{18}\text{F}$ ]-FDOPA data was analysed using Patlak reference plots with  $t^*$  set at 30 minutes as the time for the tracer to equilibrate between the plasma and brain free compartments. Based on the atlas by Watanabe (2001)<sup>50</sup>, we created custom-made masks of the cerebellum to obtain the cerebellar tissue radioactivity as a region of non-displaceable binding and extracted time activity curves for the cerebellum and striatum.

## Histology

Deeply anesthetized (Zoletil® 50 Vet.) minipigs were euthanised 15 months post MPTP with an intracardial injection of 20 mL sodium pentobarbital 400 mg/mL (Exagon Vet, Richter Pharma, Austria) followed by transcardial perfusion with 5 L of 4% formaldehyde prior to brain removal as previously described<sup>51,52</sup> and stored in 4% formaldehyde at 4°C. Brains were then embedded in a sectioning chamber (Quick Slicer 100 mm, HistOtech, Aarhus, Denmark) filled with HistOmer mixed with water. Following polymerization of the HistOmer, 2 cm thick sections were cut and transferred to a 30% sucrose in isotonic buffer solution for 1–2 weeks. Brain slabs were then frozen in isopentane cooled to  $-40^\circ\text{C}$  for 1–2 min before being cut into eight series of coronal sections (50  $\mu\text{m}$ ) on a CryoStar NX70 (Thermo Scientific) and stored free-floating in DeOlmos at  $-20^\circ\text{C}$ . One series was mounted directly onto 0.5% gelatin-coated slides and stained with 0.1% toluidine blue in citrate buffer (pH 4) for 9 min followed by a rinse in distilled water, dehydration in 99% alcohol (2 x 30 s) and clearing with xylene before being coverslipped with Pertex®.

Sections covering the substantia nigra were stained with anti-TH as in Lillethorup, et al.<sup>11</sup> using TBS containing 0.25% Triton X-100 (TBS-T, 0.05 M, pH 7.4) as a buffer, rabbit anti-TH (1:1000, ab112, Abcam)

as primary antibody, a horseradish peroxidase (HRP) labeled secondary antibody (HRP-labeled anti-rabbit, 1:400, Dako) and 0.05% 3,3'-diaminobenzidine (DAB; Alfa Aesar, Cat.no J60972), TBS and 0.01% H<sub>2</sub>O<sub>2</sub> for visualization. Sections were then mounted on large 0.5% gelatin-coated slides, cleared in xylene for 5 min and coverslipped with Pertex®. Images were taken using a Leica camera (DFC480) connected to a Leica microscope (DM5000B). The TH-stained sections were semi-quantified as described in Lillethorup, et al.

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## Statistics

Statistical tests were performed using Prism8 (GraphPad Software, California, USA). To compare PET and behavioral data in animals injected with MPTP at each timepoint, a one-way ANOVA was used with a Bonferroni correction for multiple comparisons where each post-MPTP timepoint was compared to baseline. The statistical significance level was set at  $p < 0.05$  and normal distribution of the data was checked. In order to test the hypothesis that the animals recovered significantly from MPTP treatment, a two-tailed paired t-test was performed on the data acquired 1 month vs 9 months after the last MPTP treatment. Furthermore, an unpaired two-tailed t-test was performed comparing nigral TH-immunopositive area of saline- and MPTP-treated animals. Pearson correlation was performed to determine the relationship between FDOPA uptake and DTBZ binding at 1, 3, 9 and 12 months after MPTP and between the measures of each of the two PET tracers at 12 months and the TH-immunopositive area.

## Declarations

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### Competing interests

The authors declare no competing interests.

### Author contribution statement

AML, KS, DJB and JCS designed the studies. KS, MBT, HZ, DO and ANG did the MPTP injections and behavioral testing and TPL analysed the gait data. EHTN and AS synthesized the radioligand, and AKOA, KS and MBT imaged the minipigs. AML, ON and MW conducted the PET analysis. TWM, DO and TPL did the immunohistochemical stainings and analysis. AML wrote the first draft of the manuscript with support from TPL and DJB. All authors have provided suggestions and approved the final version of the manuscript.

