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In vivo imaging of neuromelanin in Parkinson's disease using ^{18}F-AV-1451 PET

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The tau tangle ligand ^{18}F-AV-1451 (^{18}F-T807) binds to neuromelanin in the midbrain, and may therefore be a measure of the pigmented dopaminergic neuronal count in the substantia nigra. Parkinson's disease is characterized by progressive loss of dopaminergic neurons. Extrapolation of post-mortem data predicts that a ~30% decline of nigral dopamine neurons is necessary to cause motor symptoms in Parkinson's disease. Putamen dopamine terminal loss at disease onset most likely exceeds that of the nigral cell bodies and has been estimated to be of the order of 50–70%. We investigated the utility of ^{18}F-AV-1451 positron emission tomography to visualize the concentration of nigral neuromelanin in Parkinson's disease and correlated the findings to dopamine transporter density, measured by ^{123}I-FP-CIT single photon emission computed tomography. A total of 17 patients with idiopathic Parkinson's disease and 16 age- and sex-matched control subjects had ^{18}F-AV-1451 positron emission tomography using a Siemens high-resolution research tomograph. Twelve patients with Parkinson's disease also received a standardized ^{123}I-FP-CIT single photon emission computed tomography scan at our imaging facility. Many of the patients with Parkinson's disease displayed visually apparent decreased ^{18}F-AV-1451 signal in the midbrain. On quantitation, patients showed a 30% mean decrease in total nigral ^{18}F-AV-1451 volume of distribution compared with controls ($P = 0.004$), but there was an overlap of the individual ranges. We saw no significant correlation between symptom dominant side and contralateral nigral volume of distribution. There was no correlation between nigral ^{18}F-AV-1451 volume of distribution and age or time since diagnosis. In the subset of 12 patients, who also had a ^{123}I-FP-CIT scan, the mean total striatal dopamine transporter signal was decreased by 45% and the mean total ^{18}F-AV-1451 substantia nigra volume of distribution was decreased by 33% after median disease duration of 4.7 years (0.5–12.4 years). ^{18}F-AV-1451 positron emission tomography may be the first radiotracer to reflect the loss of pigmented neurons in the substantia nigra of parkinsonian patients. The magnitude of the nigral signal loss was smaller than the decrease in striatal dopamine transporter signal measured by dopamine transporter single photon emission computed tomography. These findings suggest a more severe loss of striatal nerve terminal function compared with neuronal cell bodies, in accordance with the post-mortem literature.

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Abbreviations: DaT = dopamine transporter; SPECT = single photon emission computed tomography; SUVR = standardize uptake value ratio; UPDRS = Unified Parkinson's Disease Rating Scale; V_d = volume of distribution

Introduction

Parkinson's disease is characterized by progressive loss of dopaminergic neurons in the substantia nigra and midbrain tegmentum. Different dopaminergic cell groups are affected to variable degrees (Damier *et al.*, 1999). The progression rate of the dopaminergic cell loss in Parkinson's disease and the symptomatic threshold are still uncertain. Previously, it was held that motor symptoms do not appear until 50–70% of the substantia nigra dopamine neurons are lost (Marsden, 1990; Lang and Lozano, 1998; Dauer and Przedborski, 2003). However, more recent studies have argued that only a ~30% decline of nigral dopamine neurons is sufficient to cause motor symptoms (Fearnley and Lees, 1991; Ma *et al.*, 1997; Greffard *et al.*, 2006; Cheng *et al.*, 2010). The loss of putamen dopamine terminals at disease onset most likely exceeds that of the nigral cell bodies *per se* and has been estimated to be of the order of 50–70% (Riederer and Wuketich, 1976; Scherman *et al.*, 1989; Cheng *et al.*, 2010). These estimates were based on cross-sectional post-mortem studies and the symptomatic threshold was calculated by extrapolating neuronal loss at death back to time of diagnosis.

Imaging studies of dopamine terminal function have used radiotracer ligands for the dopamine and vesicular monoamine transporters and DOPA decarboxylase. A 30–70% putaminal signal loss is seen at the time of Parkinson's disease diagnosis (Morrish *et al.*, 1998; Tissingh *et al.*, 1998; Lee *et al.*, 2000; Filippi *et al.*, 2005; Bohnen *et al.*, 2006). In contrast, no radiotracers have been validated as imaging markers of neuromelanin concentration in dopaminergic cell bodies of the nigra. The ability to perform direct *in vivo* imaging of nigral dopaminergic cell density is an important goal for elucidating the symptomatic threshold and the progression rate of dopaminergic nigral cell loss.

Recently, Marquié *et al.* (2015) reported that the tau tangle ligand ^{18}F-AV-1451 (previously known as ^{18}F-T807) displays off-target binding to neuromelanin in the midbrain. Nigral neuromelanin has been previously reported to reflect the dopaminergic neuronal count at post-mortem (Gibb and Lees, 1991). In the present study, we investigated the ability of *in vivo* ^{18}F-AV-1451 PET to detect loss of neuromelanin in the midbrain of Parkinson's disease patients in comparison to healthy control subjects. Nigral ^{18}F-AV-1451 binding was correlated with dopamine transporter (DaT) single photon emission computed tomography (SPECT) in patients with Parkinson's disease. The dopaminergic neurons of non-primates do not contain neuromelanin and should be devoid of specific ^{18}F-AV-1451 binding (Barden and Levine, 1983; Nielsen *et al.*, 2009). To confirm the specificity of ^{18}F-AV-1451 binding to neuromelanin, we performed *in vitro* autoradiography studies in pig and rat brain tissue to demonstrate a lack of retention in the midbrain of these species.

Materials and methods

Autoradiography

Post-mortem brain tissue was obtained from a 3-month-old male Danish Landrace pig and two female Sprague-Dawley rats (Taconic). Tissue was fresh frozen with isopentane cooled to −40°C with dry ice and stored at −80°C. Sectioning at −20°C through the brainstem into 20 µm sections was performed using a CryoStar NX70 (Thermo Scientific). Brain slices were thaw mounted onto Thermo Scientific Polylysine slides and stored at −80°C. Eye tissue was obtained from a Landrace pig and processed similarly.

