6-hydroxydopamine-induced Parkinson’s disease-like degeneration generates acute microgliosis and astrogliosis in the nigrostriatal system but no bioluminescence imaging-detectable alteration in adult neurogenesis

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Abstract

Parkinson’s disease (PD) is a slowly progressing neurodegenerative disorder caused by loss of dopaminergic neurons in the substantia nigra (SN), leading to severe impairment in motor and non-motor functions. Endogenous subventricular zone (SVZ) neural stem cells constantly give birth to new cells that might serve as a possible source for regeneration in the adult brain. However, neurodegeneration is accompanied by neuroinflammation and dopamine depletion, potentially compromising regeneration. We therefore employed in vivo imaging methods to study striatal deafferentation (N-\textit{o}-fluoropropyl-2[\beta\textit{]-carbomethoxy-3[\beta\textit{]-4-[123\textit{I}]iodophenyl)nortropane single photon emission computed tomography, DaTscan™) and neuroinflammation in the SN and striatum (N,N-diethyl-2-[2-(4-[18\textit{F}]fluoroethoxy)phenyl]-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)acetamide positron emission tomography, [18\textit{F}]DPA-714 PET) in the intranigral 6-hydroxydopamine Parkinson’s disease mouse model. Additionally, we transduced cells in the SVZ with a lentivirus encoding firefly luciferase and followed migration of progenitor cells in the SVZ–olfactory bulb axis via bioluminescence imaging under disease and control conditions. We found that activation of microglia in the SN is an acute process accompanying the degeneration of dopaminergic cell bodies in the SN. Dopaminergic deafferentation of the striatum does not influence the generation of doublecortin-positive neuroblasts in the SVZ, but generates chronic astrogliosis in the nigrostriatal system.

Introduction

Parkinson’s disease (PD) is characterized by loss of dopaminergic neurons in the substantia nigra (SN) and their striatal projections. PD is usually diagnosed after 50% of dopaminergic neurons in the SN and 80% of striatal dopamine have been lost (Fearnley & Lees, 1991). As only symptomatic treatment is available, novel endogenous neuroregeneration approaches are desired.

The subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone of the dentate gyrus generate progenitor cells that migrate to the olfactory bulb (OB) or granular cell layer. In the SVZ, slowly dividing radial glia-like cells give rise to transient amplifying cells, which themselves generate neuroblasts. Neuroblasts migrate long distances along the rostral migratory stream towards the OB, maturing and integrating into existing...
neural circuits (Ming & Song, 2011). Therefore, SVZ neuroblasts might serve as a source for new neurons in the diseased brain. However, endogenous neuroregeneration is insufficient or nonexistent in PD, potentially due to disease-associated alterations in neurogenesis.

Different studies report decreased (Baker et al., 2004; Höglinger et al., 2004), unchanged (van den Berge et al., 2011), or even increased (Aponso et al., 2008) progenitor cell proliferation after striatal dopamine depletion in patients with PD and animal models. Moreover, dopaminergic neurodegeneration is accompanied by increased numbers of microglia in post-mortem patients with PD (McGeer et al., 1988; Imamura et al., 2003) as well as PD animal models (Czlonkowska et al., 1996). Depending on their plasticity, microglia can have favourable or detrimental effects on neurogenesis, and neuron survival (Ekdahl et al., 2003; Walton et al., 2006; Bastos et al., 2008; Sierra et al., 2010). Activated microglia, reactive astrocytes and infiltrating peripheral macrophages produce a variety of cytokines, chemokines, neurotransmitters, and reactive oxygen species, which affect the proportion of neurogenesis and gliogenesis, and the amount of progenitor cell proliferation.

Compared with conventional histological techniques, in vivo imaging reduces experimental animal numbers and allows for longitudinal studies. Single photon emission computed tomography (SPECT) using the tracer N-3-fluoropropyl-2β-carbomethoxy-3β-(4-123I)iodophenyl)nortropine ([123I]Ioflupane), which has high binding affinity for the pre-synaptic dopamine transporters (DaTs) (Booij et al., 1997a,b), allows for detection of pathological changes in dopaminergic projections in patients and pre-clinical PD models. Translocator protein (TSPO) expression in healthy brain tissue is low (Giatzakis & Papadopoulos, 2004). High levels of TSPO expression in activated microglia and reactive astrocytes (Chen & Guilarte, 2008; Cosenza-Nashat et al., 2009; Scarf & Kassiou, 2011; Lavisse et al., 2012) allow for imaging of brain inflammation with positron emission tomography (PET) tracers targeting TSPO (Jacobs & Tavitian, 2012), such as N,N-diethyl-2-(2-(4-(2-[18F]fluoroethyl)pyrenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)acetamide ([18F]DPA-714) (James et al., 2013). Bioluminescence imaging (BLI) was described as a tool to follow and quantify the migration of inflammation, and progenitor cell migration in a PD mouse model employing non-invasive multimodal imaging.

Materials and methods

Cell culture

Human HEK293T (kind gift of Dr R. Thomas, Max Planck Institute for Metabolism Research, Cologne, Germany) and Gli36ΔEGFR (kind gift of Dr David Louis, Molecular Neuro-Oncology Laboratory, Massachusetts General Hospital, Boston, MA, USA) cells were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium high glucose GlutaMAX (DMEM) (Gibco, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1 x penicillin/streptomycin (penicillin, 1000 IU; streptomycin, 1000 µg/mL; PAA Laboratories, Colbe, Germany) at 37 °C and 5% CO2/95% air.