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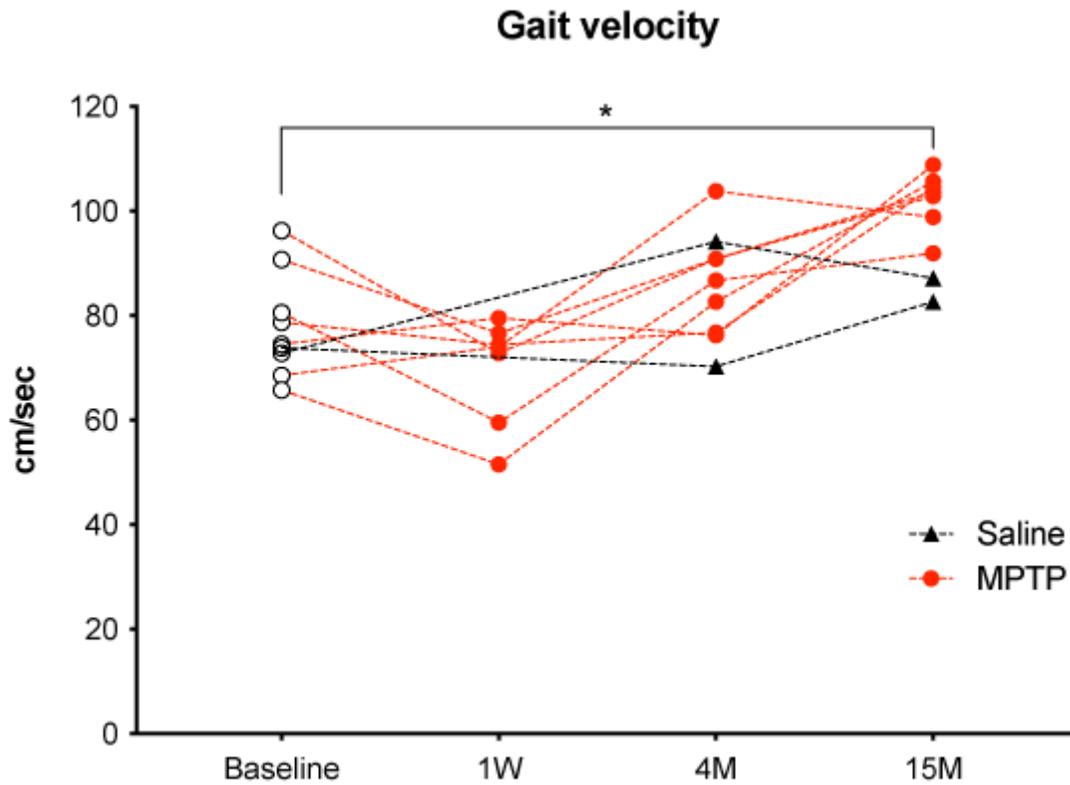
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