^{18}F-AV-1451 (Avid Radiopharmaceuticals) was synthesized on site as described by Shoup *et al.* (2013). ^{18}F-AV-1451 autoradiography was then performed as previously described (Marquié *et al.*, 2015) with slight modifications. Brain sections were thawed at room temperature for 20 min prior to fixation in 100% methanol for 20 min. They were incubated in 10 mM phosphate-buffered saline (PBS) pH 7.4 containing 35–50 MBq of ^{18}F-AV-1451. Separate baths containing 1, 10 or 20 µM unlabelled AV-1451 were used to incubate adjacent sections to assess non-specific binding in the presence of the same high specific activity tracer concentration. Post-incubation washes were done in 100% 10 mM PBS (1 min), 70% ethanol / 30% 10 mM PBS (2 min), 30% ethanol / 70% 10 mM PBS (1 min) and 100% 10 mM PBS (1 min). Sections were placed under a stream of cool air for drying and transferred to multisensitive phosphor screens (Fujifilm) for 15 min in complete darkness prior to development using a Fujifilm BAS-5000 phospho-imager. Autoradiograms were visualized using ImageJ software (http://imagej.nih.gov/ij/).

Nissl staining was performed in parallel sections with 0.1% toluidine blue in citrate buffer pH 4.0 for 4 min at room temperature. Sections were rinsed in distilled water and dehydrated in three baths of 99% alcohol. Sections were cleared with xylene and coverslips were appended using Depex® mounting media. The Nissl stained slices were used to confirm that the tissue sections used for autoradiography were in fact at the level of the substantia nigra.

Study subjects

A total of 17 patients with idiopathic Parkinson's disease and 16 age- and sex-matched control subjects were recruited (Table 1). All patients were diagnosed by movement disorder specialists according to the UK Brain Bank criteria (Hughes *et al.*, 2001). Study participants were aged 50 to 85 years and able to give informed consent. All Parkinson's disease

Table 1 Subject demographics

	Control	Parkinson's disease
n	16	17
Age (mean ± SD)	69.4 ± 7.3	67.8 ± 6.5
Gender (female/male)	4 / 12	4 / 13
MMSE	29 (26–30)	29 (26;30)
MoCA	27 (25–29)	26 (21;29)
Olfaction (mean ± SD)	11.1 ± 1.4[a]	6.6 ± 3.8
UPDRS part III	-	23.9 ± 12.6[b]
Hoehn and Yahr stage	-	2 (1–3)
Disease duration, years	-	5.2 (0.5–12.4)

Median (range) unless otherwise stated. MMSE = Mini-Mental State Examination; MoCA = Montreal Cognitive Assessment. [a]*n* = 11; [b]*n* = 16.

patients included had previously had a DaT SPECT as part of their clinical work-up and this showed typical loss of putamen signal.

Exclusion criteria for both groups included previous or current depression with a raised score on the Geriatric Depression Scale (Yesavage *et al.*, 1983), past history of more than one concussive head injury with loss of consciousness, <8 years of education, past history of schizophrenia, schizoaffective disorder, bipolar disorder or electroconvulsive therapy and contraindication to MRI. The project was approved by the regional Ethics Committee.

The Montreal Cognitive Assessment (MoCA) and Mini-Mental State Examination (MMSE) were performed on all patients. Healthy controls had either MMSE ($n = 14$), MoCA ($n = 11$), or both ($n = 9$). Olfaction was examined using Sniffin' Sticks (Burghardt) (Hummel *et al.*, 1997). Motor disability was evaluated using the MDS-UPDRS (Movement Disorder Society Unified Parkinson's Disease Rating Scale) Part III (Goetz *et al.*, 2007) while the patients were receiving medication.

MRI

In all subjects, a high resolution 3D T_1-weighted sequence was performed using a 32 channel head coil on a standard clinical 3 T MAGNETOM Trio system (Siemens Healthcare) using a 3D T_1 MPRAGE with 176 slices, $1 \times 1 \times 1$ voxel size, field of view 256 mm, echo time = 4.58 ms, repetition time = 2420 ms, inversion time = 1110 ms, flip angle = 9, normal water excitation, one acquisition and with an acquisition time of 10:55.

PET

All subjects had PET with a Siemens High-Resolution Research Tomograph (ECAT HRRT; CTI /Siemens) (Heiss *et al.*, 2004). Subjects received an intravenous injection of 300–370 MBq ^{18}F-AV-1451. A transmission scan from 74 to 80 min and an emission scan from 80 to 120 min post-injection in list-mode were performed. The PET data were binned into eight frames of 5 min each. Image based (AIR) frame-by-frame motion correction was performed when needed. Scans were reconstructed using 3D OSEM (ordered subsets expectation maximization) (Liu *et al.*, 2001) and resolution recovery modelling (PSF) with 10 iterations and 16 subsets. The reconstructed image volume consists of 207 axial image slices with a 1.22 mm voxel size. The reconstructed images were corrected for random and scatter events, detector efficiency variations, and dead time. Final resolution was 2.5 mm full-width at half-maximum isotopic.

PET analysis

PET data were analysed using PMOD v.3.608. In brief, PMOD's built-in tool PNEURO segments white/grey matter in the anatomical T_1-weighted magnetic resonance sequence and transforms it into MNI stereotaxic space where it defines anatomical volumes of interest using Hammer's probabilistic atlas (Hammers *et al.*, 2003). The volumes of interest were then transformed back into subject PET space, after co-registration.

For this study, we enlarged the atlas substantia nigra volumes of interest to encompass the peduncles (Fig. 2A). This method is advantageous when investigating very small anatomical structures, as it is less sensitive than smaller substantia nigra volumes of interest to partial volume effects and patient head movement during the scan. It was assumed that only two tissue types were present in the substantia nigra volume of interest, a specific signal from neuromelanin and non-specific background signal estimated using a cerebellar cortex reference region. This assumption is supported by prior ^{18}F-AV-1451 autoradiographical results (Marquié *et al.*, 2015). The reference volume of interest was defined avoiding CSF, blood vessels, choroid plexus, or the anterior cerebellar lobe. We noted that blood vessels and choroid plexus showed increased activity in most subjects. The cerebellar cortex was chosen as a reference on the assumption that it is devoid of both neuromelanin and paired helical tau filaments. All volumes of interest were visually inspected in PET and magnetic resonance space and adjusted manually to ensure that the entire nigral signal was included. No post-filtering of the PET data was used in the volume of interest analyses.