Generation of reporter vectors

The pLKO.1-CMV-1Luc-IRESp-mCherry plasmid (pLKO.1-CMV-LIC, Supporting Information Fig. S1) was constructed in two steps starting from the pLKO.1shControl-Luc plasmid created by G. Jungwirth (AG Jacobs, Max Planck Institute for Metabolism Research) from the pLKO.1 vector (kind gift of Dr R. Thomas, Max Planck Institute for Metabolism Research). The first step consisted of introducing the mCherry gene from Kl13-pCDNA (created by Dr K. Kruttwig, Max Planck Institute for Metabolism Research) from the pLKO.1 vector (kind gift of Dr R. Thomas, Max Planck Institute for Metabolism Research) using the following primers (Nhel restriction site in bold): forward: 5'-CGGATGCTAGCGAGACGTTGCA-3'; reverse: 5'-TGGATGCTAGCTCAGGCTACAGAG-3'.

After restriction with Nhel, pLuc-IRESp was inserted into the Nhel restriction site of pLKO.1shControl-Luc plasmid by homologous recombination. The plasmid was transfected into HEK293T cells using Lipofectamine LTX (Life Technologies, Carlsbad, CA, USA) and OptiMEM I Reduced Serum Media (Gibco). Transfection efficiency was 1.7 x 10^5 transducing units/µL.

Lentiviral vector particle production

Lentiviral vector particles were produced as described elsewhere (Palm et al., 2013; Viel et al., 2013). In brief, after medium exchange to fresh DMEM without serum and antibiotics, HEK293T cells were transfected with a mixture of three plasmids. pLKO.1-CMV-LIC plasmid (2.6 µg) was mixed with 3.8 µg of a second-generation packaging plasmid [pCMV-dR8.2 dvpr; provided by Dr R. Thomas (Max Planck Institute for Metabolism Research)] and 0.76 µg of a plasmid encoding the glycoprotein G of vesicular stomatitis virus (pCMV-VSV-G; Dr R. Thomas) in 250 µL OptiMEM I Reduced Serum Media (Gibco). Plus Reagent (7.16 µL) Life Technologies, Carlsbad, CA, USA) was added and the solution was vortexed and incubated for 10 min at room temperature (20–25 °C). In a 24-well plate, 250 µL OptiMEM I Reduced Serum Media was mixed with 21.7 µL Lipectofectamine LTX (Life Technologies) and 250 µL of the DNA Mix. After 30 min incubation at room temperature, the transfection mix was added to the cells. Supernatant was replaced on the next day by DMEM supplemented with 10% fetal bovine serum without penicillin/streptomycin. Supernatants were harvested and pooled on days 2 and 3, cleared through a 0.45 µm filter and vector particles were concentrated by low-speed centrifugation (5 h, 26 000 g). In a 24-well plate, 250 µL OptiMEM I Reduced Serum Media was mixed with 21.7 µL Lipectofectamine LTX (Life Technologies) and 250 µL of the DNA Mix. After 30 min incubation at room temperature, the transfection mix was added to the cells. Supernatant was replaced on the next day by DMEM supplemented with 10% fetal bovine serum without penicillin/streptomycin. Supernatants were harvested and pooled on days 2 and 3, cleared through a 0.45 µm filter and vector particles were concentrated by low-speed centrifugation (5 h, 26 000 g) at 4 °C (Heraeus Biofuge Stratos, Thermo Fisher Scientific, Waltham, MA, USA). After resuspension in DMEM supplemented with 8 µg/mL polybrene (hexadimethrine bromide; Sigma-Aldrich, St Louis, MO, USA), 500-fold concentrated vector particles were stored at −80 °C.

Titration of lentiviral particles

For titration of lentiviral particles, 0.8 x 10^4 Gli36ΔEGFR cells were seeded in black 96-well plates with transparent bottom in 200 µL of DMEM supplemented with 10% fetal bovine serum and 1 x penicillin/streptomycin. After cells reached confluence, medium was replaced by serial dilutions of concentrated viral particles in DMEM supplemented with 10% fetal bovine serum, 1 x penicillin/streptomycin and 8 µg/mL polybrene. The next day, the medium was replaced and mCherry-positive cells were counted on the following day (AxioCam MRm, Carl Zeiss, Oberkochen, Germany). The mean transduction efficiency was 1.7 x 10^5 transducing units/µL.
Animal experiments

All animal experiments were performed in accordance with the German laws for animal protection and were approved by the local bureau for animal care (LANUV, Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). C57Bl6 and FVB mice (Janvier, Saint-Berthevin, France) were housed at constant temperature (23 °C) and relative humidity (40%), under a 12 h light/12 h dark schedule. Mice were given ad-libitum access to food and water.

6-Hydroxydopamine Parkinson’s disease model

For stereotactic 6-hydroxydopamine (6-OHDA) injections, C57Bl6 mice (11–15 weeks old) were anaesthetized with 150 mg/kg ketamine and 6 mg/kg xylazine (i.p.) and fixed into a stereotactic frame (Kopf Instruments, Tujunga, CA, USA). A small skin incision was made and a hole was drilled into the skull. The needle was placed in position and, after 1 min, 2 μL of 5 mg/mL 6-OHDA (Sigma-Aldrich) in 0.01% ascorbic acid (Carl Roth, Karlsruhe, Germany) and 0.9% NaCl (Carl Roth) or vehicle (0.01% ascorbic acid in 0.9% NaCl) was injected into the left SN using a Hamilton 7005KH 5 μL syringe. The syringe was kept in place for 5 min in order to allow the solution to diffuse into the surrounding tissue and was then retracted slowly. The following stereotactic coordinates in relation to bregma were used for the SN: lateral, −1.5 mm; anterior–posterior, −3.0 mm; dorsal–ventral (DV), −4.4 mm.

Injection of lentiviral particles

For the stereotactic injection of lentiviral particles, FVB mice (6–7 weeks old) were anaesthetized with 180 mg/kg ketamine and 9.6 mg/kg xylazine (i.p.). The surgical procedure is described above. The stereotactic coordinates for the SVZ were: lateral, −1.4 mm; anterior–posterior, +0.8 mm; DV, −2.5 mm (−2.7 mm). The two coordinates for DV indicate that the needle was placed at DV −2.7 mm, kept in place for 1 min, retracted to DV −2.5 mm and kept in place for 1 min before 2 μL of concentrated lentivirus particle solution was injected.