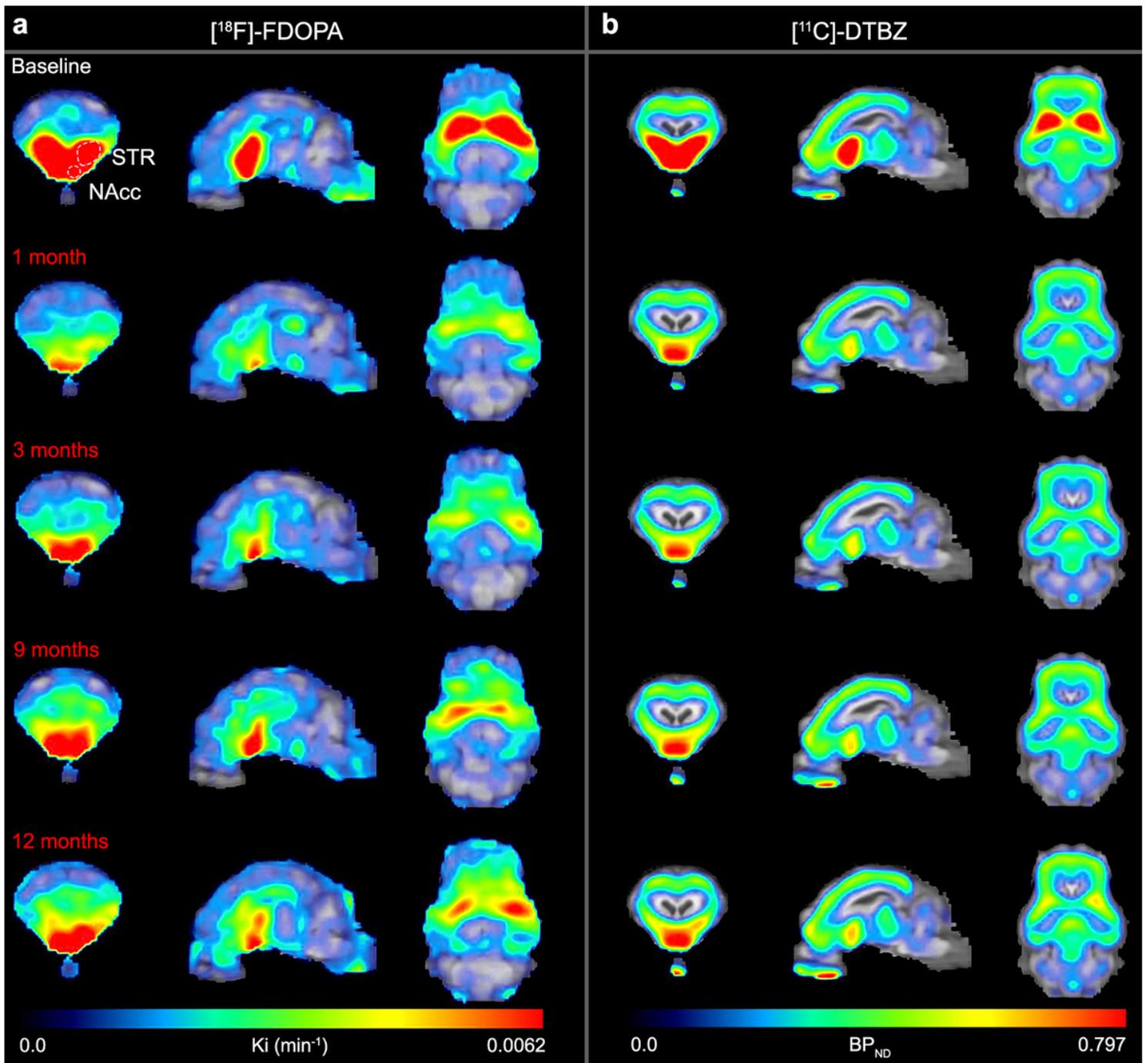
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## Figures



