Evaluation of Imidazoline 2 binding sites reflecting astroglia pathology in Parkinson's Disease: An *in vivo* [^{11}C]BU99008 PET study

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Abbreviations: I_2BS: Imidazoline 2 binding sites; PET: Positron Emission Tomography; MRI: Magnetic Resonance Imaging; V_T: Volume of Distribution; LED: Levodopa Equivalent Dose

ABSTRACT

Astroglia are multifunctional cells which regulate neuroinflammation and maintain homeostasis within the brain. Astroglial α-synuclein-positive cytoplasmic accumulations have been shown *postmortem* in patients with Parkinson's disease and therefore astroglia may play an important role in the initiation and progression of Parkinson's disease. The imidazoline 2 binding sites are expressed on activated astroglia in the cortex, hippocampus, basal ganglia and brainstem; therefore, by measuring imidazoline 2 binding site levels we can indirectly evaluate astrogliosis in Parkinson's disease patients. Here, we aimed to evaluate the role of astroglia activation *in vivo* in patients with Parkinson's disease using [^{11}C]BU99008 PET, a novel radioligand with high specificity and selectivity for imidazoline 2 binding sites. Twenty-two patients with Parkinson's disease and 14 healthy controls underwent 3T MRI and a 120 min [^{11}C]BU99008 PET scan with volume of distribution (V_T) estimated using a two-tissue compartmental model with a metabolite corrected arterial plasma input function. Parkinson's disease patients were stratified into early (n=8) and moderate/advanced (n=14) groups according to disease stage. In early Parkinson's disease, increased [^{11}C]BU99008 V_T uptake was observed in frontal (P=0.022), temporal (P=0.02), parietal (P=0.026) and occipital (P=0.047) cortical regions compared with healthy controls. The greatest [^{11}C]BU99008 V_T increase in early Parkinson's patients was observed in the brainstem (52%; P=0.018). In moderate/advanced Parkinson's patients, loss of [^{11}C]BU99008 V_T was observed across frontal (P=0.002), temporal (P<0.001), parietal (P=0.039), occipital (P=0.024), and insula (P<0.001) cortices; and in the subcortical regions of caudate (P<0.001), putamen (P<0.001) and thalamus

(*P*<0.001); and in the brainstem (*P*=0.018) compared with healthy controls. In Parkinson's patients, loss of [^{11}C]BU99008 V_T in cortical regions, striatum, thalamus and brainstem correlated with longer disease duration (*P*<0.05) and higher disease burden scores, measured with Movement Disorder Society Unified Parkinson's Disease Rating Scale (*P*<0.05). In the subgroup of moderate/advanced Parkinson's patients, loss of [^{11}C]BU99008 V_T in the frontal (r=0.79; *P*=0.001), temporal (r=0.74; *P*=0.002) and parietal (r=0.89; *P*<0.001) cortex correlated with global cognitive impairment. This study demonstrates *in vivo* the role of astroglia in the initiation and progression of Parkinson's disease. Reactive astroglia observed early in Parkinson's disease could reflect a neuroprotective compensatory mechanisms and pro-inflammatory upregulation in response to α-synuclein accumulation. However, as the disease progresses and significant neurodegeneration occurs, astroglia lose their reactive function and such loss in the cortex has clinical relevance in the development of cognitive impairment.

INTRODUCTION

Astroglia play a pivotal role in synaptic plasticity, neuroprotection and regulation of the blood-brain barrier; as well as maintaining metabolic and ionic homeostasis and providing trophic support for surrounding neurons (Maragakis and Rothstein, 2001; Volterra and Meldolesi, 2005; Rodriguez *et al.*, 2009). Although astroglia are not strictly immune cells, they contribute together with microglia to the modulation of the innate and chronic immune brain response (Maragakis and Rothstein, 2006). When activated, astroglia change their morphology from a so-called surveying state to an activated state by enlarging their cell body and thickening their processes. Astroglial activation is characterized by the upregulation of intermediate filament proteins, such as the glial fibrillary acidic protein. Imidazoline 2 binding sites (I_2BS), localised in the outer membrane of mitochondria in glial cells (Parini *et al.*, 1996), have been shown to regulate the expression of glial fibrillary acidic protein (Sastre and Garcia-Sevilla, 1993). Preclinical studies showed rats treated with specific imidazoline antagonists induced parallel increases in I_2BS and glial fibrillary acidic protein (Garcia-Sevilla *et al.*, 1999); indicating a direct physiological role for I_2BS in the regulation of glial fibrillary acidic protein levels, and a link between I_2BS and neuroinflammation (Head, 1998; Head and Mayorov, 2006). I_2BS are expressed on reactive astroglia in the cortex, hippocampus, basal ganglia and brainstem (De Vos *et al.*, 1991; De Vos *et al.*, 1994). Therefore, the quantification of I_2BS could be a useful indirect marker of reactive astroglia *in vivo*. Astroglia exert neuroprotective functions by clearing excess glutamate from the extracellular space *via* the glutamate transporter-1 to protect against excitotoxicity (Maragakis and Rothstein, 2001), suggesting that astroglia dysfunction and loss of this neuroprotective function may be particularly relevant to neuronal loss in neurodegeneration disorders.