Single photon emission computed tomography

Animals were anaesthetized with 1.5% isoflurane (Abbott Animal Health, IL, USA) in 100% O2 and the lateral tail vein was cannulated using a 26 Ga catheter (Vasculon Plus, BD, Heidelberg, Germany) connected to polyethylene tubing (15 cm) (27 Ga, Smith Medical, Kent, UK). [123I]Ioflupane (16 MBq) (DaTscan™, GE Healthcare, Chalfont St Giles, UK) was injected i.v. and a 15 min SPECT scan was conducted 60 min post-injection in a combined SPECT/computed tomography (CT) imaging system (NanoSPECT/CT pre-clinical camera; Mediso Medical Imaging Systems, Budapest, Hungary), followed by a CT acquisition for acquiring anatomical information. Images were reconstructed by an ordered-subsets expectation maximization algorithm software (HiSPECT™, SciVis GmbH, Göttingen, Germany).

Single photon emission computed tomography data analysis

Image data analysis of SPECT/CT data was performed using the in vivo Research Workspace software package (Siemens Healthcare, Erlangen, Germany). Volumes of interest (VOIs) of equal size and orientation were applied in order to quantify tracer uptake in the left and right striatum as well as in the cerebellum. A 50% threshold of the VOI maximum was applied to the VOI for the right striatum, and the resulting VOI50% right was mirrored to the left brain hemisphere in order to quantify the left striatum (VOI50% left). [123I]Ioflupane uptake was quantified as mean specific tracer uptake [(mean uptake striatum50% left : right – mean uptake cerebellum)/mean uptake cerebellum] and the specific uptake ratio left : right was calculated. Representative images show SPECT and magnetic resonance images that were co-registered using the contour of the mouse skull.

Positron emission computed tomography

Radiosynthesis of [18F]FDPA-714 was conducted as described elsewhere (Damont et al., 2008; James et al., 2008). Animals were anaesthetized with 1.5% isoflurane (Abbott Animal Health) in 100% O2 and the lateral tail vein was cannulated as described above. PET studies were performed on a high-resolution small animal scanner (32 module quadHIDAC, Oxford Positron Systems Ltd, Oxford, UK). Data reconstruction was performed using a one-pass listmode EM algorithm (EMRecon) (Kösters et al., 2011). Animals were injected with 10 MBq [18F]FDPA-714 i.v. and images were acquired at 45–75 min post-injection. Following the PET acquisition, the animal bed was transferred to the CT scanner (Inveon, Siemens Healthcare) for acquiring anatomical information. The CT images were co-registered to PET images using three spheres (Acros Organics, Geel, Belgium) rinsed in radiotracer prior to image acquisition as landmarks on the animal bed.

Magnetic resonance imaging

Mice were anaesthetized with 1.5% isoflurane (DeltaSelect, Dreieich, Germany) in O2/compressed air (30/70, 1 L/min). Magnetic resonance imaging was performed with a 9.4 T small animal magnetic resonance scanner with 20 cm bore size (Bio-Spec 94/20; Bruker BioSpin MRI GmbH, Ettlingen, Germany), operated with the PARAVISION 5.1 software (Bruker BioSpin MRI GmbH). Using a helium-cooled cryoprobe (Bruker BioSpin MRI GmbH), we obtained anatomical two-dimensional T2w rapid acquisition with relaxation enhancement brain images in three imaging planes (repetition time/echo time, 3000–5000 ms/50 ms; 12–28 slices; slice thickness, 0.5 mm; field of view, 2 cm2; matrix, 2562; in plane resolution 78 μm2).

Positron emission tomography data analysis

The PET and magnetic resonance image data were analysed using VInCI software (Vollmar et al., 2004). Fusion of PET and CT images was performed using the landmark tool of the VInCI software, and PET/CT and magnetic resonance images were co-registered using the contour of the mouse skull. Co-registered images were matched to a mouse brain template generated from the mouse brain atlas of Swanson (2001) and VOIs for the SN and striatum were defined based on the brain atlas. Quantification was based on mean tracer uptake values for the respective VOIs. The VOI for the right unlesioned striatum was used as the background VOI.

Bioluminescence imaging

After virus injection, BLI was performed on a weekly basis using the ivis Spectrum Imaging System and Living Image 4.0 software (PerkinElmer, Waltham, MA, USA). The day before measurement,
fur on the head was removed using depilatory cream (Pilca) under isoflurane (Abbott Animal Health) anaesthesia. Mice were injected i.p. with 300 mg/kg D-luciferin in phosphate-buffered saline (PBS) without calcium and magnesium (PAA Laboratories). At 3 min after D-luciferin injection, mice were anaesthetized with 2.5% isoflurane in 100% oxygen and placed in the imaging system before two 10 min time frames were recorded at 8 and 18 min post-injection (field of view, B; subject height, 1.5 cm; binning, 4; f/stop, 1). Grayscale photographic images and bioluminescence colour images were superimposed. Regions of interest were drawn for the right and left SVZ and OB to determine the signal intensity [average radiance (p/s/cm²/ster)]

**Immunohistochemistry**

Mice were deeply anaesthetized with 5% isoflurane and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde. Brains were isolated and post-fixed overnight in 4% paraformaldehyde. After paraffin embedding, 5-μm-thick coronal microtome sections were cut. Following deparaffinization and rehydration, sections were boiled in citrate buffer (pH 6, 25 °C) for antigen retrieval and stained according to one of the following protocols using primary antibodies against tyrosine hydroxylase (TH) (Chicken α TH, 1 : 1000; ab76442, Abcam, Cambridge, UK), ionized calcium-binding adapter molecule 1 (Iba1) (Rabbit α Iba1, 1 : 250; no. 019-19742, Wako Chemicals, Neuss, Germany), doublecortin (Dcx) (Guinea pig α Dcx, 1 : 400; AB2253, Millipore, Billerica, MA, USA), glial fibrillary acidic protein (GFAP) (Chicken α GFAP, 1 : 1000; ab13970, Abcam) or TSPO (Rabbit α peripheral benzodiazepine receptor, 1 : 250; EPR5384, Novus Biologicals, Cambridge, UK).