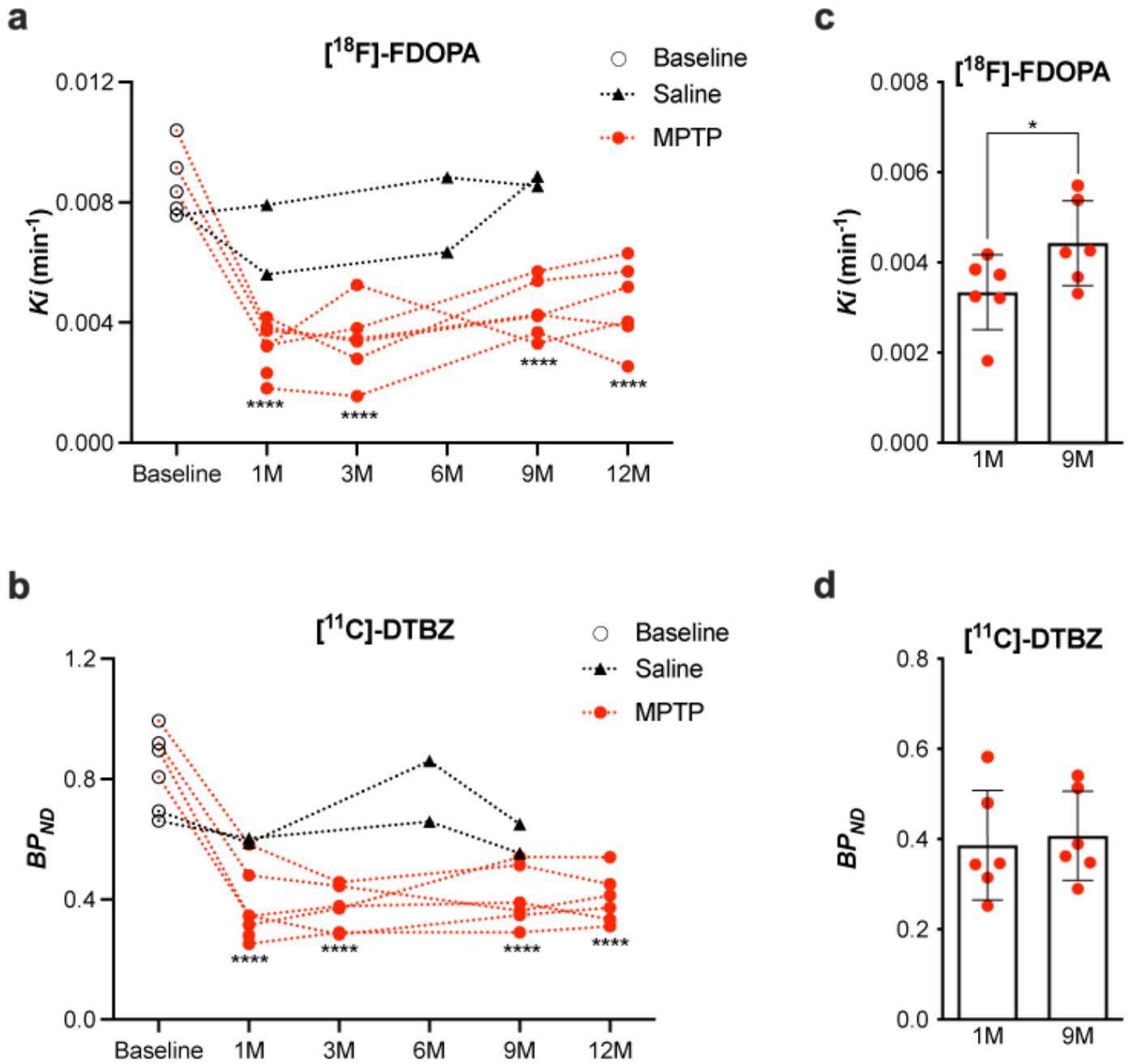
**Figure 1**

Velocity on the gait mat after MPTP. Each data point represents an average of minimum 3 quality walks on the GAIT4Dog® walking mat for each minipig at baseline, 1 week, 4 months and 15 months. Empty circles display baseline recordings (n=9), grey triangles show recordings 4 and 15 months after saline injections (n=2) and red circles represent recordings at 1 week, 4 months and 15 months after MPTP (n=7). Statistical analysis comparing the MPTP animals at baseline vs post-MPTP timepoints revealed a significant increased velocity at 15 months post-MPTP compared to baseline (AVOVA with Bonferroni correction, \*p<0.05).