In Parkinson's disease, neuroinflammation in the substantia nigra pars compacta, including the presence of reactive astrogliosis was originally hypothesised as a downstream response arising as a consequence of dopaminergic neuronal death (Miklossy *et al.*, 2006; Koprich *et al.*, 2008). Accumulating evidence suggests that activated astroglia may play a key role early in the pathophysiology of Parkinson's disease. Elevated levels of glial fibrillary acidic protein, which is regulated by I_2BS, has been reported in the cerebrospinal fluid of early Parkinson's patients suggesting the presence of astrogliosis early in the course of disease pathology (Sussmuth *et al.*, 2010). It has been suggested that altered α-synuclein, released by axon terminals, is taken up by astroglial cells surrounding the synapses (Braak *et al.*, 2007), supporting the hypothesis of neuron-to-astroglia propagation of α-synuclein (Lee *et al.*, 2010). Moreover, a recent study provides evidence for the transmission of pathogenic α-synuclein from astrocytes to neurons (di Domenico *et al.*, 2019). Thus, the accumulation of α-synuclein in astroglial cells, prior to neuronal loss in the substantia nigra pars compacta, may function as a key factor in the initiation and progression of Parkinson's disease (Halliday and Stevens, 2011). The upregulation of reactive astroglia, expressing I_2BS, could be an early neuroprotective phenomenon, in response in the accumulation of α-synuclein, in the pathophysiology of Parkinson's disease. However, the role of reactive astroglia in Parkinson's disease has not been in studied *in vivo.*

The novel PET radioligand [^{11}C]BU99008 has high specificity and selectivity for I_2BS, which is expressed on reactive astroglia (Tyacke *et al.*, 2012; Parker *et al.*, 2014; Tyacke *et al.*, 2018); therefore, providing an invaluable tool to elucidate the role of astroglial activation in Parkinson's disease pathophysiology and its relationship with disease stage and burden, and symptom severity. Here, we investigated the role of reactive astroglia *in vivo* in early and moderate/advanced patients with Parkinson's disease using [^{11}C]BU99008 PET imaging.

MATERIALS AND METHODS

Participants

Twenty-two patients diagnosed with idiopathic Parkinson's disease according to the UK Brain Bank criteria were recruited from specialist Movement Disorders clinics at King's College Hospital NHS Foundation Trust, London, UK (Table 1). Fourteen healthy individuals with no history of neurological or psychiatric disorders served as the control group. Examples of specific exclusion criteria for all participants included (1) alcohol or drug dependency or abuse; (2) contraindication for PET or MRI scanning; (3) use of medications with known actions on I_2BS (e.g. idaxozan, efaroxan, yohimbine, atomoxetine, atipamezole, mianserin, mirtazapine, clonidine, guanfacine, guanabenz, guanethidine, xylazine, tizanidine, tedetomidine, methyldopa, fadolmidine, dexmedetomidine); and (4) a history of other neurological or psychiatric disorders. Imidazoline-2 receptors have been implicated to play a role in neuropathic pain, with studies showing Imidazoline-2 receptor ligands increasing the analgesic effects of opioids in chronic and persistent pain (Jett *et al.*, 1999; Li and Zhang, 2011; Li *et al.*, 2014; Li, 2017). Therefore, participants with the presence of neuropathic pain were excluded from the study. All participants had adequate visual and auditory acuity to complete the psychological testing. Parkinson's disease patients who were receiving Parkinson's drugs stopped any dopaminergic medications 24 hours before the scans, and had imaging in the practically defined OFF state, and clinical assessments in both OFF and ON states. All participants screened successfully to undertake PET and MRI scanning under scanning safety criteria (http://www.mrisafety.com; https://www.gov.uk/government/publications/arsac-notes-for-guidance). The study was approved by the institutional review boards and the research ethics committee. Permission to use radioactive substances was obtained by the Radioactive Substances Advisory Committee,

Department of Health and Social Care, United Kingdom. Written informed consent was obtained from all study participants in accordance with the Declaration of Helsinki.