**Immunofluorescence staining**

After washing with PBS and pre-incubation with blocking solution (4% goat serum, 0.25% Triton-X in PBS) for 20 min, sections were incubated with primary antibody in blocking solution at 4 °C overnight, washed with PBS and incubated with the respective secondary antibody (Alexa Fluor 488/555, 1 : 800, Life Technologies) in night, washed with PBS and incubated with the respective secondary antibodies in PBS for 45 min at room temperature in a dark chamber. After washing with PBS, sections were incubated with 0.5 μg/mL 4',6-diamidino-2-phenylindole (Carl Roth) in PBS for 7 min, washed again and mounted with Mowiol (Sigma-Aldrich).

**Immunoperoxidase staining**

Blocking was performed with peroxidase-blocking solution (S2023, Dako, Hamburg, Germany) for 10 min followed by washing with PBS. Sections were then incubated in primary antibody diluted in antibody diluent (S3022, Dako), washed in PBS, incubated with biotinylated secondary antibody (DSB-π/biotin goat anti-chicken IgG, 1 : 800, Life Technologies) in antibody diluent, washed in PBS and incubated with horseradish peroxidase–strepavidin conjugate (1 : 600 in PBS, P0397, Dako) for 45 min. After final washing in PBS, sections were placed in 2% 3,3'-diaminobenzidine and 0.0012% H2O2 until a good staining intensity was reached. Sections were counterstained with haematoxylin for 5–10 s (1 : 3 in distilled water), dehydrated and mounted in entellan (Millipore).

**Microscopy**

Stained sections were analysed using a Nikon ECLIPSE Ni-E microscope operated by the NIS-Elements AR software. Z-stacks (± 2 μm in 0.5 μm steps) were recorded and combined to a focused image using the extended depth of focus function.

For quantification purposes, cells from three images from the respective region of every animal were manually counted.

**Statistical analysis**

Statistical analysis was performed in Sigma Plot 13.0 (Systat Software Inc., San Jose, CA, USA). SPECT data and Iba1+ cell counts were analysed using a two-way ANOVA followed by a pairwise multiple comparison procedure (Holm–Sidak method). PET data were analysed using a Mann–Whitney rank sum test. BLI data were analysed using a two-way repeated-measures ANOVA. For data analysed using ANOVA, values are shown as mean (M) with SD. For data analysed using the Mann–Whitney rank sum test, values are shown as median (Mdn) with upper and lower percentiles. A P-value below 0.05 was considered as significant.

**Results**

Neurodegenerative processes in PD were reported to be accompanied by neuroinflammatory processes, which can potentially favour or reduce disease progression and regeneration. We therefore investigated neurodegeneration, neuroinflammation, and stem cell properties in the unilateral 6-OHDA injection mouse model for PD. The detailed experimental setup including the exact numbers of studied animals and the timing of the various imaging approaches is illustrated in Supporting Information Figs S2 and S3.

### 6-Hydroxodopamine-induced neurodegeneration

In order to ensure degeneration of dopaminergic nigrostriatal projections in 6-OHDA-treated animals and integrity of the nigrostriatal system in vehicle-injected animals, [123I]Ioflupane SPECT was performed at different time points post-injection (Fig. 1A and Supporting Information Fig. S2). Tracer uptake in the left striatum was observed to be reduced in 6-OHDA-treated (n\_day 3 = 5; n\_day 7 = 4; n\_day 18 = 7) compared with vehicle-injected (n\_day 3 = 3; n\_day 7 = 5; n\_day 18 = 8) mice at all studied time points (Fig. 1A and B), whereas tracer uptake in the right striatum did not change (Fig. 1C), indicating degeneration of ipsilateral striatal projections without compensation on the contralateral side. Both the left and right striatum showed a very variable absolute tracer uptake between the different time points, which did not allow for statistical testing with a two-way ANOVA due to violation of the equal variance assumption. As pre-synaptic DaT levels can vary between individuals, and specific activities can vary between tracer syntheses, we calculated the ratio of the mean tracer uptake in the left : right striatum (Fig. 1D). The specific [123I]Ioflupane uptake ratio between vehicle-injected and 6-OHDA-injected animals differed significantly (two-way ANOVA, F\_2\_6 = 117.67, P < 0.001) and was significantly reduced in 6-OHDA-treated compared with vehicle-treated animals at all time points (day 3: M\_6-OHDA = 0.08, SD\_6-OHDA = 0.30, M\_vehicle = 0.96, SD\_vehicle = 0.08, t = 6.34, P < 0.001; day 7: M\_6-OHDA = 0.34, SD\_6-OHDA = 0.11, M\_vehicle = 1.09, SD\_vehicle = 0.06, t = 5.83, P < 0.001; day 18: M\_6-OHDA = 0.09, SD\_6-OHDA = 0.12, M\_vehicle = 0.77, SD\_vehicle = 0.20, t = 6.94, P < 0.001; pairwise multiple comparison procedure, Holm–Sidak method). No statistically significant interaction between group and time point could be observed (two-way ANOVA, F\_2\_6 = 0.68, P = 0.52), suggesting that nigrostriatal degeneration is a fast process taking a maximal time of a few days to reach the final state of degeneration. Intramural application of
6-OHDA induced a fast degeneration of axonal projections towards the striatum, which was also visible in histological staining for TH, the rate-limiting enzyme in the synthesis of dopamine (Nagatsu et al., 1964). TH staining intensity was clearly reduced in the left striatum as well as numbers of TH+ cell bodies in the left SN, compared with the unlesioned contralateral side (Fig. 2A and B). The observed amount of degeneration was similar at all studied time points, again stressing the short time frame needed for neurodegeneration in this model.