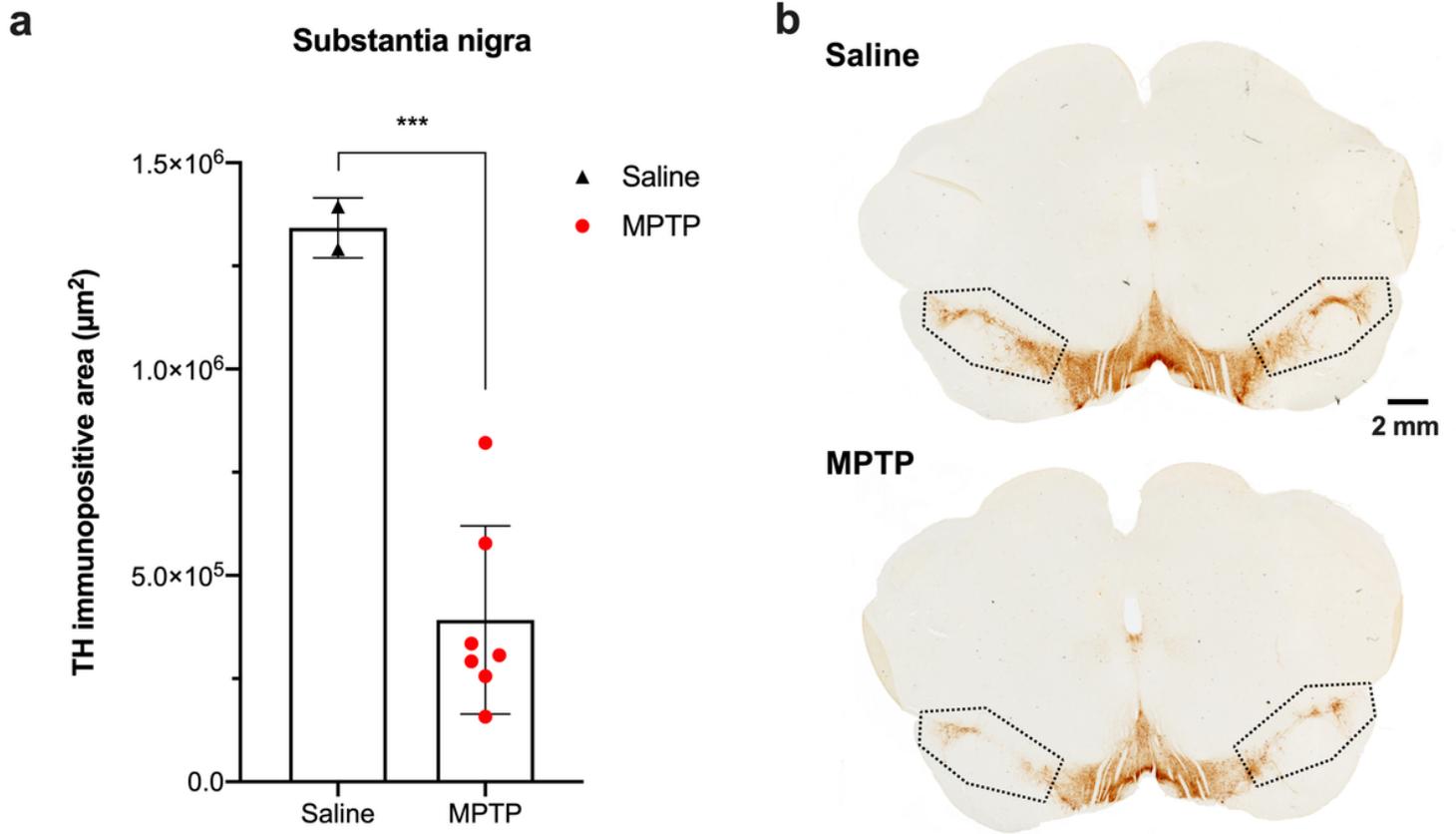
**Figure 2**

Average parametric maps of [18F]-FDOPA Ki (a) and [11C]-DTBZ BPND (b) for minipigs at baseline (top row), 1 month (second row), 3 months (third row), 9 months (fourth row) and 12 months (fifth row) after MPTP injections (n=5-7). Coronal (left column), sagittal (middle column) and axial/transverse (right column) views are shown for both Ki (min<sup>-1</sup>) and BPND maps. NAcc = Nucleus accumbens, STR = striatum.



