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***In vivo* Quantification of Glial Activation in Minipigs Overexpressing human α -synuclein**

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Conflict of interest

The authors have no conflict of interest to declare.

Abstract

Parkinson's disease is characterized by a progressive loss of substantia nigra (SN) dopaminergic neurons and the formation of Lewy bodies containing accumulated alpha-synuclein (α -syn). The pathology of Parkinson's disease is associated with neuroinflammatory microglial activation, which may contribute to the ongoing neurodegeneration. This study investigates the *in vivo* microglial and dopaminergic response to overexpression of α -syn. We used positron emission tomography (PET) and the 18 kDa translocator protein radioligand, [^{11}C](R)PK11195, to image brain microglial activation and (+)- α -[^{11}C]dihydrotetrabenazine ([^{11}C]DTBZ), to measure vesicular monoamine transporter 2 (VMAT2) availability in Göttingen minipigs following injection with recombinant adeno-associated virus (rAAV) vectors expressing either mutant A53T α -syn or Green Fluorescent Protein (GFP) into the SN (4 rAAV- α -syn, 4 rAAV-GFP, 5 non-injected control minipigs). We performed motor symptom assessment and immunohistochemical examination of tyrosine hydroxylase (TH) and transgene expression. Expression of GFP and α -syn was observed at the SN injection site and in the striatum. We observed no motor symptoms or changes in striatal [^{11}C]DTBZ binding potential *in vivo* or striatal or SN TH staining *in vitro* between the groups. The mean [^{11}C](R)PK11195 total volume of distribution was significantly higher in the basal ganglia and cortical areas of the α -syn group than the control animals. We conclude that mutant α -syn expression in the SN resulted in microglial activation in multiple sub- and cortical regions, while it did not affect TH stains or VMAT2 availability. Our data suggest that microglial activation constitutes an early response to accumulation of α -syn in the absence of dopamine neuron degeneration.

Keywords

Alpha-synuclein, [^{11}C](R)PK11195, Inflammation, Parkinson's disease, Minipig, animal model, adeno-associated viral vectors

Introduction

Aberrant alpha-synuclein (α -syn) aggregation to form Lewy bodies and neurites in association with degeneration of dopaminergic neurons in the substantia nigra (SN) is the defining pathology of Parkinson's disease (PD). This neuronal degeneration is accompanied by intrinsic inflammation in the form of microglial activation and proliferation that leads to expression of various proteins, including the 18 kDa translocator protein (TSPO) (McGeer et al. 1988; Venneti et al. 2006). There is increasing evidence that this neuroinflammation drives disease progression (Bartels & Leenders 2007; Orr et al. 2002).

Brain microglial activation can be imaged with the TSPO positron emission tomography (PET) radioligand, [^{11}C](R)PK11195 (Banati 2002). TSPO is a transporter of cholesterol and anions which is expressed by the outer mitochondrial membrane of activated microglia (Papadopoulos et al. 2006). While TSPO shows low expression under normal conditions, any injury to the brain, such as trauma, stroke, and neurodegenerative disorders, increases its expression and its isoquinoline binding sites available for [^{11}C](R)PK11195 rise (Bonsack et al. 2016; Cagnin et al. 2001; Ouchi et al. 2005). [^{11}C](R)PK11195 PET has detected brainstem, basal ganglia, and association cortex inflammation in cases of established PD (Gerhard et al. 2006; Ouchi et al. 2005) supported by post-mortem studies detecting reactive microglia using HLA-DR immunohistochemistry (McGeer et al. 1988). Furthermore, increased neuroinflammation has been detected in the monkey with HLA-DR and minipig with [^{11}C](R)PK11195 PET following 1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) administration (Cumming et al. 2006; McGeer et al. 2003). While the dopamine depletion in MPTP intoxicated minipigs was restored following grafting, the study observed a significant elevation of [^{11}C](R)PK11195 volume of distribution (V_T) in the striatum of the grafted pigs (Cumming et al. 2001).