**Neuroinflammatory processes accompanying neurodegeneration**

As several studies reported neuroinflammation accompanying degeneration of dopaminergic neurons in PD, we aimed to investigate the presence of neuroinflammation after 6-OHDA lesion. To assess this parameter, we performed PET with the TSPO ligand $[^{18}F]$DPA-714 at 7 ($n_{\text{vehicle}} = 6$; $n_{\text{6-OHDA}} = 7$), 14 ($n_{\text{vehicle}} = 12$; $n_{\text{6-OHDA}} = 12$) and 21 ($n_{\text{vehicle}} = 11$; $n_{\text{6-OHDA}} = 9$) days post-lesion (Supporting Information Fig. S2). Tracer uptake was clearly visible in the left SN as well as in the injection tract region (Fig. 3A). At 7 days post-injection (dpi), tracer uptake was higher in the left SN compared with right SN in 6-OHDA-treated and vehicle-treated animals, but without reaching statistical significance between groups (Fig. 4B, ratio $\text{SN}_{\text{left}} : \text{SN}_{\text{right}}$, $Mdn_{\text{6-OHDA}} = 1.29$, $Mdn_{\text{vehicle}} = 1.09$, $U = 12.0$, $P = 0.234$; Mann–Whitney rank sum test), demonstrating enhanced inflammatory processes in both groups due to the injection procedure. At 14 dpi, the signal : background ratio and $\text{SN}_{\text{left}} : \text{SN}_{\text{right}}$ ratio were increased in 6-OHDA-lesioned compared with vehicle-injected animals (Fig. 4A and B), showing increased inflammation after neurotoxic degeneration ($\text{SN}_{\text{left}} : \text{background}$: $Mdn_{\text{6-OHDA}} = 1.60$, $Mdn_{\text{vehicle}} = 1.26$, $U = 29.0$, $P = 0.014$; $\text{SN}_{\text{left}} : \text{right}$: $Mdn_{\text{6-OHDA}} = 1.27$, $Mdn_{\text{vehicle}} = 1.08$, $U = 36.0$, $P = 0.040$; Mann–Whitney rank sum test). This neuroinflammatory response abated at 21 dpi, as measured by not significantly altered signal : background and $\text{SN}_{\text{left}} : \text{SN}_{\text{right}}$ ratios (signal : background: $Mdn_{\text{6-OHDA}} = 1.28$, $Mdn_{\text{vehicle}} = 1.42$, $U = 39.0$, $P = 0.447$; $\text{SN}_{\text{left}} : \text{right}$: $Mdn_{\text{6-OHDA}} = 1.11$, $Mdn_{\text{vehicle}} = 1.17$, $U = 49.0$, $P = 1.0$; Mann–Whitney rank sum test).

The median left : right tracer uptake ratio for the striatum was similar at days 7 and 21 (Figs 3B and 4E), whereas it was slightly, but significantly, higher in the 6-OHDA-lesioned striatum at day 14 (day 7: $Mdn_{\text{6-OHDA}} = 1.08$, $Mdn_{\text{vehicle}} = 1.01$, $U = 7.0$, $P = 0.051$; day 14: $Mdn_{\text{6-OHDA}} = 1.03$, $Mdn_{\text{vehicle}} = 0.95$, $U = 31.0$, $P = 0.019$; day 21: $Mdn_{\text{6-OHDA}} = 0.98$, $Mdn_{\text{vehicle}} = 1.01$, $U = 45.0$, $P = 0.761$; Mann–Whitney rank sum test).
As the injection procedure itself might cause increased inflammation and thereby influence quantification of tracer uptake in 6-OHDA vs. vehicle, we calculated the ratio of injection tract : background and mean percent injected dose tracer uptake in the injection tract at 7 (n_{vehicle} = 6; n_{6-OHDA} = 8) and 14 (n_{vehicle} = 9; n_{6-OHDA} = 10) dpi (Fig. 4C and D). Due to advanced healing processes, the injection tract was not visible in all cases on day 21 in the magnetic resonance imaging data and was therefore not quantified. Median tracer uptake in the injection tract did not differ significantly between 6-OHDA-injected and vehicle-injected brains (injection tract : background: day 7: Mdn_{6-OHDA} = 1.64, Mdn_{vehicle} = 1.55, U = 18.0, P = 0.491; day 14: Mdn_{6-OHDA} = 1.49, Mdn_{vehicle} = 1.49, U = 44.0, P = 0.967; percent injected dose injection tract: day 7: Mdn_{6-OHDA} = 2.21, Mdn_{vehicle} = 1.92, U = 19.0, P = 0.573; day 14: Mdn_{6-OHDA} = 1.89, Mdn_{vehicle} = 1.63, U = 39.0, P = 0.653; Mann–Whitney rank sum test), and was lower at day 14 in general, but without reaching statistical significance.

**Histological analysis of microglial and astrocytic markers**

We performed immunohistological staining of the SN and striatum for the microglial markers Iba1 and TSPO, as well as for the astrocytic marker GFAP at 7, 14 and 21 dpi. Increased staining for Iba1+ microglia was observed in the ipsilateral 6-OHDA-injected SN compared with the vehicle-injected SN at all time points (Fig. 5A). The vehicle-injected SN displayed a certain amount of microgliosis, but without reaching the magnitude of the 6-OHDA condition. A peak in Iba1+ microgliosis in the SN could be observed at day 14 post-lesion. Microgliosis in the lesioned SN displayed an activated phenotype, as determined by their increased cell body size and reduced ramifications (arrows in Fig. 5A). TSPO staining displayed the same regional patterning as Iba1 staining, although the staining was speckled over the cell body, prohibiting determination of the cell morphology (Fig. 5B). Astrogliosis, as shown by GFAP positivity, was persistent over time in the ipsilateral SN in 6-OHDA-injected animals, but was also observed in the ipsilateral SN of vehicle-injected mice, indicating the role of astrocytes in wound healing (Fig. 5B).