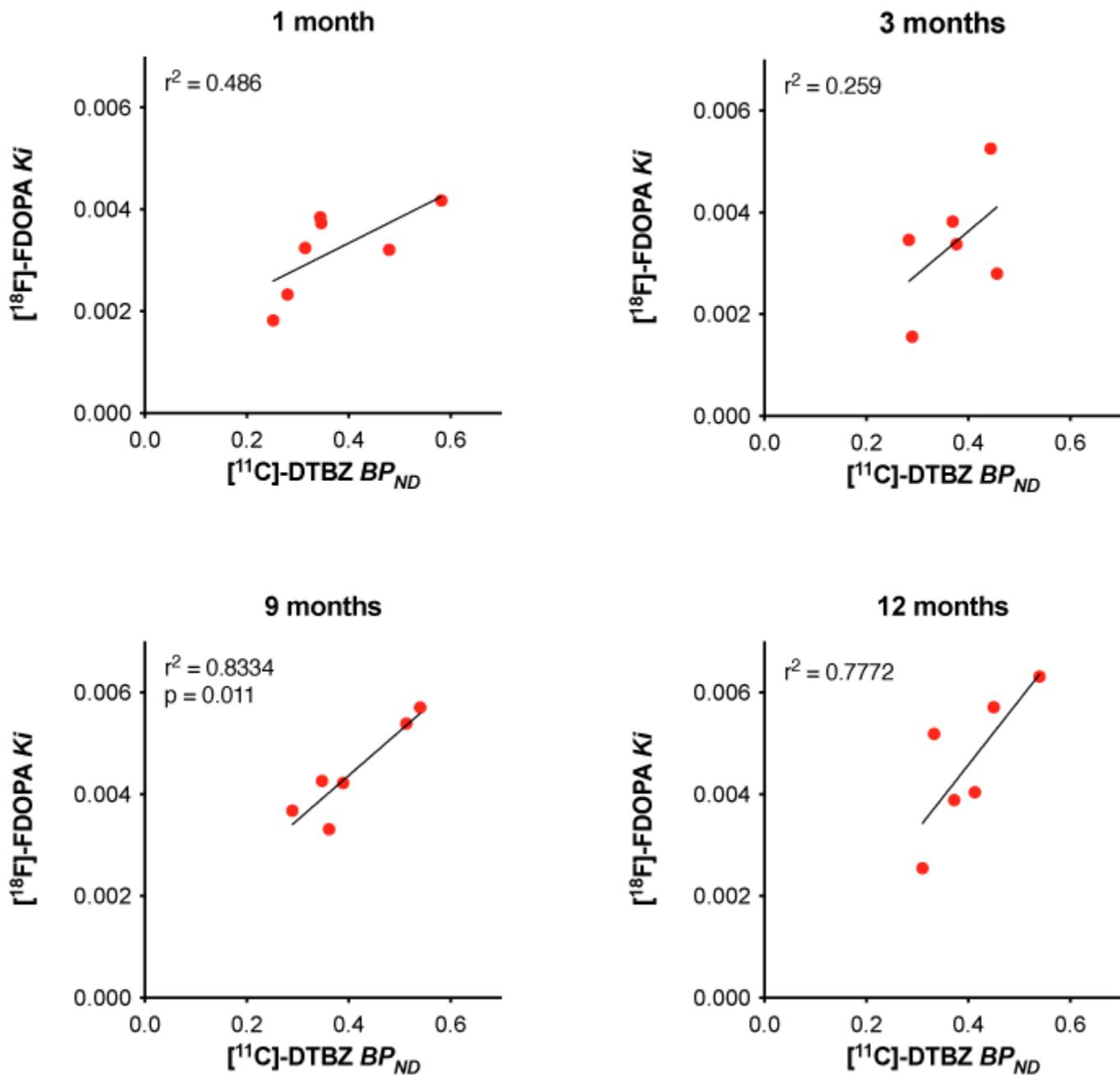
**Figure 3**

Longitudinal effects of MPTP on [<sup>18</sup>F]-FDOPA Ki and [<sup>11</sup>C]-DTBZ BPND. Empty circles show the a) striatal Ki (min<sup>-1</sup>) and b) striatal BPND values for each animal at the time of the baseline scan (n=5-6), black triangles represent the animals injected with saline and scanned after 1, 6 and 9 months (n=2), and the red circles represent the striatal values at 1, 3, 9 and 12 months after MPTP (n =5-7). Statistical analysis comparing the MPTP animals at baseline vs post-MPTP timepoints revealed a significant decreased binding of each radioligand at 1, 3, 6, and 9 months post-MPTP compared to baseline (AVOVA with Bonferroni correction, \*\*\*\*p<0.0001). c) Regional analysis of striatal [<sup>18</sup>F]-FDOPA at 1 and 9 months after MPTP showing a significant increase in Ki (n=6, two-tailed paired t-test, \*p<0.05). d) Regional analysis of striatal [<sup>11</sup>C]-DTBZ showing no differences between 1 and 9 months after MPTP.