In our study, mutant α -syn was overexpressed in the substantia nigra (SN) of the minipig brain to generate a large animal model with PD pathology. This α -syn overexpression approach extends similar studies in mice, rats and marmosets where recombinant adeno-associated viral (rAAV) vectors encoding wild-type or mutant human α -syn genes were inoculated into nigral dopamine neurons (Kirik et al. 2003; Maingay et al. 2005; Phan et al. 2017; Ulusoy et al. 2010). These transduction studies led to abnormal accumulation and aggregation of α -syn associated with loss of dopaminergic function, axonal pathology and progressive neurodegeneration of tyrosine hydroxylase (TH)-positive neurons in the SN (Kirik et al. 2003; Kirik et al. 2002; van der Putten et al. 2000). Furthermore, early and persistent activation of microglia has previously been reported in AAV-mediated α -syn overexpression models in both rodents and primates (Barkholt et al. 2012; Chung et al. 2009; Sanchez-Guajardo et al. 2010).

For the first time, we translate the rodent transfection model to the Göttingen minipig, which presents a superior model for human brain due to the large gyrencephalic porcine brain. Its size (69 g at 12 months) allows for good quantification of magnetic resonance imaging (MRI) and PET markers using commercial/clinical scanners (Bjarkam et al. 2017a; Lind et al. 2007). Here we investigate the distribution of activated microglia and the dopamine terminal integrity in response to nigral inoculation of rAAV vector overexpressing mutant α -syn in minipig brain using [^{11}C](R)PK11195 and [^{11}C]DTBZ PET, respectively.

Materials and Methods

Animals

The Danish Animal Experiments Inspectorate approved this study (2013–15–2934–00820) and we followed the 2010/63/EU directive for protection of animals used for scientific purpose. Thirteen adult female Göttingen minipigs (10.6 ± 0.8 months; control $n = 5$; GFP $n = 4$; α -syn $n = 4$) from Ellegaard Minipigs ApS (Dalmoose, Denmark) were housed in pairs in adjacent pens (4.6 m^2) at the Aarhus University animal farm with 50–55% humidity, 20°C air ventilation and with a day/night cycle of 12 h. They were fed a restricted diet with free access to tap water and were weighed every second week. Prior to surgery, the minipigs had acclimatized for at least one month and they were fasted for 16 h before either PET or surgery.

rAAV vector design and production

Recombinant AAV vectors contained a cytomegalovirus enhancer element/chicken beta-actin promoter (CBA) and either a human A53T α -syn or green-fluorescent protein (GFP) gene followed by an h-SV40 polyA sequence. These cassettes were flanked by inverted terminal repeats (ITR2) and packaged using rAAV serotype 5 capsids.

HEK293 cells at 70–80% confluence were transfected using the calcium-phosphate precipitation method including the rAAV plasmid and helper plasmids encoding for essential adenoviral packaging and rAAV5 capsid genes as previously described (Grimm et al. 1998). After 3 days of incubation, cells were harvested and lysed by performing three freeze-thaw cycles in a dry ice/ethanol bath. After treatment with benzonase (Sigma, Stockholm, Sweden) the lysate was purified using a discontinuous iodixanol gradient followed by Separose Q column chromatography (Zolotukhin et al. 1999) and then concentrated with a 100

kD cut-off column (Millipore Amicon Ultra, Millipore, Solna, Sweden). To determine the titer of the viral stock solutions, quantitative PCR with primers and probes targeting the ITR sequence was performed. Stock titers were $4.16 \cdot 10^{14}$ and $6.6 \cdot 10^{14}$ genome copies/mL for α -syn and GFP vectors, respectively. Prior to use in experiments, vectors were diluted in phosphate buffered saline. All diluted batches were re-titered and values obtained ranged from $1.04 \cdot 10^{14}$ to $1.16 \cdot 10^{14}$ genome copies/mL.

Stereotaxic surgery

Animals were pre-medicated with 6 mg/kg S-Ketamine (Pfizer) and 1.3 mg/kg Midazolam (Hamelin) IM, intubated and connected to a ventilator. Anesthesia was induced and maintained using 10 mg/kg/hr propofol IV (B. Braun) during the whole procedure. Bupivacaine hydrochloride (Marcain®, 0.5%, 0.8 mg/kg, AstraZeneca) was locally injected on the periosteum at the site of surgical incision and at the MR localizer box fixation points. The stereotaxic localizer box (Mark 2.5, Neurologic, Denmark) was fixed to the animal's head and through a scalp incision an MRI-visible fiducial marker was placed in a skull drill hole at bregma, as described previously (Glud et al. 2017; Glud et al. 2011). Animals underwent a 3D T1 MPAGE MRI scan (3T Trio, Siemens) to permit the calculation of injection coordinates (1x1x1 mm reconstruction) in the SN relative to the localizer box and the fiducial marker. Anesthetized animals were transported to a Class II virus facility and a stereotaxic frame was attached to the localizer box. After the scalp incision was reopened, a hole was drilled in the skull using a Midas Rex high-speed drill. When the brain was exposed a small incision was made in the dura. Using a Hamilton syringe (VWR), 20 μ L of rAAV- α -syn ($n = 4$) or rAAV-GFP ($n = 4$) was injected into the right SN guided by the calculated injection coordinates over 20 minutes (1 μ L/min). The assembly was left in place for an extra 5 minutes before being slowly withdrawn. The scalp incision was closed with 2.0 resorbable sutures. After the surgery, minipigs were placed in a quarantine facility for genetically modified animals for 72 h. Animals received pre- and postoperative antibiotics and analgesics including buprenorphine (Temgesic®, 0.01 mg/kg IM) 3 times on the surgery day with 8 hour