Clinical assessments and Stratification

Global Parkinson's disease clinical burden was assessed with the Movement Disorders Society (MDS) Unified Parkinson's Disease Rating Scale (MDS-UPDRS), including MDS-UPDRS-III for the examination of motor symptoms, MDS-UPDRS-II for motor experiences of daily living and MDS-UPDRS-I for non-motor experiences of daily living. Disease severity was also classed with the Hoehn and Yahr (H&Y) scale. Daily dopamine agonist equivalent dose (LED_{DA}) and daily levodopa equivalent dose ($LED_{L\text{-}DOPA}$) unit calculations were based on theoretical equivalence to levodopa as described previously (Politis *et al.*, 2010).

Parkinson's disease patients were stratified according to disease stage into early (n=8) and moderate/advanced (n=14) Parkinson's disease subgroups in order to investigate I_2BS level in different clinical stages of Parkinson's disease (Table 1). Early Parkinson's patients had less than 5 years of disease duration, average MDS-UPDRS-III OFF of 13.63 (±5.10), average H&Y OFF of 1.25 (±0.71). Five early Parkinson's patients were naïve to treatment for Parkinson's symptoms *(de novo)* and 3 early Parkinson's patients were taking dopamine agonists for less than 3 months but not on levodopa treatment, and had mean daily LED_{DA} of 53.38 mg (±84.14). Moderate/advanced Parkinson's patients had more than 6 years of disease duration, average MDS-UPDRS-III OFF of 44.93 (±18.49), average H&Y OFF of 2.50 (±1.40), were more than two years on levodopa treatment, and had mean daily LED_{TOTAL} of 418.57 mg (±451.63).

Global cognitive function was assessed with the Mini Mental Status Examination (MMSE) and the Montreal Cognitive Assessment (MoCA). Olfactory function was assessed with the University of Pennsylvania Smell Identification Test (UPSIT). Total non-motor symptom burden was assessed with the Non-motor Symptom Scale (NMSS). Depression levels were assessed with the Beck Depression Inventory-II (BDI-II) and autonomic symptoms with the Scales for Outcomes in Parkinson's disease–Autonomic (SCOPA-AUT). Pain was assessed with the King's Pain Scale (Table 1).

Imaging assessments

Participants underwent PET and MR imaging, which was performed at Invicro LLC, London, UK. Participants were scanned on a Siemens Biograph Hi-Rez 6 PET-CT scanner (Erlangen, Germany). A mean dose of 316.56 MBq (±13.98) [^{11}C]BU99008 was administered intravenously as a slow bolus injection over 20s.

Dynamic emission data were acquired continuously for 120 minutes following the injection of [^{11}C]BU99008. The dynamic images were reconstructed into 26 frames (8 x 15 s, 3 x 60 s, 5 x 120 s, 5 x 300 s, and 5 x 600 s), using a filtered back projection algorithm (direct inversion Fourier transform) with a 128 matrix, zoom of 2.6 producing images with isotropic voxel size of 2 x 2 x 2 mm^3, and smoothed with a transaxial Gaussian filter of 5 mm. Blood sampling was performed for [^{11}C]BU99008 through an arterial line inserted in the radial artery to generate arterial plasma input data. For the initial 15 minutes radioactivity levels in blood was continuously measured through an automatic blood sampling system at 5ml/min, followed by samples at 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 110 and 120 min during the scan. Plasma samples and the fraction of unchanged radioligand was determined according to previously described procedures (Tyacke *et al.*, 2018). There were no differences in the plasma free

fraction of [^{11}C]BU99008 between the groups of Parkinson's disease patients and healthy controls.

MRI scans were acquired with a 32-channel head coil on a Siemens Magnetom MAGNETOM TrioTim syngo MR B17 (Erlangen, Germany), 3T MRI scanner. MRI acquisition included a T1-weighted Magnetization Prepared Rapid Gradient Echo sequence [MPRAGE; time repetition = 2300 ms; time echo = 2.98 ms, flip angle of 9°, time to inversion = 900 ms; matrix = 240 x 256 x 160; voxel size = 1.0 mm x 1.0 mm x 1.0 mm; slice thickness 1.0 mm].