Average volume of interest activity and size were extracted for each subject. For the right and left substantia nigra volumes of interest, we calculated specific V_d (volume of distribution), proportional to the amount of neuromelanin present, using the following equation:

$$V_d = [(C_{SN} \times V_{SN}) - (C_{ref} \times V_{SN})]/C_{ref}$$

where C_{SN} and C_{ref} denote activity concentrations in the substantia nigra and reference volume of interest, and V_{SN} the volume of the substantia nigra volume of interest. We compared left, right, and total (left + right) substantia nigra V_d values between patients with Parkinson's disease and control subjects and also the minimum substantia nigra V_d values (left or right). In the Parkinson's disease group, we also defined ipsi- and contralateral substantia nigra V_d values with reference to the predominant side of motor symptoms.

Basal ganglia uptake of ^{18}F-AV-1451 was calculated as standardized uptake value ratios (SUVRs) ($C_{basal\ ganglia}/C_{ref}$) from volume-weighted averages of the nucleus accumbens, pallidum, and putamen as identified by the Hammer's probabilistic atlas divided by the cerebellar reference volume of interest. Simple SUVR calculations were applied to the basal ganglia data, as the volumes of interest were much larger and head motion artefacts and partial volume effects were not a major concern. The caudate nucleus was omitted due to close proximity to the choroid plexus, which showed high activity.

Dopamine transporter SPECT

Twelve patients had had DaT SPECT at our imaging facility using previously described methodology (Borghammer *et al.*, 2014). Subjects were scanned 3 h post intravenous injection of 150–180 MBq ^{123}I-FP-CIT on a Siemens Symbia T16 SPECT/CT camera with a low-energy high-resolution collimator, 128 × 128 matrix, 64 steps of 35 s. Image data were reconstructed (OSEM, Chang attenuation correction and Butterworth post-filtering) using Hermes software (HERMES Medical Solutions, Stockholm, Sweden). Qualitative and semi-quantitative evaluations were performed by an experienced nuclear medicine physician. Data were analysed with Hermes BRASS, automatically defining volumes of interest in putamen and caudate nucleus bilaterally as well as an occipital reference region. Specific tracer binding in each region was defined as (region-occipital)/occipital and subsequently compared with in-house, age-matched reference data. Ten of 12 patients had their DaT SPECT and ^{18}F-AV-1451 PET within 1 year, the remaining two patients had 4.7 and 7.3 year gaps between DaT and PET. For the purpose of comparing DaT SPECT with ^{18}F-AV-1451 PET results, a −5% per year reduction correction factor was applied to the specific DaT binding ratios (Pirker *et al.*, 2003). The remaining five patients had had DaT scans performed at other imaging facilities. These scans were abnormal, but the data were not quantitatively comparable to our data and therefore not used for correlations in the present study.

Statistical analysis

Statistical analyses were performed in Stata IC 13 (StataCorp LP, Texas, USA). Categorical data were evaluated using a two-sample test of proportions. Means were compared with two-tailed Student's *t*-test unless otherwise stated, after assumption of normality was checked using Q-Q plot and histograms and equal variances using F-test. For unequal variances, Welch's approximation was used. For non-normal distributions, Wilcoxon rank-sum (Mann-Whitney) test was used. *P*-values < 0.05 were considered statistically significant. Linear regression was used for interrogating correlations, followed by model verification by diagnostic plots of residuals. The Holm correction for multiple comparisons was applied to the analysis of the PET data.

Results

Autoradiography

Figure 1 shows the autoradiography results. We saw no ^{18}F-AV-1451 binding in the nigra of rats or the pig. In contrast, strong ^{18}F-AV-1451 binding was seen in the retinal pigment epithelium of a pig eye, which was partially displaceable by 20 μM of unlabelled AV-1451.

PET and SPECT

Table 1 summarizes the demographic data of the two groups. There were no significant differences in age ($P = 0.49$), gender distribution ($P = 0.92$) or dementia scores (Wilcoxon rank-sum, MMSE: $P = 0.95$, MoCA: $P = 0.68$) between patients and healthy controls. The quantitative imaging results are summarized in Table 2.

Substantia nigra

Representative ^{18}F-AV-1451 images are shown in Fig. 2. Many of the Parkinson's disease patients displayed visually apparent decreased ^{18}F-AV-1451 signal in the midbrain. The Parkinson's disease patients displayed a 31% decrease in minimum substantia nigra V_d compared with controls ($P = 0.008$), but there was an overlap of the individual ranges (Fig. 3A). The patients with Parkinson's disease also demonstrated significant average decreases in the left ($P = 0.003$), right ($P = 0.01$), and total [$P = 0.004$, 30% decrease, 95% confidence interval (CI) (10%, 50%)] substantia nigra V_d values. The subregional volume of interest analysis of medial/lateral substantia nigra showed a 33.1% [95% CI (11.2%, 54.9%); $P = 0.004$] decrease in the lateral substantia nigra and 25.7% [95% CI (4.9%, 46.6%); $P = 0.02$] decrease in the medial substantia nigra of patients with Parkinson's disease. Comparing left and right substantia nigra V_d in controls, the average left substantia nigra V_d was significantly larger ($P = 0.004$). This was not the case for the patients ($P = 0.06$).

We hypothesized that motor symptom severity and substantia nigra V_d would show an inverse relationship in patients with Parkinson's disease. An inverse correlation with UPDRS-III score was seen, but the finding was not significant [Fig. 3B; linear regression, $r^2 = 0.13$, $P = 0.09$ (one-sided), mean slope −0.005, 95% CI (−0.0134, 0.0027)]. We saw no significant correlation between symptom dominant side and contralateral substantia nigra V_d ($P = 0.05$). Indeed, the ipsilateral substantia nigra V_d values were on average lower compared to the contralateral side.

There was no correlation between age and minimum substantia nigra V_d among controls (linear regression, $P = 0.78$) or patients ($P = 0.92$).

Dopamine transporter SPECT correlation

In the 12 patients with Parkinson's disease with in-house DaT SPECT data, the mean total striatum DaT signal was decreased to 55.0% [lag time corrected, unequal variances, 95% CI (46.0%, 63.9%)] of the reference mean after a median disease duration of 4.7 years, range 0.5 to 12.4 years. In this subgroup of patients, the mean total ^{18}F-AV-1451 substantia nigra V_d was decreased to 66.5% [95% CI (43.8%, 89.3%)] (Fig. 4A).