intervals and benzylpenicillin procain (Penovet®, 15.000 IE/kg IM) and Flunixin (2 mg/kg oral) for 5 days.

One GFP-injected animal sustained an infection following the surgical intervention and was excluded from the study.

Analysis of behaviour

The minipigs were filmed individually from above in an enclosed cage for 30 min at the same time of the day on separate days at baseline including naïve control minipigs ($n = 9$), at 4 months (GFP $n = 4$; α -syn $n = 4$) and 6 months (GFP $n = 2$; α -syn $n = 2$) after the injection of AAV- α -syn or AAV-GFP vectors. Motor function was assessed blinded using EthoVision XT software (Noldus) by tracking their color-labeled back and measuring the distance moved in the cage during the 30 min filming.

PET data acquisition and analysis

Four months after the stereotaxic inoculation of rAAV vectors into the right SN, minipigs were imaged with PET along with 5 naïve controls. The minipigs were pre-medicated and the anesthesia induced using a mixture of midazolam (1.3 mg/kg) and ketamine (6.0 mg/kg). Animals were intubated and mechanically ventilated while anesthesia was maintained (2.1% Isofluran, Zoetis). Catheters were placed in the femoral artery for arterial blood sampling during the [^{11}C](R)PK11195 scan and in the ear vein for radiotracer injection (Ettrup et al. 2011). Prior to PET, CT scans were performed for attenuation correction of PET emission data.

A 90 min PET scan was performed in each minipig using a human Biograph64 Truepoint PET-CT scanner (Siemens Healthcare). Radiotracer injection was administered through the ear vein catheter and flushed

with 10 mL saline. The average injected dose and specific activity were 352 ± 17 MBq and 43 ± 47 GBq/ μ mol for [^{11}C](R)PK11195 and 405 ± 27 MBq and 212 ± 201 GBq/ μ mol for [^{11}C]DTBZ. PET data was acquired as 14 time frames (5×60 , 3×300 , 4×600 and 2×900 seconds) and images were reconstructed with PSF resolution modelling (TrueX, 3 iterations, 21 subsets) and 2 mm Gaussian filtering using a 3D algorithm. In some of the animals, varying amounts of activity in the ear vein catheter and tube were observed on the PET images within the field of view. One [^{11}C](R)PK11195 scan (GFP-injected animal) was excluded based on streak artifacts most pronounced in the initial time frame images most likely caused by a scatter correction error (Alstrup et al. 2018). However, blood information from this animal was used in construction of the standard blood curve.

Blood samples were manually drawn during the [^{11}C](R)PK11195 scan from the femoral artery (12×5 , 6×10 , 6×30 , 5×60 , 8×600 seconds). Blood samples were centrifuged and the plasma radioactivity was measured using a well counter (Cobra II; Packard Instrument Co.) cross-calibrated to the scanner. Plasma data were linearly interpolated. The fraction of radiolabeled parent compound was measured by radio-HPLC in plasma samples taken at 2, 5, 10, 20, 30 and 60 min. The HPLC conditions were Spherisorb ODS C18 (Phenomenex, 250 x 4.6 mm) using 80% methanol and 20% ammonium formate 0.1 M as fluent, with serial UV ($\lambda = 254$ nm) and radio detection. Due to problems with femoral artery cannulation, blood samples were drawn from 10 out of the 13 minipigs during the [^{11}C](R)PK11195 PET scans. Plasma parent radiotracer fractions were obtained for 9 minipigs and these curves were averaged and fitted to a sigmoid model (Guo et al. 2013). Metabolite fractions were not affected by rAAV injection as can be appreciated from the overlapping standard deviations (Figure 1).