Imaging data analysis

PET data analysis

The Molecular Imaging and Kinetic Analysis Toolbox software package (MIAKAT™: www.miakat.org), implemented in MATLAB® (The Mathworks, Natick, MA, USA) was used to carry out image processing and kinetic modelling. MIAKAT™ combines in-house code with wrappers for FMRIB Software Library (FSL, http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/) and Statistical Parametric Mapping (SPM, http://www.fil.ion.ucl.ac.uk/spm/) commands in order to provide state-of-the-art functionality within a coherent analysis framework. Individual PET frames were corrected for head motion using frame-by-frame rigid registration using frame 16, with high signal-to-noise ratio, as reference. The MIAKAT™ processing pipeline allows for modelling of input function data, regional sampling of PET data and creation of motion-corrected time-activity-curves. Parent plasma input functions were derived from the arterial blood measurements, the whole-blood data corrected for plasma and metabolite fractions using sigmoid models, and interpolated with a triexponential function (Tyacke *et al.*, 2018). The arterial blood samples were used to determine a plasm input function which enabled modelling of the time-activity-curves and estimation of the total volume of distribution (V_T) using the

two-tissue compartment model (2TCM), with fixed 5% blood volume, which has been shown as the most appropriate and reliable model for [^{11}C]BU99008.

Partial Volume Correction

To account for atrophy and volumetric loss on [^{11}C]BU99008 V_T measures regions of interest were segmented with grey matter mask, generated by segmenting subjects' MRI, to extract [^{11}C]BU99008 V_T from regions of interest within the grey matter. Furthermore, partial volume correction was performed using on 3-compartment (grey matter, white matter and CSF) segmentation method (Meltzer *et al.*, 1990; Muller-Gartner *et al.*, 1992). [^{11}C]BU99008 V_T values were considered with and without partial volume correction.

Region-of-interest analysis

The multi-atlas propagation with enhanced registration (MAPER) was used to define region of interests (Heckemann *et al.*, 2010). MRI scans were automatically segmented using the MAPER approach into 95 anatomic regions. This robust technique improves the quality of multi-atlas based automatic whole-brain segmentations (Heckemann *et al.*, 2010), and is applicable even to subjects with significant cortical atrophy and ventriculomegaly. The output object maps were visually checked independently by two reviewers to ensure accurate segmentation based on each subjects' structural MRI. Motion-corrected time-activity curves were generated for the regional quantification of [^{11}C]BU99008 PET data.

MRI volumetric FreeSurfer analysis

FreeSurfer image analysis suite was used to derive measures of cortical thickness and deep grey matter nuclei volume. Cortical thickness was measured as the distance from the grey and white matter boundary to the corresponding pial surface. Reconstructed data sets were visually

inspected to ensure accuracy of registration, skull stripping, segmentation, and cortical surface reconstruction. Subcortical structure volumes were derived by automated procedures, which automatically assign a neuroanatomical label to each voxel in an MRI volume based on probabilistic information automatically estimated from a manually labelled training set (Fischl *et al.*, 2002). All individual nuclei volumes were normalised for total intracranial volume automatically generated by FreeSufer (Malone *et al.*, 2015).

Voxel-based morphometry

Images were segmented into grey matter, white matter and CSF tissue classes using the statistical parametric mapping (SPM) version 12 software package (Wellcome Department of Imaging Neuroscience, London, UK). Grey and white matter images were normalized to a grey and white matter population template, generated from the complete image set using the diffeomorphic anatomical registration using exponentiated lie-algebra (DARTEL) registration method (Ashburner, 2007). This non-linear warping technique minimizes between-subject structural variations. All images were checked following spatial normalization to ensure registration accuracy. The final voxel resolution was 1·0 mm x 1·0 mm x 1·0 mm. Spatially normalized images were modulated by the Jacobian determinants so that intensities represent the amount of deformation needed to normalize the images, and then smoothed with an 8 mm full-width at half-maximum Gaussian kernel. Voxel-based multiple regression analysis (based on the general linear model) was carried out using SPM12 with voxel-wise grey and white matter volumes as the dependent variables. Age and gender were added as nuisance covariates. The threshold for statistical significance was set at $P<0{\cdot}05$ after family wise error (FWE) correction for multiple comparisons.

Statistical analysis

Statistical analysis and graph illustration were performed with SPSS (version 20 Chicago, Illinois, USA) and GraphPad Prism (version 6.0c) for MAC OS X, respectively. For all variables, variance homogeneity and Gaussianity were tested with Bartlett and Kolmogorov-Smirnov tests. We proceeded with parametric tests as our imaging and clinical data were normally distributed. Multivariate analysis of variance (MANOVA) was used to assess group differences in clinical measures and multivariate analysis of covariance (MANCOVA) with age, gender, LED_{DA}, $LED_{L\text{-}DOPA}$, BDI-II and King's pain scale as covariates was used to assess group differences in [^{11}C]BU99008 V_T values. If the overall multivariate test was significant, *P* values for each variable were calculated following Bonferroni's multiple comparisons test. We interrogated correlations between [^{11}C]BU99008 V_T values and clinical data using a covariate-adjusted spearman's rank correlation, with age, gender, LED_{DA}, $LED_{L\text{-}DOPA}$, BDI-II and King's pain scale as covariates. We set the false discovery rate cut-off at 0.05. All data are presented as mean±SD, and the level α was set for all comparisons at $P<0.05$, Bonferroni corrected.

