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

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

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Running title: Neuromelanin PET in Parkinson's disease

Abstract

The tau tangle ligand 18F-AV-1451 (18F-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 18F-AV-1451 PET to visualize the concentration of nigral neuromelanin in Parkinson's disease and correlated the findings to dopamine transporter density, measured by 123I-FP-CIT SPECT.

A total of 17 patients with idiopathic Parkinson's disease and 16 age- and sex-matched control subjects had 18F-AV-1451 PET using a Siemens High-Resolution Research Tomograph. Twelve patients with Parkinson's disease also received 123I-FP-CIT SPECT at our imaging facility.

Many of the Parkinson's disease patients displayed visually apparent decreased 18F-AV-1451 signal in the midbrain. On quantitation, patients showed a 30% mean decrease in nigral 18F-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 18F-AV-1451 volume of distribution and age or time since diagnosis. In the subset of 12 patients, who also had a 123I-FP-CIT scan, the mean total striatal dopamine transporter signal was decreased by 45% and the mean total 18F-AV-1451 substantia nigra volume of distribution was decreased by 33% after a median disease duration of 4.7 years, range 0.5 to 12.4 years.

18F-AV-1451 PET 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 SPECT. These findings suggest a more severe loss of striatal nerve terminal function compared with neuronal cell bodies, in accordance with the post-mortem literature.

Keywords: AV-1451; PET; neuromelanin; Parkinson; dopamine transporter

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Abbreviations:

DaT	Dopamine transporter
MMSE	Mini Mental State Examination
MoCA	Montreal Cognitive Assessment
PBS	Phosphate-buffered saline
SN	Substantia nigra
SPECT	Single photon emission computed tomography
UPDRS	Unified Parkinson's Disease Rating Scale.
V_d	Volume of distribution
VOI	Volume of interest

Introduction

Parkinson's disease is characterized by progressive loss of dopaminergic neurons in the SN 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 SN dopamine neurons are lost (Dauer and Przedborski, 2003; Lang and Lozano, 1998; Marsden, 1990). However, more recent studies have argued that a ~30% decline of nigral dopamine neurons is sufficient to cause motor symptoms (Cheng *et al.*, 2010; Fearnley and Lees, 1991; Greffard *et al.*, 2006; Ma *et al.*, 1997). 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% (Cheng *et al.*, 2010; Riederer and Wuketich, 1976; Scherman *et al.*, 1989). 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 utilized 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 (Bohnen *et al.*, 2006; Filippi *et al.*, 2005; Lee *et al.*, 2000; Morrish *et al.*, 1998; Tissingh *et al.*, 1998). 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 and colleagues reported that the tau tangle ligand 18F-AV-1451 (previously known as 18F-T807) displays off-target binding to neuromelanin in the midbrain (Marquié *et al.*, 2015). 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* 18F-AV-1451 PET to detect loss of neuromelanin in the midbrain of Parkinson's disease patients in comparison to healthy control subjects. Nigral 18F-AV-1451 binding was correlated with DaT SPECT in Parkinson's disease patients. The dopaminergic neurons of non-primates do not contain neuromelanin and should be devoid of specific 18F-AV-1451 binding (Barden and Levine, 1983; Nielsen *et al.*, 2009). To confirm the specificity of 18F-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.

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Materials and methods

Autoradiography

Postmortem brain tissue was obtained from a 3-month old male Danish Landrace pig and two female Sprague-Dawley rats (Taconic, Denmark). Tissue was fresh frozen with isopentane cooled to -40ºC with dry ice and stored at -80ºC. Sectioning at -20ºC 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 -80ºC. Eye tissue was obtained from a Landrace pig and processed similarly.