Reconstructed images were processed using PMOD 3.6 software (PMOD Technologies Ltd., Zurich, Switzerland). PET images were spatially transformed to an average MRI template based on 22 Göttingen

minipig brains (Watanabe et al. 2001) using a rigid transformation. Time-activity curves (TACs) for VM (ventral midbrain), STR (striatum), THA (thalamus), pons, FC (frontal cortex), TC (temporal cortex), Cer (cerebellum) and WhBr (whole brain) were generated based on volumes of interest from a Göttingen minipig PET atlas (Watanabe et al. 2001).

Quantification of [^{11}C](R)PK11195 binding

The dynamic PET brain TACs were kinetically analysed using a reversible 1-tissue compartmental model (Gunn et al. 2001). Due to the incomplete blood dataset, a population based plasma input function (PBIF) was derived from the existing datasets, corrected for injected dose, weight and metabolites (Shiozaki et al. 2000). Final fits were performed for a 60 min dataset and vB was kept as a free model parameter, as this provided the most optimal goodness-of-fit assessed by the residual mean squared error, Akaike Information Criterion, and the coefficient of variation. As part of the validation, data was also investigated using a simplified reference tissue model and SUV ratio with cerebellum as reference, which provided similar group binding trends. However, due to uncertainty if cerebellum would represent a valid, non-displaceable binding region, the 1-tissue compartment model with blood input was preferred. Parametric maps were calculated from 2 mm Gaussian smoothed PET images using a 1-tissue compartment model.

Quantification of [^{11}C]DTBZ binding

The [^{11}C]DTBZ TACs were obtained in left and right striatal and cerebellar regions and the binding potential of specific compared to non-displaceable binding (BP_{ND}) was computed using Logan graphical analysis (Logan et al. 1996) with the cerebellum providing a reference tissue input and setting t^* to 20 min to define the linear portion of the scan. For each animal, k_2' was initially estimated using the simplified reference tissue model (SRTM) (Lammertsma and Hume, 1996). The BP_{ND} values were generated by subtracting 1 from the estimated volume of distribution ratios.

Histological analysis

Seven months after viral injection, the minipigs were deeply anesthetized with 5 mg/kg S-Ketamine and 1.5 mg/kg Midazolam before they received an IC overdose injection of sodium pentobarbital (20 mL 400 mg/mL, Exagon Vet, Richter pharma). Blood was flushed out using 5 L of 0.9% cold NaCl to permit collection of fresh striatal punches for analysis of metabolites for another study. Brain was removed and immediately sliced into 1 cm thick sections and the striatal hole punches were collected. The remaining sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 4 days (Bjarkam et al. 2017b; Bjarkam et al. 2001). Next, brains were transferred to 10% sucrose solution for 2 days followed by a 30% sucrose solution for 7 days. Brains were then cut into 50 μ m thick coronal sections using a microtome (Microm HM450) - 12 series for each region - and stored in antifreeze solution (0.5 M phosphate buffer, 30% glycerol, 30% ethylene glycol) at -20°C until further processing.

Brain sections were rinsed from antifreeze solution with Tris-buffered saline (TBS) (5 mM Tris, 15 mM NaCl) twice for 10 min. For antigen retrieval, sections stained against tyrosine hydroxylase (TH) were placed into Tris / EDTA buffer pH 9.1 for 30 min at 80°C and were rinsed again afterwards with buffer. Sections were then incubated in 3% H₂O₂ and 10% Methanol in TBS for 30 min. Quenching solution was washed off with TBS for 10 min followed by two washes using 0.05% Triton X-100 in TBS (TBS-T). Sections were incubated for 30 min in TBS-T containing 5% of normal serum from the same animal species from which the secondary antibody was raised. Primary antibody solutions were applied overnight at room temperature (RT): 1% BSA in TBS-T containing either rabbit anti-TH (AB1542, Millipore, USA) at 1:2,000; mouse anti-human α -syn (610787, BD Biosciences, USA) at 1:30,000; rabbit anti-GFP (ab290, Abcam, UK) at 1:100,000. After 16 – 20 h,