There was no correlation between striatal DaT signal and time since diagnosis [Fig. 4B, $r^2 = 0.16$, slope −0.92 per cent points/year, 95% CI (−2.4, 0.5)]. There was also no correlation between total substantia nigra ^{18}F-AV-1451 V_d and time since diagnosis [$r^2 = 0.013$, slope −0.84 per cent points/year, 95% CI (−4.9, 3.2), $P = 0.66$].

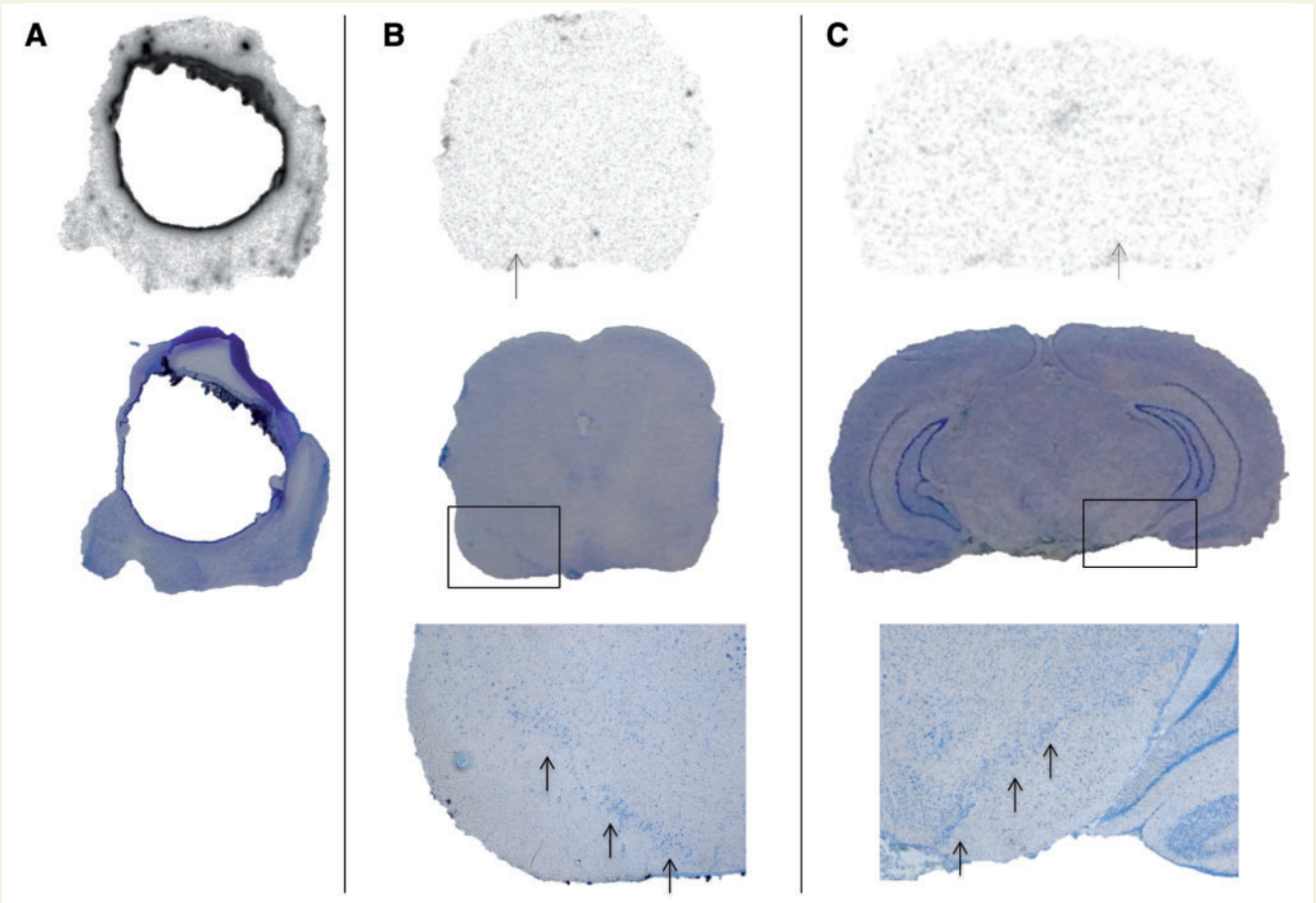

Figure 1 ^{18}F-AV-1451 autoradiography in pig and rat brain. The *top row* depicts ^{18}F-AV-1451 autoradiograms. *Middle* and *bottom rows* show Nissl stained sections adjacent to the autoradiograms. Arrows on the insets designate the substantia nigra and ventral tegmental area. Strong ^{18}F-AV-1451 binding was seen in the retinal pigment epithelium of the pig eye (**A**). No ^{18}F-AV-1451 binding was seen in the substantia nigra of the pig (**B**) or in rats (**C**).

Table 2 PET and SPECT results

	Control		Parkinson's disease	
n	16		17	
Basal ganglia (SUVR)	1.44 ± 0.13		1.36 ± 0.15	
Susbstantia nigra V_d (ml)				
Total	1.53 ± 0.48		1.07 ± 0.37	
Minimum	0.71 ± 0.25		0.49 ± 0.19	
Ipsilateral			0.51 ± 0.18	
Contralateral			0.56 ± 0.20	
	Left	**Right**	**Left**	**Right**
Total	0.81 ± 0.25	0.72 ± 0.24	0.56 ± 0.19	0.51 ± 0.19
DaT binding ratio[a]	1.90 ± 0.35	1.84 ± 0.29	1.00 ± 0.17	1.05 ± 0.21

Values are mean ± SD. Total substantia nigra (left + right). Minimum substantia nigra (left or right). Ipsilateral and contralateral values are given with reference to predominant symptom side. L/R = Left/Right.
[a]The normal DaT ratio values were derived from an in-house reference material of 26 healthy controls.

Basal ganglia

The majority of study subjects showed increased ^{18}F-AV-1451 activity in the basal ganglia (Fig. 5), and this basal ganglia activity increased linearly with age in the Parkinson's disease group [$r^2 = 0.45$, slope 0.015 SUVR/year, 95% CI (0.006, 0.025), $P = 0.003$], but not in the control group [$r^2 = 0.097$, slope 0.006 SUVR/year, 95% CI (−0.004, 0.016), $P = 0.24$] (Fig. 6B). There was however, no significant difference in the slopes between Parkinson's disease and controls ($P = 0.11$).