18F-AV-1451 (Avid Radiopharmaceuticals, Philadelphia, PA) was synthesized on site as described by Shoup *et al.*, 2013. 18F-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 minutes prior to fixation in 100% methanol for 20 minutes. They were incubated in 10 mM PBS pH 7.4 containing 35-50 MBq of 18F-AV-1451. Separate baths containing 1, 10 or 20 µM unlabeled 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% 10mM PBS (1 min), 70% ethanol / 30% 10mM PBS (2 min), 30% ethanol / 70% 10mM PBS (1 min) and 100% 10mM PBS (1 min). Sections were placed under a stream of cool air for drying and transferred to multisensitive phosphor screens (Fujifilm) for 15 minutes 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 minutes 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 SN.

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

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85 and able to give informed consent. All Parkinson's disease patients included had previously had 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, less than 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 MoCA and 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, Wedel, Germany*) (Hummel *et al.*, 1997). Motor disability was evaluated using the MDS-UPDRS Part III (Goetz *et al.*, 2007) while the patients were receiving medication.

MRI

In all subjects, a high resolution 3D T1 weighted sequence was performed using a 32 channel head coil on a standard clinical 3T MAGNETOM Trio system *(Siemens Healthcare, Erlangen, Germany)* using a 3D T1 MPRAGE with 176 slices, 1 x 1 x 1 voxel size, FOV 256 mm, TE=4.58 ms, TR=2420 ms, TI=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, Knoxville, TN, USA)* (Heiss *et al.*, 2004). Subjects received an intravenous injection of 300 to 370 MBq 18F-AV-1451. A transmission scan from 74 to 80 minutes and an emission scan from 80 to 120 minutes post-injection in list-mode were performed. The PET data was binned into 8 frames of 5 minutes 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 modeling (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.

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PET Analysis

PET-data were analyzed using PMOD v. 3.608 *(PMOD, Zürich, Switzerland)*. In brief, PMOD's built-in tool PNEURO segments white/gray matter in the anatomical T1 weighted MR sequence and transforms it into MNI stereotaxic space where it defines anatomical VOIs using Hammer's probabilistic atlas (Hammers *et al.*, 2003). The VOIs were then transformed back into subject PET space, after co-registration.

For this study, we enlarged the atlas SN VOIs to encompass the peduncles (**Fig. 2 A**). This method is advantageous when investigating very small anatomical structures, as it is less sensitive than smaller SN VOIs to partial volume effects and patient head movement during the scan. It was assumed that only two tissue types were present in the SN VOI, a specific signal from neuromelanin and non-specific background signal estimated using a cerebellar cortex reference region. This assumption is supported by prior 18F-AV-1451 autoradiographical results (Marquié *et al.*, 2015). The reference VOI 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 VOIs were visually inspected in PET and MR space and adjusted manually to ensure that the entire nigral signal was included. No post-filtering of the PET data was used in the VOI analyses.

Average VOI activity and size were extracted for each subject. For the right and left SN VOIs, we calculated specific V_d, 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 SN and reference VOI, and V_{SN} the volume of the SN VOI. We compared left, right, and total (left+right) SN V_d values between Parkinson's disease patients and controls and also the minimum SN V_d values (left or right). In the Parkinson's disease group, we also defined ipsi- and contralateral SN V_d values with reference to the predominant side of motor symptoms.

Basal ganglia uptake of 18F-AV-1451 was calculated as 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 VOI. Simple SUVR calculations were applied to the basal ganglia data, since the VOIs were much larger and head motion artefacts and partial

volume effects were not a major concern. The caudate nucleus was omitted due to its close proximity to the choroid plexus, which showed high activity.

A voxel-based analysis of the nigral AV1451 signal was performed using statistical parametric mapping (SPM12). The cerebellum-normalized SUVr PET volumes were co-registered by affine and subsequent nonlinear deformation to an atlas brain in MNI space. A 4 mm isotropic Gaussian filter was applied to the PET data prior to analysis, and a linear contrast was defined to reveal between-group difference in mesencephalic AV1451 uptake.