sections were rinsed three times with TBS-T for 10 min and incubated with the appropriate biotinylated secondary antibody (1:200, Vector Laboratories) in 1% BSA in TBS-T for 1 h. Sections were rinsed three times with TBS-T for 10 min. The avidin-biotin-peroxidase complex solution (Vectastain Elite ABC kit, Vector Laboratories Inc, Burlingame, USA) was then applied for 1 h. After rinsing sections again with TBS-T for 10 min then twice using TBS for 10 min, staining was visualized using 0.5 mg/ml 3,3'-diaminobenzidine (DAB), 0.01 % H₂O₂ in TBS for 5-10 min. Sections were washed with TBS and mounted on chromatin-gelatin coated glass slides, dehydrated in first 70%, then 95% followed by 3x 100% Ethanol (2 min each) and cleared in 3x xylene solution (5 min each). Immediately afterwards, sections were coverslipped using Depex (Sigma-Aldrich, Stockholm, Sweden). Images were taken on a Zeiss microscope (Axio Zoom.V16) for low power images or a Nikon microscope (eclipse 90i) for higher magnification images.

Statistics

Statistical tests were performed using Prism7 (GraphPad Software, California, USA). Values are presented as means $\pm$ standard deviation (SD) if not otherwise specified. A kruskal-wallis one-way ANOVA was used to compare behavioural data. To compare mean regional V_T and BP_{ND} values, a repeated measures two-way ANOVA with animal groups and regions (repeated within-subject) as factors were performed with Tukey's multiple comparisons test. The statistical significance level was set at $P < 0.05$.

Results

Response of motor behaviour

From the EthoVision XT tracking analysis, the mean distance moved for the minipigs measured at baseline was 29.2 ± 17.3 meters. Four months following injection of AAV-GFP or AAV- α -syn vectors, the mean distance moved for the GFP ($n = 4$) and α -syn ($n = 4$) animals had decreased to 23.9 ± 7.6 and 28.9 ± 11.2 meters, respectively (Figure 2). A further minor decrease was observed after 6 month to 21.3 ± 6.5 meters (α -syn) and 17.7 ± 0.8 meters (GFP), although recordings were only obtained for 2 animals in each group at this time-point. Statistical analysis showed no significant difference between any of the groups ($P > 0.05$).

[¹¹C](R)PK11195 volume of distribution following rAAV transduction

Regional estimates of [¹¹C](R)PK11195 V_T were obtained for the three groups of minipigs (α -syn $n = 4$; GFP $n = 2$; Control $n = 5$) using a population based input function and a 1-tissue compartment model with three free parameters (K_1 , k_2 and v_B) which was preferred over a 2-tissue compartment model as it provided more robust parameter estimates. A comparison between findings from the left and the right sides of the brain within the groups revealed no differences larger than 2% in any of the regions sampled in either of the groups ($P > 0.05$). Therefore, left and right side regions of interest were merged and bilateral V_T values were estimated again and compared between groups.

A repeated measures two-way ANOVA analysis found a significant effect of region ($F(7, 56) = 84.98$, $P < 0.0001$), treatment ($F(2, 8) = 6.4$, $P = 0.02$), matching ($F(8, 56) = 43.54$, $P < 0.0001$) and interaction ($F(14, 56) = 2.99$, $P = 0.002$). Tukey's multiple comparisons test revealed that α -syn subjects had significantly higher V_T values than control animals in all brain regions sampled ($P < 0.05$) except the cerebellum (Figure

3A). The largest effect of rAAV- α -syn transduction on [^{11}C](R)PK11195 binding was observed in regions closest to the nigral injection site (Figure 3A, B).

Symmetric [^{11}C]DTBZ binding

Analysis of the vesicular monoamine transporter 2 (VMAT2) [^{11}C]DTBZ PET data, showed no effect of group or region on BP_{ND} values between the α -syn ($n = 4$), the GFP ($n = 3$) or the control group ($n = 5$) (Figure 4).

Moreover, when comparing left and right striatal BP_{ND} values within groups, no statistical differences ($P > 0.05$) were observed.