RESULTS

Clinical assessments

There was no difference in age between early Parkinson's disease patients ($P=0.64$) and healthy controls, and between early Parkinson's patients and moderate/advanced Parkinson's patients ($P=0.094$). However, the moderate/advanced Parkinson's disease patients were older compared with the healthy controls ($P=0.036$); therefore, age was included as covariate in all the statistical analysis. Compared with early Parkinson's disease patients, moderate/advanced Parkinson's disease patients had longer disease duration ($P<0.001$), higher total daily LED

intake (*P*=0.036), greater burden of motor symptoms (MDS-UPDRS-III OFF: *P*<0.0001; H&Y OFF: *P*=0.012) and greater total disease burden (MDS-UPDRS Total OFF: *P*=0.001; Table 1).

The moderate/advanced Parkinson's disease patients had greater olfactory dysfunction compared with the cohort of early Parkinson's disease patients (UPSIT: *P*=0.003); while global burden of non-motor symptoms (NMSS Total: *P*=0.54), depression levels (BDI-II: *P*=0.23), pain (King's Pain Scale: *P*=0.13), autonomic dysfunction (SCOPA-AUT: *P*=0.21) and global cognitive function (MMSE: *P*=0.92; MoCA: *P*=0.24) were not significantly different between early and moderate/advanced Parkinson's disease patients (Table 1).

Region-of-interest based analysis

Multivariate analysis across the three groups revealed significant differences in cortical [^{11}C]BU99008 V_T uptake ($F(10,42)=14.85$, $P<0.001$, Wilks' $\Lambda=0.049$) after covarying for age, gender, LED_{DA}, $LED_{L\text{-}DOPA}$, BDI-II and King's pain scale. Between the three groups, significant differences in [^{11}C]BU99008 V_T were observed in the frontal ($F(2,25)=17.81$, $P<0.001$), parietal ($F(2,25)=12.80$, $P<0.001$), temporal ($F(2,25)=20.37$, $P<0.001$), occipital ($F(2,25)=11.73$, $P<0.001$), and insula ($F(2,25)=19.25$, $P<0.001$), cortices after covarying for age, gender, LED_{DA}, $LED_{L\text{-}DOPA}$, BDI-II and King's pain scale.

[^{11}C]BU99008 V_T findings in early Parkinson's disease patients

In early Parkinson's patients, increased [^{11}C]BU99008 V_T uptake (following partial volume correction) was observed in frontal cortical regions, including the straight frontal gyrus (*P*=0.014), precentral gyrus (*P*=0.023, uncorrected), inferior (*P*=0.018) and superior (*P*=0.05) frontal gyrus, medial (*P*=0.017) and lateral (*P*=0.10), anterior (*P*=0.024), and posterior (*P*=0.025, uncorrected) orbital gyrus; temporal cortical regions, including the superior

temporal gyrus middle part (*P*=0.031, uncorrected), middle and inferior temporal gyrus (*P*=0.037, uncorrected), posterior temporal lobe (*P*=0.045, uncorrected), anterior temporal lobe lateral part (*P*=0.025, uncorrected) and the anterior temporal lobe medial part (*P*=0.045, uncorrected); parietal cortical regions including the supramarginal gyrus (*P*=0.039), postcentral gyrus (*P*=0.016), superior parietal gyrus (*P*=0.014) and the angular gyrus (*P*=0.02, uncorrected); and occipital cortical regions including the cuneus cortex (*P*=0.029), the lingual gyrus (*P*=0.05, uncorrected) and the remaining occipital lobe (*P*=0.029, uncorrected; Figures 1 & 2). Early Parkinson's disease patients showed also increased [^{11}C]BU99008 V_T uptake in the anterior cingulate gyrus (*P*=0.036) and in the brainstem (*P*=0.018). No differences were found in the insula cortex or in subcortical regions in early Parkinson's disease compared with healthy controls. The greatest increase in [^{11}C]BU99008 V_T was observed in the brainstem showing approximately 52% increase in early Parkinson's disease compared with healthy controls (Table 2).