DaT SPECT

Twelve patients had had DaT SPECT at our imaging facility using previously described methodology (Borghammer *et al.*, 2014). Subjects were scanned three hours post i.v. injection of 150-180 MBq ^{123}I-FP-CIT on a Siemens Symbia T16 SPECT/CT camera (*Siemens AG, Erlangen, Germany*) with a low-energy high-resolution collimator, 128 x 128 matrix, 64 steps of 35 sec. 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 analyzed with Hermes BRASS, automatically defining VOIs 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 the 12 patients had their DaT SPECT and 18F-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 18F-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 so not used for correlations in the present study.

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Statistical analysis

Statistic analyses were performed in Stata IC 13 *(StataCorp LP, Texas)*. 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 less than 0.05 were considered statistically significant. Linear regression was used for interrogating

correlations, followed by model verification by diagnostic plots of residuals. As this was an exploratory study, we did not perform corrections for multiple comparisons.

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Results

Autoradiography

Fig. 1 shows the autoradiography results. We saw no 18F-AV-1451 binding in the nigra of rats or the pig. In contrast, strong 18F-AV-1451 binding was seen in the retinal pigment epithelium of a pig eye, which was partially displaceable by 20 µM of unlabeled 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 18F-AV-1451 images are shown in **Fig. 2.** Many of the Parkinson's disease patients displayed visually apparent decreased 18F-AV-1451 signal in the midbrain. The Parkinson's disease patients displayed a 31% decrease in minimum SN V_d compared with controls (p=0.008), but there was an overlap of the individual ranges (**Fig. 3 A**). The Parkinson's disease patients also demonstrated significant average decreases in the left (p = 0.003), right (p = 0.01), and total (p=0.004, 30% decrease, 95% CI [10%, 50%]) SN V_d values. The sub-regional VOI analysis of medial/lateral SN showed a 33.1% (95% CI [11.2%, 54.9%]; p=0.004) decrease in the lateral SN and 25.7% (CI [4.9%, 46.6%]; p=0.02) decrease in the medial SN of Parkinson patients. The voxel-based analysis localised the most significant decrease to the lateral part of the left nigra and more central part of the right nigra (p<0.001, uncorrected; **Fig. 2C**). Comparing left and right SN V_d in controls, the average left SN 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 SN V_d would show an inverse relationship in the Parkinson's disease patients. An inverse correlation with UPDRS-III score was seen, but the finding did not reach significance (**Fig. 3 B**; 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

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dominant side and contralateral SN V_d (p=0.05). Indeed, the ipsilateral SN V_d values were on average lower compared to the contralateral side.

There was no correlation between age and minimum SN V_d among controls (linear regression, p = 0.78) or patients (p = 0.92).

DaT SPECT correlation.

In the 12 Parkinson's disease patients 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 18F-AV-1451 SN V_d was decreased to 66.5% (95% CI [43.8%, 89.3%]) (**Fig. 4 A**).

There was no correlation between striatal DaT signal and time since diagnosis (**Fig. 4 B**, r^2 = 0.16, slope -0.92 %-points/year, 95% CI [-2.4, 0.5], p = 0.19). There was also no correlation between total SN 18F-AV-1451 V_d and time since diagnosis (r^2 = 0.013, slope -0.84 %-points/year, 95% CI [-4.9, 3.2], p = 0.66).

Basal ganglia.

The majority of study subjects showed increased 18F-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. 6 B**). There was however no significant difference in the slopes between Parkinson's disease and controls (p = 0.11).

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 = 0.096).

Discussion

To our knowledge, this is the first radiotracer imaging study to measure neuromelanin concentration in the SN of patients with Parkinson's disease. Initially, we performed 18F-AV-1451 phosphor screen autoradiography in rat and pig brain, since 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

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human SN (Marquié *et al.*, 2015). We saw strong 18F-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 18F-AV-1451 binds to neuromelanin in human catecholaminergic neurons.