The TSPO staining was barely detectable in the ipsilateral striatum of 6-OHDA-injected and vehicle-injected animals, suggesting weak or no activation of striatal microglia. In the ipsilateral striatum of 6-OHDA-lesioned animals, Iba1 staining was unchanged (Fig. 5C, E and F) and the left : right ratio of Iba1+ cells was not significantly different between the 6-OHDA and vehicle conditions (two-way ANOVA, F{subscript,1,15} = 3.737, P = 0.072). In contrast, increased numbers of GFAP+ cells were observed (Fig. 5D) compared with...
the striatum of vehicle-injected specimens. Likewise, vehicle injection led to a slight increase in GFAP$^+$ cells compared with the contralateral site, but not to the same extent as after 6-OHDA injection.

**Effect of neurodegeneration and inflammation on neural stem cell proliferation and migration**

In order to study the effect of neurodegeneration and neuroinflammation on neural stem cell proliferation and migration in the unilateral 6-OHDA injection...
PD model, we first established injections of virus particles into the SVZ of FVB mice (the experimental setup is illustrated in Supporting Information Fig. S3). FVB mice were used because the pigmented fur and skin of C57Bl6 mice do not allow for reliable quantification of optical imaging data due to light absorption and scattering. The plasmid used for production of lentiviral particles encodes mCherry and firefly luciferase protein and is driven by a cytomegalovirus (CMV) promoter (Supporting Information Fig. S1). BLI was conducted weekly in order to follow migration of infected neuroblasts, and proliferation and migration of progeny of infected neural stem cells in the same animal over time. At 1 week post-injection, BLI signal was detected at the site of injection in 24 out of 24 animals (100%), but could not be quantified accurately due to remaining fur. At 2–4 weeks post-injection, signals showed anterior extension in 12 out of 24 animals (50%), demonstrating migration of labelled neuroblasts towards the OB (Fig. 6A). Animals that showed no anterior light signal extension were excluded from the study as virus particles in these cases failed to infect progenitor cells probably due to mislocated injections. Over time, the OB : SVZ ratio changed significantly (Fig. 6C; one-way repeated-measures ANOVA, $F_{3,40} = 8.62, P < 0.001$) and, from week 5 after virus injection onwards, the mean OB : SVZ ratio was significantly higher compared with week 2 (Holm–Sidak pairwise multiple comparison; week 5 vs. week 2: $t = 3.33, P = 0.021$; week 6 vs. week 2: $t = 4.36, P = 0.001$; week 7 vs. week 2: $t = 5.65, P < 0.001$) clearly showing signal accumulation in the OB over time. At 7.5 weeks after virus injection, 6-OHDA and vehicle injections into the SN were validated after the first BLI acquisition by immunohistochemistry for TH in all animals included in the BLI data analysis (data not shown).

**Histological analysis of neural progenitor cells after 6-hydroxydopamine lesion**

As the detection of very small alterations in progenitor cell migration is challenging when using BLI, we additionally analysed sections from the C57Bl6 mouse SVZ at different time points after intranigral 6-OHDA/vehicle injection. We used Dcx as a marker for neurogenesis (Fig. 7). Dcx is a microtubule-associated protein transiently expressed in neuronal progenitor cells and immature neurons (Brown et al., 2003) mainly in the SVZ, dentate gyrus, rostral migratory stream and OB. Visual inspection at 4, 8, 15 and 21 days post-lesion indicated no difference in Dcx staining between the contralateral and ipsilateral SVZ of 6-OHDA-lesioned and vehicle-injected animals, leading to the assumption that neuroblast generation was not affected by the pathophysiological changes in the 6-OHDA-lesioned brain (Fig. 7). Furthermore, only single or no Dcx+ cells were observed at more lateral positions of the striatum.
Fig. 5. Histological markers validate the presence of microgliosis and astrogliosis in the SN, but lack of substantial microgliosis in the striatum. (A) Co-staining for dopaminergic neurons (TH) and microglia (Iba1) in the ipsilateral SN at 8, 15 and 22 dpi. Arrows show microglia cells displaying an activated phenotype. (B) GFAP/TSPO co-staining in the ipsilateral SN at 8, 15 and 22 dpi. (C) Microglia (Iba1) staining in the striatum at 8, 15 and 22 dpi. (D) GFAP staining in the striatum at 8, 15 and 22 dpi. (E) Quantification of left: right Iba1 cell ratio in the striatum. Two-way ANOVA. Significance level: $P < 0.05$. Error bars: SD. (F) Co-staining for GFAP (astrocytes) and TSPO (activated microglia) in the striatum at 15 days post-lesion. Scale bars: 100 μm.
Discussion

This study sought to shed light on the complex interplay of neurodegeneration, neuroinflammation and neurogenesis in the unilateral intranigral 6-OHDA injection model of PD.

Employing \([^{123}I]\)Ioflupane SPECT, we observed significantly reduced tracer binding in the lesioned striatum at 3, 7, and 18 days post-lesion, indicating degeneration of ipsilateral striatal projections with loss of DaTs in a time frame of several days.

At 14 dpi, increased uptake ratios for \([^{18}F]\)DPA-714 were measured in 6-OHDA-lesioned compared with vehicle-injected animals via PET. Striatal \([^{18}F]\)DPA-714 uptake ratios were similar at day 7 and day 21, whereas they were slightly, but significantly, higher in the 6-OHDA-lesioned striatum at day 14.

Histological analysis revealed a peak in Iba1+ microgliosis in the SN at day 14 post-lesion, but no increased numbers of microglia in the striatum. Astroglisis, as shown by GFAP-positive staining, was persistent over time in the ipsilateral SN in 6-OHDA-injected animals, but it was also observed in the ipsilateral SN of vehicle-injected mice. Increased astroglisis was also detectable in the ipsilateral striatum of 6-OHDA-injected mice.