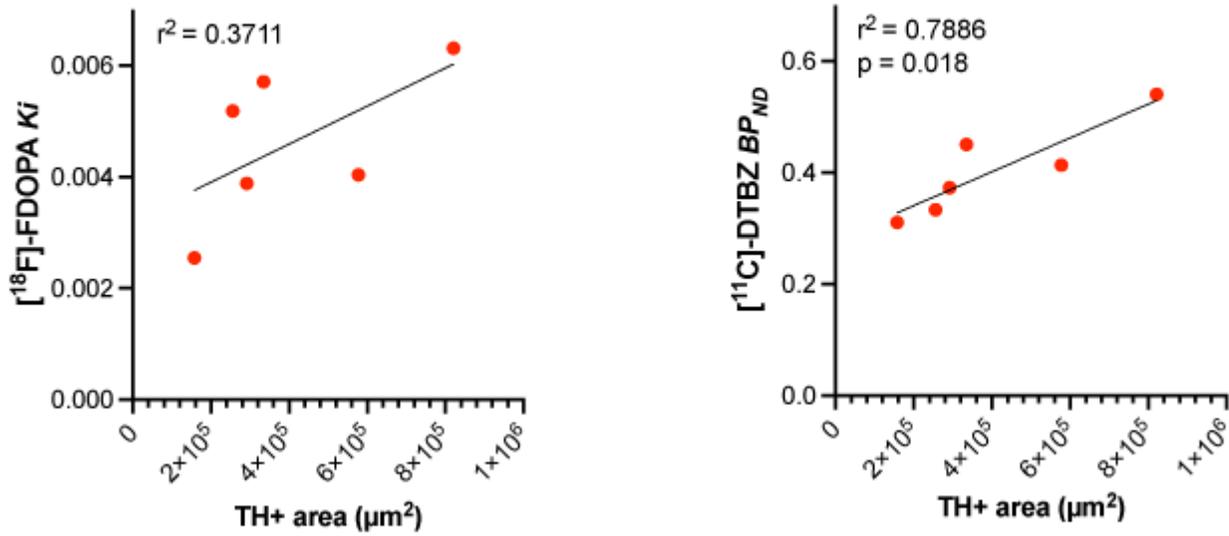
**Figure 4**

TH immunopositive area measured bilaterally in the substantia nigra (SN) of the two saline- and seven MPTP-injected minipigs. a) Each point in the graph represent the mean of 10 measurements done in 5 sections covering the SN pars compacta for each minipig. b) Representative image of the SN from a saline (top) and MPTP (bottom) injected minipig. The connected dots mark the outline of the SN.



**Figure 5**

Correlations between [18F]-FDOPA Ki and [11C]-DTBZ BPND imaging data at 1, 3, 9 and 12 months after MPTP (n = 6-7). Each data point represents one minipig and the lines are linear fits of the data. [18F]-FDOPA Ki and [11C]-DTBZ BPND measures exhibited a significant, positive, linear relationship only at 9 months after MPTP ( $r = 0.9131$ ,  $n=6$ ,  $p<0.05$ ).  $r^2$  is shown for each comparison.



**Figure 6**

Correlations between PET imaging data at 12 months after MPTP ([<sup>18</sup>F]-FDOPA Ki left panel, [<sup>11</sup>C]-DTBZ BPND right panel) and area of TH immunoreactivity. Each data point represents one minipig and the lines are linear fits of the data. [<sup>11</sup>C]-DTBZ BPND and TH+ area significantly correlated ( $r=0.888$ ,  $n=6$ ,  $p<0.05$ ), while [<sup>18</sup>F]-FDOPA Ki and TH+ area did not.  $r^2$  is shown for each comparison.