Histological examination of viral vector injections

rAAV 2/5 containing a CBA promoter and either the human A53T α -syn transgene, or GFP as a control, were unilaterally injected into minipig right SN. To visualize targeting and successful transduction of the rAAV, immunohistochemical stainings of matched selected sections of the minipig brain were performed to detect human- α -syn (Figure 5 G,H,K,L) and GFP (Figure 5 E,F,I,J). Immunostaining for human α -syn or GFP showed expression of the transgenes in the striatum (Figure 5 E,G) as well as in the SN, which confirmed the correct targeting of our injections into the right ventral midbrain. In both groups, backflow of the vector was seen around the injection tract into the midbrain / thalamic regions (Figure 5 F, H, I-J). Smaller cell bodies were transduced in the nigral region (Figure 5 J, L) suggesting a high affinity of the rAAV5 for glial cells. In the striatum, bigger neurons, presumably medium spiny neurons (MSN) were labeled, especially visible in the GFP expressing animals (Figure 5 I) compared to the human α -syn staining (Figure 5 K) as GFP is a cytosolic protein while α -syn appears mostly in terminals as puncta. TH staining in both striatum and midbrain sections showed no apparent fiber or cell loss, respectively, in the α -syn group when compared to GFP expressing brains (Figure 5 A-D).

Discussion

In this study we have delivered rAAV2/5 vectors expressing mutant α -syn unilaterally into the SN of minipig brains and found a bilaterally increased [^{11}C](R)PK11195 binding across all brain regions sampled 4 months after transduction, suggesting a widespread neuroinflammatory response which was highest around the injection site. As only smaller cells, possibly glial cells, rather than dopaminergic cells were transduced in the SNc, it was not surprising that the TH staining visually showed no dopaminergic cell or fiber loss in the SNc 7 months after transduction. In the striatum, MSN were transduced by the rAAV5 vector, which indicates that it was mainly taken up by either glial cells or by MSN axons in the ventral midbrain and then retrogradely transported to the striatum via the nigrostriatal pathway. From the TH staining, no dopaminergic terminal loss was observed in the striatum and similarly, results from [^{11}C]DTBZ PET showed no changes in VMAT2 binding, a marker of dopamine terminal functional integrity, in the striatum of the α -syn group compared to either the GFP or the control group 4 months after transduction. Furthermore, the distance moved by the animals in their cages was similar in the α -syn and GFP group at 4 and 6 months after surgery, which corresponds with the presumable intact dopamine system in both groups.

Statistically significant increased [^{11}C](R)PK11195 binding was detected in the striatum, thalamus, pons and the ventral midbrain (containing the SN) of the α -syn inoculated minipigs, regions that are all affected by α -syn pathology in PD patients (Braak et al. 2003). Damage to the neurons in the SN is considered the hallmark of PD leading to deafferentation of the striatum, which in turn sends strong projections to pallidum and thalamus (Braak et al. 2003; Gibb & Lees 1991). As activated microglia release cytokines, such as $\text{TNF}\alpha$, they may well be a contributor to the ongoing degeneration of dopamine neurons in PD (Boka et al. 1994; Chung et al. 2009; McGuire et al. 2001). Indeed, increased [^{11}C](R)PK11195 binding has been reported in the brainstem and cortical association areas of PD patients and it has been suggested that microglial activation may be an early event in the disease process (Gerhard et al. 2006; Ouchi et al. 2005).

Glial pathology may be occurring before loss of nigral dopamine neurons in PD (Stokholm et al. 2017), and our minipigs show an early inflammatory response, which is most likely caused by the accumulation of aberrant α -syn in glia and MSN neurons of the basal ganglia. Indeed, glial cells have been shown to scavenge and accumulate α -syn in their cytoplasm in early PD, which normally leads to tissue destruction, but has also been shown to be beneficial in order to attenuate α -syn pathology when preconditioned with cytokines (Halliday & Stevens 2011; Koller et al. 2017; Lee et al. 2008; Lee et al. 2010). Furthermore, extracellular α -syn is able to activate microglia through interaction with Toll-like receptors (Kim et al. 2013; Stefanova et al. 2011). However, [^{11}C](R)PK11195 does not distinguish between TSPO expressed on micro- or astroglia and different activation states (i.e. pro- versus anti-inflammatory processes) and reactive astroglia have been shown to contribute to the TSPO PET signal, so further neurochemical and expression analysis would be needed to assess this (Lavis et al. 2012). It is also possible that activated microglia vary in their protein expression profiles depending on their role in the various stages of neurodegeneration, which may complicate the association between increased TSPO binding and microglial activation (Gerhard 2016). Finally, the expression of TSPO on blood vessel endothelium may also be a confounding factor (Turkheimer et al. 2015).