We could visualize migration of labelled neuroblasts towards the OB using BLI after virus injection, with a stable signal distribution at week 7. After 6-OHDA lesion, no significantly different OB or OB : SVZ signal was observed compared with the vehicle group.

Histological analysis of Dcx expression in the SVZ underlined unaltered neuroblast generation.

Intranigral 6-hydroxydopamine injection induces a fast degeneration of the nigrostriatal system

Induction of dopaminergic neurodegeneration by 6-OHDA injection into the SN allows the study of SVZ neurogenesis after degeneration of striatal projections without direct influence from the damage caused by the injection procedure itself. After intranigral injection, we observed degeneration of dopaminergic neurons taking place over several days, as also described after SN or medial forebrain bundle neurotoxin administration (Jeon et al., 1995; Walsh et al., 2011). The majority of dopaminergic neurons in the SN were reported to die during the first 10 days post-lesion in rats, whereas fibre degeneration in the striatum was observed between day 1 and 7 (Jeon et al., 1995). We could observe this reduction in dopaminergic innervation as a strong reduction in DaT ligand accumulation in the ipsilateral striatum and reduced TH staining intensity at early and late time points. DaT density in the striatum is directly correlated with the number of dopaminergic cell bodies in the SN, making \([^{123}I]\)Ioflupane SPECT an excellent read-out for dopaminergic neurodegeneration in the SN (Bäck et al., 2013). The detected amount of tracer uptake in the contralateral striatum was similar at
all time points, indicating that the contralateral site does not compensate for loss of innervation on the ipsilateral site.

**Degeneration of dopaminergic cell bodies is accompanied by acute neuroinflammation**

Degeneration of dopaminergic neurons was reported to be accompanied by neuroinflammatory processes in patients (McGeer et al., 1988; Imamura et al., 2003), and in animal models of PD (Akiyama & McGeer, 1989; Czlonkowska et al., 1996). Our in vivo PET study showed an acute increase in TSPO-ligand accumulation at the direct lesion site in 6-OHDA-injected compared with vehicle-injected animals, whereas tracer accumulation in the striatum was significantly, but very slightly (Mdn<sub>6-OHDA</sub> = 1.03, Mdn<sub>vehicle</sub> = 0.95), increased. However, visual inspection of the PET datasets as well as analysis of the corresponding immunohistochemistry for Iba1 and TSPO gave no evidence for significant microglial activation in the striatum. De-novo microglial activation, whereas degenerating cell bodies strongly trigger inflammation, whereas degenerating cell bodies strongly trigger inflammatory cells.

As numbers of Iba1<sup>+</sup> cells were unchanged in the striatum and TSPO reactivity was barely detectable, our data indicate that degeneration of nerve terminals is not a trigger for microglial activation, whereas degenerating cell bodies strongly trigger inflammatory cells.

In line with this, previous studies demonstrated increased microglial activation at the direct 6-OHDA lesion site in the SN, but only weak or missing microglial activation in the striatum (Kitamura et al., 2010; Walsh et al., 2011). In contrast, intrastriatal injections led to increased inflammation at the primary and secondary lesion site (He et al., 2001; Cicchetti et al., 2002; Maia et al., 2012). Concerning the time course of microglial activation, a peak in microglial activation in the SN at 2 weeks post-lesion was reported for intrastriatal 6-OHDA injection in rats (Cicchetti et al., 2002; Maia et al., 2012) and mice (He et al., 2001), further underlining our observation that microglial activation is an acute process in this PD model. It remains elusive whether microglial activation appears only after the neurodegenerative process is complete, or during the neurodegenerative process. Due to the invasive nature of the intranigral injection, we cannot distinguish between early degeneration-induced neuroinflammation and the inflammatory response caused by the injection procedure itself. Therefore, we cannot rule out that inflammation is already present during the neurodegenerative process and not only after completion of this process. In addition, blood–brain barrier integrity might be crucial for the quantification of tracer uptake, as the mechanical damage caused by the injection procedure certainly increases blood–brain barrier permeability early after lesion. However, this effect will influence tracer uptake in both vehicle and 6-OHDA-lesioned animals and therefore does not interfere with our analysis. Moreover, our histological findings confirmed our PET imaging findings. As the variability of tracer uptake and interindividual differences are major challenges for PET quantification, longitudinal imaging and injection of 6-OHDA and vehicle in the same animal might reduce group variability in future studies. However, this experimental setup lacks the possibility of direct histological validation and does not allow the evaluation of the integrity of the nigrostriatal system following vehicle injection by in vivo imaging.

**Degeneration of the nigrostriatal system leads to persistent astrogliosis**

Despite the missing striatal microgliosis, we found severe and persistent astrogliosis in the SN and striatum after 6-OHDA lesion, demonstrated by GFAP immunoreactivity. Increased numbers of nigral and striatal astrocytes were previously shown in several models of 6-OHDA administration (Akiyama & McGeer, 1989; Sheng et al., 1993; Maeda et al., 2008). Striatal astrocytes might derive from resident astrocytes that undergo de-differentiation (Buffo et al., 2008), as well as from SVZ progenitor cells (Levison & Goldman, 1993). After intraventricular application of 6-OHDA in rats, numbers of Ki67<sup>+</sup> proliferating cells were reported to be unchanged in...
the SVZ, whereas proliferation was increased in the striatum and cortex (Wachter et al., 2010). These proliferating cells did not co-localize with the microglial marker Iba1, but with the astrocytic marker GFAP, identifying them as locally proliferating astrocytes. Persistent astrogliosis potentially reflects residual astrocytic scarring, which remains after the phagocytotic activity has been completed (Akiyama & McGeer, 1989).