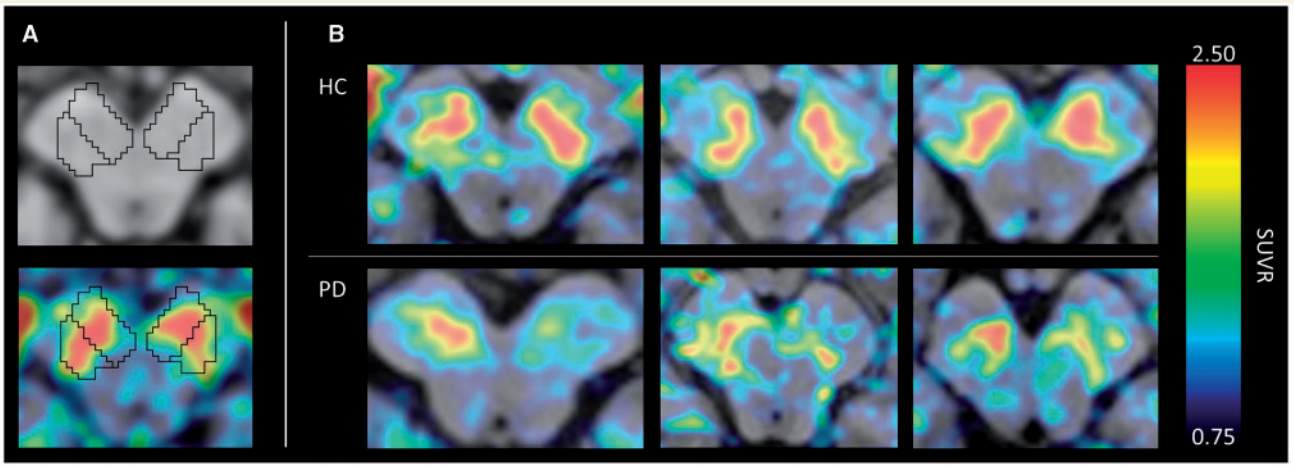

Figure 2 ^{18}F-AV-1451 PET images of the substantia nigra. (**A**) Automated midbrain volumes of interest superimposed on magnetic resonance (*top*) and fused PET/magnetic resonance images (*bottom*) of a 76-year-old female control (minimum substantia nigra V_d 0.95 ml). (**B**) Six representative fused PET/magnetic resonance examples. Healthy controls (HC), *top row from left*: 68-year-old male (minimum substantia nigra V_d 0.61 ml), 73-year-old male (minimum substantia nigra V_d 0.68 ml), 72-year-old male (minimum substantia nigra V_d 0.92 ml). Parkinson's disease (PD); *bottom row from left*: 66-year-old female minimum (substantia nigra V_d 0.35 ml), 57-year-old male (minimum substantia nigra V_d 0.49 ml), 65-year-old male (minimum substantia nigra V_d 0.58 ml), all Hoehn and Yahr stage II. Colour scale shows SUVR with cerebellum as reference.

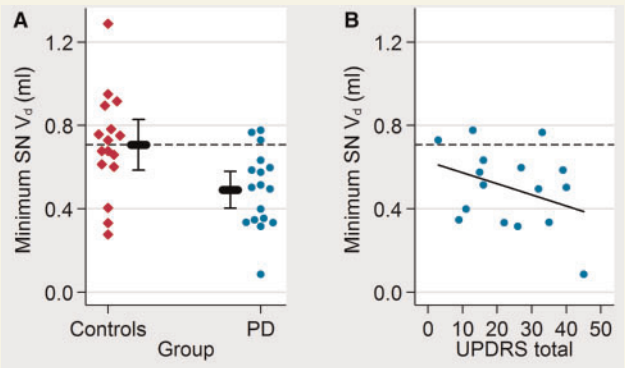

Figure 3 ^{18}F-AV-1451 PET data in the substantia nigra. (**A**) Minimum ^{18}F-AV-1451 V_d in the substantia nigra of controls and Parkinson's disease (PD) patients. The bars show mean group values and 95% CI, $P = 0.008$. (**B**) Susbstantia nigra V_d values as function of total UPDRS part III score in the Parkinson's disease group. Dashed line is mean of healthy controls. Solid line is best linear fit ($P = 0.09$, one-sided).

There was no significant difference in the basal ganglia activity between controls and Parkinson's disease using age-correction ($P = 0.14$), or without age-correction ($P = 0.10$).

Discussion

To our knowledge, this is the first radiotracer imaging study to measure neuromelanin concentration in the substantia nigra of patients with Parkinson's disease. Initially, we performed ^{18}F-AV-1451 phosphor screen autoradiography in rat and pig brain, as the nigra in these non-primate species does not contain neuromelanin (Barden and Levine, 1983; Nielsen *et al.*, 2009). As predicted, we did not see any binding in the nigra of either species, in contrast to the strong nigral signal seen in human substantia nigra (Marquié *et al.*, 2015). We saw strong ^{18}F-AV-1451 binding in the pig retinal pigment epithelium in concordance with previous findings in humans (Marquié *et al.*, 2015). Taken together, these results demonstrate that our autoradiography protocol was adequate and provide further evidence that ^{18}F-AV-1451 binds to neuromelanin in human catecholaminergic neurons.

Our PET results support the suggestion that ^{18}F-AV-1451 PET provides an *in vivo* marker of neuromelanin in the substantia nigra. The group of patients with Parkinson's disease displayed a 30% reduction in total nigral ^{18}F-AV-1451 signal compared to age-matched controls. Overlap was seen in Parkinson's disease and control ^{18}F-AV-1451 nigral signal ranges, which will limit the utility of ^{18}F-AV-1451 as a primary diagnostic tool. A large number of post-mortem studies have investigated the cell loss in mesencephalic regions of patients with Parkinson's disease. The substantia nigra pars compacta is most heavily affected, and within the pars compacta, the ventro-lateral region is the most severely affected subregion (Fearnley and Lees, 1991; Ma *et al.*, 1996). Most post-mortem studies investigated brains from patients with Parkinson's disease with extended disease duration. However, at least three studies estimated that the symptomatic threshold of dopaminergic neuron loss in the nigral pars compacta is ~30% (Fearnley and Lees, 1991; Ma *et al.*, 1997; Greffard *et al.*, 2006). Moreover, several studies have reported that other mesencephalic regions, including the ventral tegmental area, central grey substance, medial and medioventral groups, are less severely affected compared to the pars compacta (Hirsch *et al.*, 1988; German *et al.*, 1989; Kastner *et al.*, 1992; Damier *et al.*, 1999).