Our PET results support the suggestion that 18F-AV-1451 PET provides an *in vivo* marker of neuromelanin in the SN. The group of Parkinson's disease patients displayed a ~30% reduction in the nigral 18F-AV-1451 signal compared to age-matched controls. Overlap was seen in PD and control 18F-AV-1451 nigral signal ranges, which limits the utility of 18F-AV-1451 as a primary diagnostic tool. A large number of post-mortem studies have investigated the cell loss in mesencephalic regions of Parkinson's disease patients. The SN 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 Parkinson's disease patients with extended disease duration. However, at least three studies estimated that the symptomatic threshold of dopaminergic neuron loss in the nigral pars compacta is approximately 30% (Fearnley and Lees, 1991; Greffard *et al.*, 2006; Ma *et al.*, 1997). 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 (Damier *et al.*, 1999; German *et al.*, 1989; Hirsch *et al.*, 1988; Kastner *et al.*, 1992).

Thus, we believe that our finding of a 30% reduction in the total midbrain 18F-AV-1451 signal is consistent with the post-mortem literature. First, our midbrain 18F-AV-1451 measurements included both the ventral tegmental area and the SN 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 & Yahr stage 2 in the "on" state. In our sub-regional analysis, we detected an average 33.1% decrease in the lateral and 25.7% decrease in the medial substantia nigra. Although the medial vs. 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 (Kordower *et al.*, 2013; Ma *et al.*, 1997). Recently, Dijkstra and colleagues investigated the relationship between Braak alpha-synuclein stages of the brain and neuron loss in the nigra (Braak *et al.*, 2003;

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Dijkstra *et al.*, 2014). 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 and colleagues reported similar findings (Milber *et al.*, 2012), 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 Parkinson's disease patients, 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, since 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 Parkinson's disease patients the mean content of neuromelanin in surviving neurons of the SN 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 18F-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 (Kordower *et al.*, 2013; Ma *et al.*, 1997; Pakkenberg *et al.*, 1991). 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 the 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 alpha-synuclein stage, at least if incidental Lewy body cases are also included (Dijkstra *et al.*, 2014; Milber *et al.*, 2012).

In our data set, we saw no significant inverse correlation between UPDRS motor scores and nigral 18F-AV-1451 signal (**Fig. 3 B**). 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

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severity. It will be important for future 18F-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 18F-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 123I-FP-CIT signal compared to that of nigral 18F-AV-1451 signal (**Fig. 4**). The mean striatal DaT value in the patients was ~55% of reference mean. Thus, our SPECT and PET findings 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 SN (reviewed in Cheng *et al.*, 2010). We did not see a significant correlation between the nigral 18F-AV-1451 and striatal 123I-FP-CIT values in the patient group. One explanation for this lack of correlation could be the unavoidable inclusion of the VTA in the midbrain PET VOIs. 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, very 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 Parkinson's disease patients (Kordower *et al.*, 2013). The authors did not report a DaT vs nigral neuron correlation, and it seems unlikely that such a correlation was present in their data, since the putaminal DaT loss at 5 to 27 years of disease duration displayed very 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 Parkinson's disease patients and control subjects displayed visible 18F-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. 6 B**). 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 18F-AV-1451 binding constitutes

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additional binding to neuromelanin, but further studies are needed to confirm this hypothesis. The primary application of 18F-AV-1451 PET is to measure the presence of pathological paired helical filament tau protein, so our finding of age-dependent signal increases in subcortical regions is important for the correct interpretation of 18F-AV-1451 in the context of tau protein imaging.

Several studies have suggested that T1-weighted fast spin echo MRI sequences or magnetization transfer ratios may be able to estimate the neuromelanin content of the nigra (Ohtsuka *et al.*, 2013; Reimão *et al.*, 2015; Sasaki *et al.*, 2006; Schwarz *et al.*, 2011). 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 MRI studies detected a 35% increase in the free-water signal of the substantia nigra in PD patients (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 PD patients is comparable to our current data.