[^{11}C]BU99008 V_T findings in moderate/advanced Parkinson's disease patients

In moderate/advanced Parkinson's disease patients, global decrease of [^{11}C]BU99008 V_T uptake was observed across frontal (*P*=0.002), temporal (*P*<0.001), parietal (*P*=0.039), occipital (*P*=0.024), insular (*P*<0.001) cortices and the anterior (*P*=0.008) and posterior (*P*=0.003) cingulate compared with healthy controls (Figures 1 & 2). Within subcortical regions, loss of [^{11}C]BU99008 V_T was observed in the caudate (*P*<0.001), putamen (*P*<0.001), thalamus (*P*<0.001), and in the brainstem (*P*=0.018; Table 2).

We also evaluated [^{11}C]BU99008 V_T data without partial volume error correction to identity any differences in the results. Without partial volume correction, [^{11}C]BU99008 V_T was increased in the frontal (*P*=0.035), temporal (*P*=0.048) and parietal (P=0.015) cortex and in the brainstem (*P*=0.045) in early Parkinson's disease patients; and decreased in the frontal

(*P*<0.001), temporal (*P*<0.001), parietal (*P*<0.001), insula (*P*<0.001) and occipital (*P*<0.001) cortex and in caudate (*P*<0.001), putamen (*P*<0.001), thalamus (*P*<0.001) and brainstem (*P*<0.001) in moderate/advanced Parkinson's disease patients compared with healthy controls (Table S1). With the exception of the occipital and cingulate cortex which came significant only after partial volume correction, the results without partial volume correction were consistent with findings after partial volume correction (Table 2).

Correlations with clinical data

Lower [^{11}C]BU99008 V_T values in the frontal (r=-0.64; *P*=0.001), temporal (r=-0.96; *P*<0.001), parietal (r=-0.59; *P*=0.007), occipital (r=-0.58; *P*=0.01) and insula (r=-0.60; *P*=0.006) cortices, and in the caudate (r=-0.63; *P*=0.004), putamen (r=-0.62; *P*=0.005), thalamus (r=-0.61; *P*=0.006) and brainstem (r=-0.59; *P*=0.004) correlated with longer disease duration in all Parkinson's patients (Figure 3A). Lower [^{11}C]BU99008 V_T values in the frontal (r=-0.76; *P*<0.001), temporal (r=-0.61; *P*=0.003), parietal (r=-0.55; *P*=0.011), occipital (r=-0.51; *P*=0.026) and insular (r=-0.56; *P*=0.009) cortices, and in the caudate (r=-0.59; *P*=0.006), putamen (r=-0.53; *P*=0.011), thalamus (r=-0.58; *P*=0.007) and brainstem (r=-0.59; *P*=0.004) correlated with higher total disease burden, measured with total MDS-UPDRS (Figure 3B). In the subgroup of moderate/advanced Parkinson's patients, loss of [^{11}C]BU99008 V_T in the frontal (r=0.79; *P*=0.001), temporal (r=0.74; *P*=0.002) and parietal (r=0.87; *P*<0.001) cortices correlated with worse global cognitive scores as assessed with MoCA (Figure 3C).

MRI volumetric analysis

FreeSurfer analysis revealed no difference in cortical thickness between groups of Parkinson's disease patients and healthy controls (Table S2). No differences in subcortical volumes were observed in basal ganglia regions or the hippocampus (Table S3). However, within the brainstem, moderate/advanced Parkinson's disease patients showed significant volumetric loss

(*P*=0.035) and early Parkinson's disease patients showed borderline volumetric loss (*P*=0.053) compared with healthy controls (Table S3). Whole brain voxel-based morphometry between groups of Parkinson's disease patients and healthy controls revealed no volume changes at a voxel level in any brain region. To address potential issues of bias from volume loss, we applied partial volume correction and report all partial volume corrected [^{11}C]BU99008 V_T values.

DISCUSSION

In this study we used non-invasive [^{11}C]BU99008 PET molecular imaging, a marker of I_2BS, to evaluate astroglia pathology *in vivo* in patients with Parkinson's disease. Our findings demonstrate increased expression of I_2BS, reflecting reactive astrogliosis, in cortical areas and the brainstem of early Parkinson's patients. However, in the moderate/advanced Parkinson's cases I_2BS expression was decreased in cortical and subcortical areas, reflecting loss of astroglia function as the disease advances and disease burden increases, and such loss in the cortical areas correlated with worse cognitive scores.