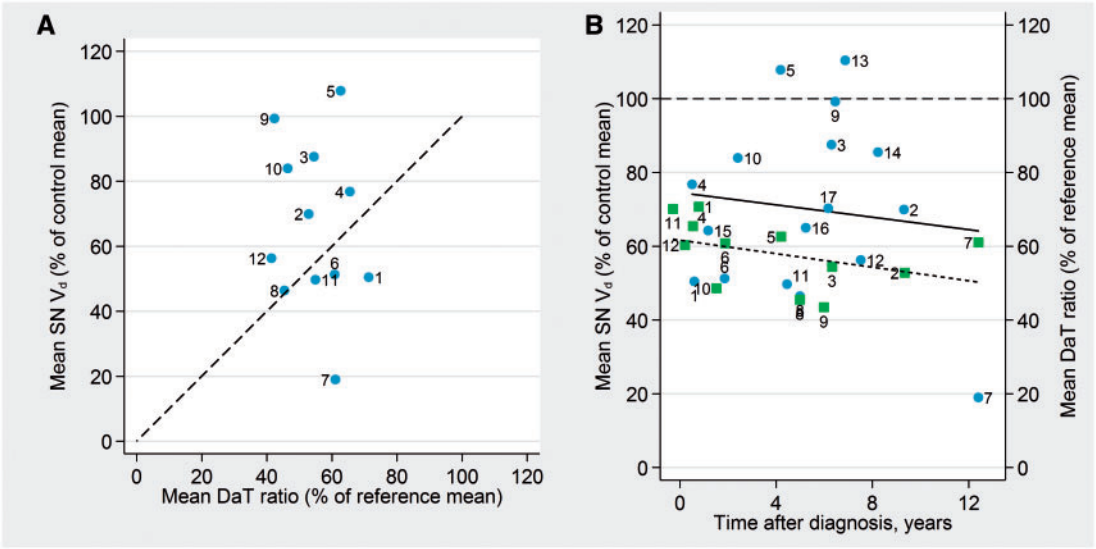

Figure 4 Substantia nigra ^{18}F-AV-1451 PET versus striatal DaT SPECT. (**A**) DAT ratios (*x*-axis) and ^{18}F-AV-1451 nigral V_d values (*y*-axis) are shown as per cent of healthy control mean. Stapled line is line of unity. (**B**) Blue circles are mean nigral ^{18}F-AV-1451 V_d as per cent of control mean. Green squares are mean striatal DaT ratios as per cent of in-house reference mean. The *x*-axis shows the time from diagnosis to scan. Horizontal dashed line is 100% of both control nigral V_d mean and reference striatum ratio. Sloping lines are best linear fits, which were not significantly different from 0. Subjects 1–12 are the same as **A**. Subjects 13–17 were DaT scanned at other centres and their DaT values were not comparable to our in-house data.

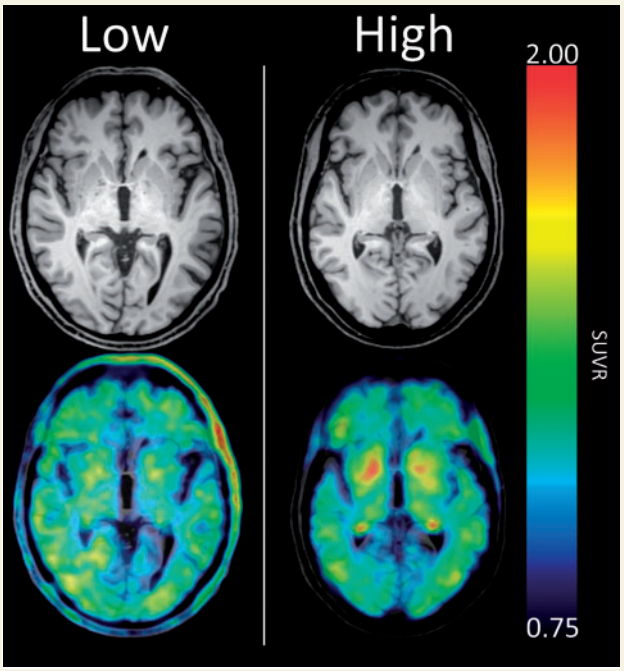

Figure 5 ^{18}F-AV-1451 uptake in basal ganglia. Representative examples of low and high basal ganglia ^{18}F-AV-1451 binding. *Left*: 52-year-old male (SUVR 1.27); *right*: 71-year-old female (SUVR 1.53). Both are healthy controls. A 6 mm Gaussian smooth was applied to the PET images.

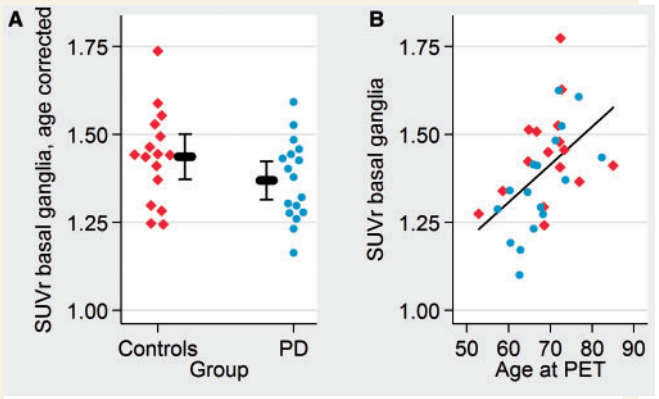

Figure 6 Basal ganglia ^{18}F-AV-1451 data. (**A**) No significant difference was seen in the basal ganglia SUVR values between controls and Parkinson's disease (PD) patients ($P = 0.14$). Data also shown as mean (95% CI). (**B**) A significant linear correlation with age was seen for the SUVR values in the basal ganglia. The line depicts the linear fit for pooled controls and Parkinson's disease patients ($r^2 = 0.25$). Red diamonds are controls, blue circles are Parkinson's disease.