A very recent study compared the neuromelanin-sensitive T1-weigthed MRI signal in the nigra to striatal FP-CIT signal of 23 patients, the majority of whom had PD (n=17) or MSA (n=3) (Kuya *et al.*, 2016). The authors detected significant linear correlations between the nigral MRI volumes and striatal DaT concentration, which is in contrast to our observed lack of correlation between AV-1451 nigral signal and striatal FP-CIT binding. A recent study of MPTP-treated monkeys demonstrated linear associations between nigral cell loss and striatal levels of 11C-carbomethoxy-3beta-(4-fluorophenyl)tropane, but only when the nigral cell loss was <50% (Karimi *et al.*, 2013). At more extreme nigral cell loss levels, the striatal PET signal was very low (~10% of control level), and no correlation was seen between the two measures, suggesting a floor effect at ~50% nigral cell loss. Our PD group was relatively early stage and did not include patients with severely pathological DaT scans, which may explain why we did not see signs of a floor effect. In contrast, the paper by Kuya et al. included several patients with very low striatal DaT signal, and they also did not see a floor effect.

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 slabs of mesencephalon (Sasaki *et al.*, 2006). To our knowledge, no studies using neuromelanin-sensitive MRI sequences have compared the SN signal in species with and without neuromelanin, which

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would be a useful way to validate the specificity of the MRI sequences. From this point of view, 18F-AV-1451 PET is at present a better validated neuromelanin marker, since very strong signal is seen in the human SN, and in pigmented retinal and skin structures, whereas no signal is seen in rat and pig SN. 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. In addition, Bohnen et al. previously showed that 11C-DTBZ can detect the decrease of vesicular monoamine transporter density in the nigra of PD patients, thus providing a marker of the functional integrity of nigral dopaminergic neurons (Bohnen *et al.*, 2006). Future multi-tracer PET studies utilizing 11C-DTBZ and AV-1451 may potentially improve our understanding of nigral degeneration in PD.

In this paper we do not report on the ability of 18F-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 vs. 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). Significant lag time was present between SPECT and PET scans in two 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 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 probably cannot be explained entirely by the mentioned factors. An additional potential limitation is that we evaluated the motor symptoms of our Parkinson's disease cases in the medicated condition, which could have obscured a true association between the nigral 18F-AV-1451 signal and motor symptoms. 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 PD patients was 68 years, so we cannot conclude whether similar decreases in nigral AV1451 signal is seen in patients with younger onset of PD.

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In summary, we have demonstrated that 18F-AV-1451 PET may provide a marker of loss of pigmented neurons in the SN of Parkinson's disease patients. As a group, the Parkinson's disease patients 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 18F-AV-1451 signal loss in Parkinson's disease patients, and could establish 18F-AV-1451 PET as a potential biomarker for neuroprotective drug trials.

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Tables

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*	6.6 ± 3.8
UPDRS part III	-	23.9 ± 12.6**
Hoehn & 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. *: N=11. **: N=16.

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Table 2. PET and SPECT results.

	Control		Parkinson's disease	
N	16		17	
Basal ganglia (SUVr)	1.44 ± 0.13		1.36 ± 0.15	
SN 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	
	L	**R**	**L**	**R**
Total	0.81 ± 0.25	0.72 ± 0.24	0.56 ± 0.19	0.51 ± 0.19
Medial	0.47 ± 0.15	0.42 ± 0.12	0.35 ± 0.14	0.31 ± 0.13
Lateral	0.30 ± 0.10	0.26 ± 0.12	0.19 ± 0.07	0.18 ± 0.07
	L	**R**	**L**	**R**
DaT binding ratio*	1.90 ± 0.35	1.84 ± 0.29	1.00 ± 0.17	1.05 ± 0.21

Mean ± SD. Total SN (left+right). Minimum SN (left or right). Ipsilateral and contralateral values are given with reference to predominant symptom side. SN: Substantia nigra. V_d: Volume of distribution. L/R: Left/Right.
* The normal DaT ratio values were derived from an in-house reference material of 26 healthy controls.