In early Parkinson's patients, we observed increased expression of I_2BS in several frontal, temporal, parietal and occipital gyri, with the highest increases (52%) observed in the brainstem. Our findings are consistent with the hypothesis that reactive astrogliosis occurs in early stage Parkinson's disease, possibly due to neuroprotective compensatory mechanisms and pro-inflammatory upregulation in response to α-synuclein accumulation (Halliday and Stevens, 2011; Barcia *et al.*, 2012). Our findings are in line with preclinical studies which have demonstrated increased expression of interferon-γ receptors on astroglia, as well as tumour necrosis factor (TNF)-α immunoreactivity related to astrogliosis, suggesting that astroglial overactivation trigged by the accumulation of α-synuclein could play a crucial role in the initiation of pathophysiological processes in Parkinson's disease (Halliday and Stevens, 2011;

Barcia *et al.*, 2012). Astroglia containing α-synuclein are thought to be more widespread throughout the brain compared with Lewy bodies (Braak *et al.*, 2007), which would explain the increased levels of I_2BS, reflecting reactive astrogliosis, observed in the present study throughout the cortex and in the brainstem of early Parkinson's patients. Astroglial cells can also activate microglial cells (Gu *et al.*, 2010; Halliday and Stevens, 2011; Schmidt *et al.*, 2011), and *vice versa* with microglial activation inducing astrogliosis (Balasingam *et al.*, 1996; Hanisch, 2002; Rohl *et al.*, 2007). Increased microglial activation has been previously demonstrated *in vivo* with [^{11}C]PK11195, [^{18}F]FEPPA and [^{11}C]DPA713 PET imaging, further supporting the role of neuroinflammation in Parkinson's pathophysiology (Ouchi *et al.*, 2005; Terada *et al.*, 2016; Ghadery *et al.*, 2017; Kang *et al.*, 2018). Such evidence taken together with our findings, suggests that astroglia and microglia are likely key players in the pathophysiology of Parkinson's disease.

In moderate/advanced Parkinson's patients we observed decreased expression of I_2BS in frontal, temporal, parietal, occipital, and insula cortices, in the brainstem and in subcortical regions such as caudate, putamen and thalamus. This was an opposite phenomenon compared with the early Parkinson's cases, and indicated loss of astroglia function as Parkinson's disease progresses. Loss of astroglial function could reflect loss of neuroprotective mechanisms and increased neurotoxic effects associated with the presence of gradually increasing neurodegeneration processes (Maragakis and Rothstein, 2001; Gu *et al.*, 2010). Reactive astroglia display a complex interplay between the neuroprotective and neurotoxic effects. In preclinical studies, the overexpression of the mutant α-synuclein in astroglial cells altered the normal function of astroglia, leading to reduced integrity of the blood-brain-barrier, a decreased homeostasis of extracellular glutamate and a significant loss of dopaminergic neurons in the midbrain (Gu *et al.*, 2010). In *post-mortem* Parkinson's brains, α-synuclein inclusions have

been reported within astroglia (Braak *et al.*, 2007). Following chronic astrogliosis to clear extracellular α-synuclein, astroglia could degenerate and become inactivated. Astroglia also help to protect against excitotoxicity by clearing excess glutamate from the extracellular space *via* the glutamate transporter-1 (Maragakis and Rothstein, 2001). Gu and colleagues (2010) showed that the presence of increasing levels of α-synuclein in reactive astrocytes disrupts the normal functioning of astrocytes which is critical for maintaining the integrity of the blood-brain barrier and homeostasis of extracellular glutamate. Therefore, reduced expression of proinflammatory cytokines, such as Interukein-1α, Interukein-1β, in response to increasing extracellular α-synuclein, in late stages of Parkinson's disease, may not only compromise anti-inflammatory functions of astrocytes, but also leave neurons more vulnerable to toxic insults. Moreover, progressive neurodegeneration has been shown to be related to astrocyte dysfunction and the expression of inflammatory molecules by astrocytes leading to microglial activation (Gu *et al.*, 2010; Lee *et al.*, 2010). It is likely that for astrocytes to function normally, they have to keep low levels of α-synuclein expression. However, astrocytes are less able to detoxify the excess α-synuclein, compared with microglia, due of their lower level of cathepsin D which has been shown to effectively degrade α-synuclein (Wootz *et al.*, 2006; Qiao *et al.*, 2008; Cullen *et al.*, 2009). A recent study provides direct evidence that astrocyte dysfunction can lead to PD-associated neurodegeneration through the impairment of chaperone-mediated autophagy in iPSC-derived astrocytes from PD patients compared with controls astrocytes (di Domenico *et al.*, 2019). Therefore, as Parkinson's disease progresses astroglial dysfunction, accompanied by increasing α-synuclein accumulation, loss of glial neuroprotective functions and increased excitotoxicity, could explain the gradual neuronal loss observed in Parkinson's disease and the increasing disease burden. While there are multiple lines of evidence to support the loss of astroglia function, we cannot fully exclude the possibility that α-synuclein astrocytic inclusions might affect the expression of I_2BS in the astrocytic membranes. Thus, further work

is required to fully elucidate the exact mechanisms underlying the loss of I_2BS, measured with [^{11}C]BU99008 PET.