Thus, we believe that our finding of a 30% reduction in the total midbrain ^{18}F-AV-1451 signal is consistent with the post-mortem literature. First, our midbrain ^{18}F-AV-1451 measurements included both the ventral tegmental area and the substantia nigra due to the limited spatial resolution of the PET camera. Second, our population of Parkinson's disease patients had early-to-moderate stage disease with median disease duration of 5.2 years and median Hoehn and Yahr stage 2 in the ON state. In our subregional analysis, we detected an average 33.1% decrease in the lateral and 25.7% decrease in the medial substantia nigra. Although the medial versus lateral difference was not statistically significant, the finding nevertheless is in line with the

many reports of the lateral nigra being the most heavily affected subregion.

It should also be noted that, although the post-mortem studies generally show a marked decrease in the average number of dopaminergic neurons of the parkinsonian pars compacta, several studies reported overlapping individual neuron counts when comparing to healthy control brains (Ma *et al.*, 1997; Kordower *et al.*, 2013). Recently, Dijkstra and colleagues (2014) investigated the relationship between Braak alpha-synuclein stages of the brain and neuron loss in the nigra (Braak *et al.*, 2003). At Braak stage 3, the dopaminergic neuron count was ~73% of normal, and at Braak stages 4–5, it was 40–45% of control mean. Milber *et al.* (2012) reported similar findings, and both studies also reported some overlap with neuronal counts in healthy control brains. Thus, even gold standard neuropathological evaluation of nigral neuron loss does not completely separate the Parkinson's disease and healthy control ranges so it is not surprising to see overlapping values when using *in vivo* PET imaging in our group of non-demented patients with Parkinson's disease, who were in the early-to-moderate disease stages.

In addition, the vast majority of post-mortem studies counted the number of remaining pigmented neurons. In contrast, a PET signal is a measure of the molecular concentration of the target molecule. This distinction is important, as pigmented and non-pigmented neurons may display differential susceptibility to neurodegeneration in Parkinson's disease, as has been demonstrated by several authors (Hirsch *et al.*, 1988; Zucca *et al.*, 2014). In patients with Parkinson's disease the mean content of neuromelanin in surviving neurons of the substantia nigra may be smaller than that of controls (Mann and Yates, 1983), and other studies demonstrated that lightly pigmented neurons are more vulnerable than heavily pigmented neurons (Gibb, 1992). Therefore, caution should be observed when comparing *in vivo* melanin PET findings to post-mortem data based on neuron counting techniques.

We did not see correlation between nigral ^{18}F-AV-1451 signals and disease duration, which could be explained by the relatively short disease duration in our Parkinson's disease patient group. The majority of our patients had had Parkinson's disease for 4–9 years. Post-mortem studies also show little or no correlation between pigmented neuron counts and disease duration, when restricting the data to <10 years of disease duration (Pakkenberg *et al.*, 1991; Ma *et al.*, 1997; Kordower *et al.*, 2013). A recent large post-mortem study of 24 Parkinson's disease patients also failed to see a correlation between pigmented neuron counts and disease duration over wide range of 4 to 26 years (Dijkstra *et al.*, 2014). In contrast, there seems to be a somewhat better correlation between pigmented neuron loss and Braak α-synuclein stage, at least if incidental Lewy body cases are also included (Milber *et al.*, 2012; Dijkstra *et al.*, 2014).

In our data set, we saw no significant inverse correlation between UPDRS motor scores and nigral ^{18}F-AV-1451 signal (Fig. 3B). This was surprising and future studies with larger sample sizes will be needed to substantiate this finding. However, given the large inter-individual variance in the total number of nigral dopamine neurons in both Parkinson's disease patients and controls, it is not surprising that a cross-sectional regression analysis does not yield a strong correlation with motor severity. It will be important for future ^{18}F-AV-1451 PET studies to be longitudinal and to include more later-stage Parkinson's disease patients to investigate if significant correlations with disease duration and motor symptom severity emerge. These studies are mandatory in order to determine if ^{18}F-AV-1451 PET has potential as a biomarker for assessing efficacy in future neuro-protective drug trials.

The majority of Parkinson's disease patients exhibited a larger decrease in the striatal ^{123}I-FP-CIT signal compared to that of nigral ^{18}F-AV-1451 signal (Fig. 4). The mean striatal DaT value in the patients was ~55% of reference mean. Thus, our SPECT and PET findings seem to support observations from post-mortem literature that the loss of striatal dopaminergic terminal function is more severe than the loss of neuronal cell bodies in the substantia nigra (reviewed in Cheng *et al.*, 2010). We did not see a significant correlation between the nigral ^{18}F-AV-1451 and striatal ^{123}I-FP-CIT values in the patient group. One explanation for this lack of correlation could be the unavoidable inclusion of the ventral tegmental area in the midbrain PET volumes of interest. Had we been able to measure the nigra V_d exclusively, it is possible that a closer correlation between striatal DaT loss and nigral neuromelanin loss would have been seen.

Surprisingly, few post-mortem studies have been done to correlate the number of pigmented nigral neurons to levels of dopamine, tyrosine hydroxylase, or other markers of synaptic function in the striatum. A recent post-mortem study investigated both putaminal DaT density and number of nigral pigmented neurons in 17 patients with Parkinson's disease (Kordower *et al.*, 2013). The authors did not report a DaT versus nigral neuron correlation, and it seems unlikely that such a correlation was present in their data, as the putaminal DaT loss at 5 to 27 years of disease duration displayed limited dynamic range. Another study found no correlation between number of pigmented nigral neurons and the amount of tyrosine hydroxylase immunoreactivity in the nigra (Gaspar *et al.*, 1983). Taken together, these previous observations and our new results suggest that there may be limited correlation between the number of remaining pigmented neuronal cell bodies and the functional state of their terminals.

Nearly all patients with Parkinson's disease and control subjects displayed visible ^{18}F-AV-1451 binding in the striatum and pallidum, but no significant difference was seen in the binding between the two groups. We did, however, see a significant increase in binding with age (Fig. 6B). It was previously demonstrated that neuronal pigment of a melanin type is also seen in non-nigral regions including the striatum and pallidum, and this pigment concentration

increases linearly with age (Zecca *et al.*, 2008). Thus, it is possible that striato-pallidal ^{18}F-AV-1451 binding constitutes additional binding to neuromelanin, but further studies are needed to confirm this hypothesis. The primary application of ^{18}F-AV-1451 PET is to measure the presence of pathological paired helical filament tau protein. Our finding of age-dependent signal increases in subcortical regions is important when considering the correct interpretation of ^{18}F-AV-1451 uptake in the context of tau protein imaging.