Interestingly, the increased [^{11}C](R)PK11195 binding in the rAAV- α -syn animals was bilateral and global despite the unilateral injection into right SN. This could in part be explained by cross-innervation of the contralateral systems that may have helped the dissemination of the α -syn protein to the contralateral hemisphere. Indeed, crossed nigrostriatal connections have been observed in non-human primates as indicated by horseradish peroxidase labeled neurons in SN contralateral to the striatal injection (Francois et al. 1984). As backflow of the viral vector along the needle tract was seen to the dorsal midbrain and thalamus, it is also possible that α -syn was transported along thalamic projections. Moreover, following

needle insertion with local tissue damage and inflammation, the rAAV vectors might have induced further immune response to an already sensitive system.

Histological analysis revealed expression of cytosolic GFP in striatal MSN suggesting a vector transduction of MSN axon fibers in the SN and a retrograde transport of the vector. In contrast to the cytosolic expression of GFP, expression of α -syn showed a more punctated structure and therefore most likely a more synaptic translocation of this protein in the MSN neurons. In the SNc, smaller cell bodies were stained which might indicate a possible preference of the vector to transduce glial cells over dopaminergic neurons. Previously, widespread transduction of the brain has been observed with the rAAV5 vector injected into thalamus and putamen of non-human primates (Samaranch et al. 2017). Samaranch et al. 2017 reported a primary transduction of neurons (both dopaminergic nigral neurons and striatal MSN) and astrocytes as well as a robust antero- and retrograde transport of AAV5 through nigrostriatal and thalamic projections (Samaranch et al. 2017). A study done in neonatal minipigs using intra-striatal injection also showed that the rAAV5-GFP vector predominantly transduced neurons in striatum, neocortex, thalamus and SN, with some GFP-positive cells showing a glial profile (Kornum et al. 2010). Interestingly, they reported a difference in rAAV5-GFP transduction between 2 day-old and 7 week-old piglets, where the older pigs showed lower spread and transduction of neuronal cells further away from the injection site (Kornum et al. 2010). As the minipig brain is fully myelinated at 6 months of age (Fang et al. 2005) and as we used Göttingen minipigs aged at least 11 months at the time of surgery, the age could have affected the vector transduction and possibly also the neuronal and glial protein expression profiles for receptors necessary to bind and internalize the vector. Furthermore, the study by Kornum et al. (2010) used intra-striatal injection whereas we used intra-nigral injection, which may have altered the transduction efficiency.

Similar studies in marmosets showed that 90-95% of TH-positive neurons in the SN were transduced with the recombinant AAV vector (Kirik et al. 2003). This clearly demonstrates interspecies differences, as delivery of rAAV5 vectors in the rat brain also resulted in effective transduction of neurons in SN, striatum, hippocampus and cortex and widespread transgene expression (Davidson et al. 2000; Kornum et al. 2010; Ulusoy et al. 2010). Alternatively, since both marmoset and rat brains are very small compared to minipig brain, it is possible that more than one injection may be necessary to produce sufficient infection in a large brain and measurable dopaminergic deficits. Different vectors should also be tested to improve the transduction of dopaminergic nigral neurons of the minipig in future studies to achieve a more effective transduction. This is supported by previous studies revealing different transduction patterns dependent on virus vector serotype, capsid, promoter sequence, titer, target region and animal species (Davidson et al. 2000; Gerits et al. 2015).

Only 2 out of 4 GFP animals were available for the [^{11}C](R)PK11195 PET quantification (1 animal sustained an immediate infection from the surgery and 1 animal had a failed PET scan). One of the PET scanned GFP animals had a [^{11}C](R)PK11195 binding comparable to the α -syn minipigs, which suggests that there might have been some inflammation caused by the surgery itself, especially in view of the opening of the dura mater. Indeed, introducing a vector carrying a non-mammal exogenous protein like GFP might trigger a widespread immune reaction. Furthermore, GFP at high concentrations has previously been reported to be associated with toxicity and inflammatory response (Landeck et al. 2017; Ulusoy et al. 2009). The higher [^{11}C](R)PK11195 signal from this animal might be a direct effect of the protein itself. The other GFP animal had binding corresponding to the higher range of the control group so more GFP controls or empty vector controls will be needed in a future study to assess the variability and mean binding in this group.