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Figure legends

Figure 1. 18F-AV-1451 autoradiography in pig and rat brain.

Top row depicts 18F-AV-1451 autoradiograms. Middle and bottom rows show Nissl stained sections adjacent to the autoradiograms. Arrows on the insets designate the SN and ventral tegmental area. Strong 18F-AV-1451 binding was seen in the retinal pigment epithelium of the pig eye (**A**). No 18F-AV-1451 binding was seen in the SN of the pig (**B**) or in rats (**C**).

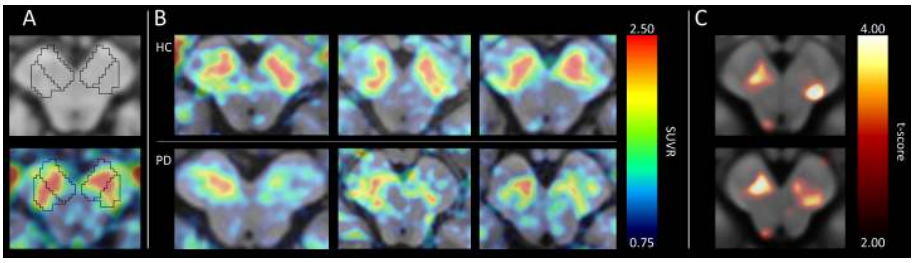

Figure 2. 18F-AV-1451 PET images of the substantia nigra.

A. Automated midbrain VOIs superimposed on MR (upper) and fused PET/MR images (lower) of a 76 year old female control (minimum SN V_d 0.95 mL).

B. Six representative fused PET/MR examples. Healthy controls (HC), upper row from left: 68 year old male (minimum SN V_d 0.61 mL), 73 year old male (minimum SN V_d 0.68 mL), 72 year old male (minimum SN V_d 0.92 mL). Parkinson's disease (PD), lower row from left: 66 year old female minimum (SN V_d 0.35 mL), 57 year old male (minimum SN V_d 0.49 mL), 65 year old male (minimum SN V_d 0.58 mL), all Hoehn & Yahr stage II. Color scale shows SUVr with cerebellum as reference.

C. Statistical t-maps superimposed on MRI at two levels of the mesencephalon. The decreases in the PD group were most significant in the lateral part of the left SN, and central part of the right SN ($p<0.001$, uncorrected).

Figure 3. 18F-AV-1451 PET data in the substantia nigra.

A. Minimum 18F-AV-1451 V_d in the SN of controls and Parkinson's disease (PD) patients. The bars show mean group values and 95% CI, p=0.008.

B. SN 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).

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Fig 4. Substantia nigra 18F-AV-1451 PET versus striatal DaT SPECT.

A. DAT ratios (x-axis) and 18F-AV-1451 nigral V_d values (y-axis) are shown as percent of healthy control mean. Stapled line is line of unity.

B. Blue circles are mean nigral 18F-AV-1451 V_d as percent of control mean. Green squares are mean striatal DaT ratios as percent 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 centers and their DaT values were not comparable to our in-house data.

Figure 5. 18F-AV-1451 uptake in basal ganglia.

Representative examples of low and high basal ganglia 18F-AV-1451 binding. Left: 52 year old male (SUVr 1.27), right: 71 year old female (SUVr 1.53). Both are healthy controls. 6mm Gaussian smooth was applied to the PET images.

Figure 6. Basal ganglia 18F-AV-1451 data.

A. No significant difference was seen in the basal ganglia SUVr values between controls and Parkinson's disease (PD) patients (p = 0.13). 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, p = 0.003). Red diamonds are controls, blue circles are Parkinson's disease.

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