Several studies have suggested that T_1-weighted fast spin echo MRI sequences or magnetization transfer ratios may be able to estimate the neuromelanin content of the nigra (Sasaki *et al.*, 2006; Schwarz *et al.*, 2011; Ohtsuka *et al.*, 2013; Reimão *et al.*, 2015). These MRI studies have sufficient resolution to investigate subregions of the nigra and usually report measures of the area of nigral hyperintensity or the contrast-ratio of nigral subregions. The lateral part of the nigra generally displays the most severe decrease, but considerable overlap with healthy control values is normally seen. Moreover, the medial part of the nigra shows nearly identical distribution to healthy control values (Ohtsuka *et al.*, 2013). Interestingly, recent diffusion tensor imaging studies using a bi-tensor model to separate the free water pool have detected a 35% increase in the free-water signal of the substantia nigra in patients with Parkinson's disease (Ofori *et al.*, 2015). Although the meaning of this nigral free water MRI signal is not fully understood, the magnitude of the signal change in patients with Parkinson's disease is comparable to our current data.

A recent study has compared the neuromelanin-sensitive T_1-weighted MRI signal in the nigra to striatal FP-CIT signal of 23 patients, the majority of whom had Parkinson's disease ($n = 17$) or multiple system atrophy ($n = 3$) (Kuya *et al.*, 2016). The authors detected a significant linear correlation between the nigral melanin volume and striatal DaT availability, in contrast to our finding of a lack of correlation between AV-1451 nigral signal and striatal FP-CIT binding. A recent study of MPTP-treated monkeys also demonstrated a linear association between nigral cell loss and striatal uptake of ^{11}C-carbomethoxy-3beta-(4-fluorophenyl)tropane, but this was only seen at low levels of nigral cell loss ($<50\%$) (Karimi *et al.*, 2013). At higher levels of nigral cell loss, the striatal PET signal became very low ($\sim$10% of control level), and no correlation was seen between the two measures, suggesting a floor effect at $\sim$50% nigral cell loss. Our Parkinson's disease group was relatively early stage and did not include patients with severe reductions in DaT binding so the poor correlation between AV-1451 nigral signal and striatal FP-CIT binding was unexpected. The paper by Kuya *et al.* (2016) included 23 patients with a mix of Parkinson's disease, essential tremor, multiple system atrophy, progressive supranuclear palsy, and spinocerebellar ataxia and the wide range of striatal FP-CIT uptake in this cohort will have given them greater power to demonstrate a correlation between nigral melanin volume and striatal DaT availability.

Whether AV-1451 and neuromelanin-sensitive MRI sequences are measures of the same underlying physiology is at present not clear. An early study did correlate the MRI signal to melanin in sections of mesencephalon (Sasaki *et al.*, 2006). To our knowledge, no studies using neuromelanin-sensitive MRI sequences have compared the substantia nigra signal in species with and without neuromelanin, which would be a useful way to validate the specificity of the MRI sequences. From this point of view, ^{18}F-AV-1451 PET is at present a better validated neuromelanin marker, as a strong signal is seen in the human substantia nigra, and in pigmented retinal and skin structures, whereas no signal is seen in rat and pig substantia nigra. Further research is necessary to validate both AV-1451 and neuromelanin-sensitive MRI, and the present PET results now provide the opportunity to perform PET-MRI cross-validation studies.

Bohnen *et al.* (2006) have previously showed that ^{11}C-DTBZ can detect a decrease of vesicular monoamine transporter density in the nigra of patients with Parkinson's disease, thus providing a marker of the functional integrity of nigral dopaminergic neurons. Similar findings were also seen with ^{18}F-DOPA PET (Rakshi *et al.*, 1999). Future multi-tracer PET studies using different markers of dopaminergic terminal function and AV-1451 may potentially improve our understanding of nigral degeneration in Parkinson's disease.

In this paper we do not report on the ability of ^{18}F-AV-1451 to visualize pathological tau protein in the brain of Parkinson's disease patients. Our study is ongoing and we will report on these aspects in a future publication.

The study has some limitations. We used pathological DaT SPECT as an inclusion criterion, which may have biased the SPECT versus PET correlation analysis. On the other hand, it has been shown that state-of-the-art DaT SPECT has a diagnostic accuracy of well above 90%, when semi-quantitative comparison to an age-matched reference material is performed (Bajaj *et al.*, 2013; Borghammer *et al.*, 2014), and a normal DaT SPECT is now considered an absolute exclusion criterion in the latest diagnostic criteria of Parkinson's disease (Postuma *et al.*, 2015). There was a significant lag time between performing SPECT and PET scans in two of our patients, which, although we implemented a correction factor, may have introduced some bias. Also, the percentage decrease in the striatal DaT signal was derived by comparing our cases with a previously scanned group of healthy controls. As a consequence, the comparison of percentage decreases of nigral AV-1451 and striatal DaT signal may have included some bias. Nevertheless, we argue that our finding of more severely decreased striatal DaT signal compared to the nigral AV-1451 signal is in line with pathological studies. An additional potential limitation is that we evaluated the motor symptoms of our Parkinson's disease cases in the medicated condition, which will have obscured a true association between the nigral ^{18}F-AV-1451 signal and motor disability. Higher UPDRS scores would certainly have been

found in some patients, had they been evaluated in the OFF state. Future studies should preferentially investigate motor symptoms in the drug-withdrawn state. Finally, the mean age of our patients with Parkinson's disease was 68 years, so we cannot conclude whether similar decreases in nigral AV1451 signal will be seen in patients with young onset Parkinson's disease.

In summary, we have demonstrated that ^{18}F-AV-1451 PET may provide a marker of loss of pigmented neurons in the substantia nigra of patients with Parkinson's disease. As a group, the patients with Parkinson's disease displayed a 30% signal loss in the midbrain compared to healthy controls. The magnitude of this loss was smaller than the decrease in striatal DaT signal measured by DaT SPECT. These findings may suggest a more severe loss of striatal nerve terminal function compared with neuronal cell bodies, which is in accordance with the post-mortem and *in vivo* animal literature and suggests a dying back pathology. Future studies are needed to explore the progression rate of nigral ^{18}F-AV-1451 signal loss in patients with Parkinson's disease, and could establish ^{18}F-AV-1451 PET as a potential biomarker for neuroprotective drug trials.

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