Second generation TSPO radioligands have been developed with a higher signal-to-noise ratio than [^{11}C](R)PK11195 but their binding affinity in humans is influenced by *TSPO* gene polymorphisms complicating quantification (Owen et al. 2012). We aimed at making our study comparable to existing human studies and for this reason selected [^{11}C](R)PK11195 for which abundant data are available (Bartels & Leenders 2007; Gerhard et al. 2006; Parbo et al. 2017). Unfortunately, microglia and TSPO sites are ubiquitously distributed throughout the brain and may become activated by any local pathology, disconnection of the region from damaged areas, and as a part of the aging process (Banati 2002; Hinz & Boellaard 2015; Kumar et al. 2012; Turkheimer et al. 2007). Lower TSPO binding in the cerebellum compared to other grey matter regions is used as a justification for its use as a tissue reference in PET imaging studies (Doble et al. 1987). An increase in cerebellum binding has been reported in some pathology cases (Gerhard et al. 2006), and as we did not know *a priori* the extent of viral dissemination and its possible effect on cerebellum, we used the alternative approach of a population based metabolite-corrected input function to obtain the binding estimate (manuscript in preparation) (Shiozaki et al. 2000; Tsuchida et al. 1999).

In conclusion, we found that the unilateral nigral injection of viral vectors overexpressing α -syn induced a significantly increased bilateral inflammatory response in sub- and cortical regions detectable with the TSPO ligand [^{11}C](R)PK11195 PET. However, no changes were detected in the function of dopaminergic neurons assessed *in vivo* with [^{11}C]DTBZ PET of the VMAT2, at post mortem with TH staining or behaviorally through motor assessment. Our study may therefore demonstrate glial activation as an early response to neuronal accumulation of α -syn in the absence of VMAT2 or TH changes. It will be of interest to test different viral vectors in future studies to find a serotype that better targets dopaminergic neurons located in the SN of the minipig for the purpose of developing a large animal model of PD in which putative treatments and promising PET biomarkers can be tested.

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Figure legends

Figure 1. No difference in parent fraction between animal groups. Unmetabolized parent fractions from the 3 groups of animals measured in arterial plasma. The plotted bars are mean $\pm$ standard deviation and the line is the fitted metabolite data using a sigmoid model.

Figure 2. Distance moved in the minipigs by video tracking. Minipigs were filmed at baseline, including naïve controls, and were again recorded 4 and 6 months after either GFP- or α -syn injection. The bars indicate the mean and standard deviation.

Figure 3. Volume of distribution of activated microglia determined by *in vivo* [^{11}C](R)PK11195. **(A)** [^{11}C](R)PK11195 volume of distribution (mean $\pm$ standard deviation) in different brain regions between the naïve control group (n = 5), GFP (n = 2) and α -syn animals (n = 4). Significant p-values are represented as * p < 0.05, *** p < 0.001 and **** p < 0.0001. Abbreviations for the volumes of interest: VM = Ventral Midbrain, STR = Striatum, THA = Thalamus, FC = Frontal Cortex, TC = Temporal Cortex, Cer = Cerebellum, WhBr = Whole Brain. **(B)** Pixel-wise parametric V_T maps. An average of the rAAV-GFP and control animals (Top row) and an average of the rAAV- α -syn animals (Bottom row) are displayed in coronal, transverse and sagittal views.

Figure 4. VMAT2 binding levels determined by *in vivo* [^{11}C]DTBZ. BP_{ND} values found four months after the rAAV injections and for naïve controls are presented with bars representing mean and standard deviation. Results are shown for 5 control scans, 3 GFP animals and 4 α -syn animals in right and left striatum.

Figure 5. Immunohistochemical staining of TH and the transgenes 7 months post-surgery. TH-positive cells in striatum and SN of GFP animal **(A, B)** and A53T α -syn animal **(C, D)**. Low magnification photos of the GFP-positive staining in striatum **(E)**, and midbrain **(F)**, and of the human α -syn expression in striatum **(G)**, midbrain **(H)**. High magnification images show GFP-positive cells in striatum **(I)** and SNc **(J)**. Also α -syn-positive cells were observed in the striatum **(K)** and SNc **(L)**. The vector transfected glial cells and neurons in the ventral (SN) and dorsal (thalamo) midbrain. The transgenes were anterogradely transported from SNc **(F/J and H/L)** to striatum **(E/I and G/K)** via the medial forebrain bundle (not shown). Scale bar A-H: 1 mm. Scale bar I-L: 50 μm .

α -syn = α -Synuclein, GFP = Green Fluorescent Protein, SN = substantia nigra, SNc = substantia nigra pars compacta, TH = Tyrosine hydroxylase.

A

B