**Subventricular neurogenesis is unaffected by striatal deafferentation**

Previous studies implemented lentiviral in vivo transduction of neural stem cells with constructs encoding for firefly luciferase as a tool to follow migration of progenitor cells in the SVZ–OB axis and showed that sensitivity of this method is sufficient to detect alterations in neurogenesis caused by bone-derived neurotrophic factor overexpression (Reumers et al., 2008) or cuprizone treatment (Guglielmetti et al., 2014). We established injections of LV-CMV-LIC into the SVZ and could follow progenitor cell migration towards the OB over time using BLI. Neurotoxin injection into the left SN led to unchanged relative OB : SVZ signal ratios. However, we observed a smaller absolute OB : SVZ ratio in 6-OHDA-lesioned compared with vehicle-injected animals. This difference was already present prior to lesion, indicating that it is independent of the lesion procedure, and is not present in the quantification of the OB : SVZ ratio relative to the mean value of the two last measurements before lesion. Taken together, our data suggest unaffected neural progenitor cell migration in FVB mice following 6-OHDA lesion. Our histological analysis of Dcx+ cells in the SVZ of C57Bl6 mice further supports this in vivo imaging-based observation. These findings are in line with results from intrastriatal 6-OHDA injections in rats, which led to increased numbers of proliferating cells in the SVZ, without affecting numbers of Dcx+ cells (Aponso et al., 2008). The characterization of these newborn cells revealed high expression of the astrocytic marker GFAP, indicative of lesion-induced astrogliosis.

Additionally, intranigral injection of 6-OHDA in mice was reported to lead to no changes in striatal 5-ethyl-2′-deoxyuridine-incorporating cells or SVZ neuroblasts, whereas inhibition of neuroinflammation by minocycline led to increased 5-ethyl-2′-deoxyuridine-positive cells and neuroblasts migrating deeply into the striatum in 6-OHDA-injected but not vehicle-injected animals (Worlitzer et al., 2012). However, a 40% reduction in proliferating neural precursors in the SVZ was reported after 6-OHDA lesion of the medial forebrain bundle and SN in mice (Baker et al., 2004). It is important to notice that these mice received double 6-OHDA injections that led to a nearly complete dopamine deprivation. As neural progenitor cells might be dopamine sensitive (O’Keeffe et al., 2009), a complete loss of striatal dopamine could affect cell proliferation in a different manner than a reduction in dopamine content. Based on our findings and the existing literature, neurodegeneration and neuroinflammation do not seem to affect neuroblast generation, but might affect the proliferation of progenitor cells and in parallel shift the ratio of neurogenesis : gliogenesis towards gliogenesis, in particular astrogliosis. One possible explanation for the lack of neurogenesis accompanying increased progenitor cell proliferation might be the lack of stimulatory cues and a restrictive microenvironment (Raponti et al., 2007).

**Conclusion**

To our knowledge, this is the first study to cover in vivo imaging of neurodegeneration, neuroinflammation, and neurogenesis in the SVZ–OB axis in the same PD mouse model. We were able to validate our imaging findings with extensive histology for TH+ dopaminergic neurons, GFAP+ astrocytes, as well as TSPO+ and/or Iba1+ microglia, showing that in vivo imaging is able to pick up changes in neurodegeneration and neuroinflammation following 6-OHDA lesion. After our careful immunohistochemical analysis, these methods can now be used to perform longitudinal studies, thereby reducing interindividual differences and, at the same time, reducing the numbers of animals. We found that activation of microglia is an acute process accompanying the degeneration of dopaminergic cell bodies, and potentially reinforcing neuronal loss. Dopaminergic deafferentation of the striatum has no impact on the generation of Dcx+ neuroblasts in the SVZ, but strongly triggers chronic astrogliosis. Our results suggest that early anti-inflammatory treatment might reduce neuronal loss in the SN, whereas at later time points, treatments that increase neural progenitor cell proliferation and shift the fate of newly generated cells towards a neuronal phenotype could be a favourable approach.

**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

I.B.F. conducted the experiments, designed the figures, and drafted the manuscript. T.V. conducted the experiments and drafted the manuscript. M.M.W. established the 6-OHDA lesion model. F.M.C. was involved in BLI measurements and analysis. A.V. assisted with the SPECT studies and analysis. A.F. synthesized [18F]DPA-714. L.W. and C.F. designed and performed the magnetic resonance imaging studies and analysis. F.D. helped in setting up the [18F]DPA-714 synthesis in Münster. M.T.K. advised on surgery and histology. K.S. and S.H. supported the PET and SPECT imaging and analysis. J.C.S. and A.H.J. designed the study, were involved in data interpretation, and drafted the manuscript.

**Supporting Information**

Additional supporting information can be found in the online version of this article:

Fig. S1. Map of the pLKO.1-pCMV-LIC reporter plasmid.

Fig. S2. Animals that underwent PET and SPECT imaging following 6-OHDA/vehicle injection.

Fig. S3. Experimental setup of the BLI experiment.

Fig. S4. Absolute signals from the BLI measurement of subventricular neurogenesis.

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**Abbreviations**

6-OHDA, 6-hydroxydopamine; BLI, bioluminescence imaging; CMV, cytomegalovirus; CT, computed tomography; D4R, dopamine transporter; Dcx, doublecortin; DMMEM, Dulbecco’s modified Eagle’s medium; [18F]DPA-714, N,N-diethyl-2-(2-(4-(2-[[18F]fluoroethoxy]phenyl)-5,7-dimethylpyrazolo[1,5-a]
pyridim-3-yl)acetamide; dpi, days post-injection; DV, dorsal-ventral; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; [18F]Fludopamine; [123I]FP-CIT; [3H]spiroperidol; [123I]iodophenyl)nortropine; M, mean; mNan, median; OB, olfactory bulb; PBS, phosphate-buffered saline; PD, Parkinson’s disease; PET, positron emission tomography; SN, substantia nigra; SPECT, single photon emission computed tomography; SVZ, subventricular zone; TH, tyrosine hydroxylase; TSPO, translocator protein; VOI, volume of interest.

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