In line with the concept that astroglial pathology may have relevance to disease burden and clinical implications, our findings show that loss of I_2BS expression correlated with longer disease duration and higher total MDS-UPDRS scores, which measures the global burden of the disease. Furthermore, in moderate/advanced Parkinson's disease, loss of I_2BS expression in cortical regions involved in cognitive function correlated with lower MoCA scores, which captures global cognitive impairment. These findings suggest that as Parkinson's disease progresses, astroglial dysfunction, accompanied by loss of glial neuroprotective functions and increased excitotoxicity, has clinical relevance in the development of cognitive impairment and increasing global disease burden. Further studies are warranted, in a cohort of Parkinson's disease patients with cognitive impairment to fully elucidate the role of I_2BS in cognitive impairment. Pharmacological manipulation of astroglia function could potentially have a therapeutic role in the alleviation of Parkinson's disease symptoms and might slow increasing disease burden. Larger longitudinal studies are required to confirm these findings.

Similarly, to the M1/M2 phenotypes of microglia, astroglia also harbour different isoforms of reactivity (Paolicelli *et al.*, 2011). A1 astroglia known as the neurotoxic phenotype, upregulate many classical complement cascade genes, such as C3, C4, C1r and C1s, which are destructive to synapses (Stevens *et al.*, 2007; Liddelow *et al.*, 2017). In contrast A2 astroglia, which is the neuroprotective phenotype, upregulate many neurotrophic factors, such as CLCF1, LIF and IL-6, which promote the survival and growth of neurons as well as synaptic repair (Zamanian *et al.*, 2012). Astroglial overactivation results in the release of pro-inflammatory cytokines, nitric oxide and reactive oxygen species which can have detrimental effects in chronic

neuroinflammation (Neumann *et al.*, 2002; Deshpande *et al.*, 2005; Mizuno *et al.*, 2005; Zhang *et al.*, 2005; Qian and Flood, 2008; Dean *et al.*, 2010; Lee *et al.*, 2010; Qian *et al.*, 2010). The majority of *post-mortem* studies in Parkinson's brains focus in microgliosis and neuroinflammatory mediators while the literature in astroglia is less extensive. From pathological examinations, an increase in the number of astroglia, as well as in glial fibrillary acidic protein expression, has been observed in Parkinson's disease (Damier *et al.*, 1993; Braak *et al.*, 2007). Recently, the A1 astroglia phenotype was reported in the substantia nigra of Parkinson's brains (Liddelow *et al.*, 2017). Therefore, the shift from an A2 to A1 phenotype could be important for disease progression.

In *post-mortem* Parkinson's disease brains, the density of GFAP-positive astrocytes has been shown to be inversely related to the magnitude of dopaminergic neuronal loss (Damier *et al.*, 1993). This suggests that dopaminergic neurons within areas where astrocytes have lost their function are more prone to degenerate. Furthermore, in Parkinson's disease the severity of substantia nigra pars compacta dopaminergic neuronal loss correlated positively with the number of α-synuclein–positive inclusions within astrocytes in the substantia nigra pars compacta (Wakabayashi *et al.*, 2000). Therefore, increasing accumulation of α-synuclein within astrocytes could subsequently result in loss of astroglia function and eventually neuronal death. While some *post-mortem* studies have reported a mild increase in the number of astrocytes these findings are not consistency observed (Forno *et al.*, 1992; Mirza *et al.*, 2000; Tong *et al.*, 2015). The presence of the A1 astroglia phenotype in the substantia nigra of Parkinson's disease brains suggests that astrocytes detected at *post-mortem* could display an A1 neurotoxic phenotype, with astrocytes shifting from an A2 to A1 phenotype in later stages of the disease (Stevens *et al.*, 2007; Liddelow *et al.*, 2017). While *post-mortem* studies provide important insights into pathological changes present at end stages of the disease, it is difficult

to disentangle from *post-mortem* studies alone if findings of increased I_2BS levels is reflective of massive neuronal death and neurodegeneration occurring at the time of death or if these changes reflect a core disease mechanism in the pathophysiology of Parkinson's disease and the extent to which they contribute to disease progression. This highlights the importance of PET imaging studies, with a specific marker for astroglia activation, which allow the investigation of molecular pathology in vivo in Parkinson's disease patients."

It is important to note that I_2BS are co-expressed with monoamine oxidase type B (Sastre and Garcia-Sevilla, 1993) however the binding site for the I_2BS is distinct from that of monoamine oxidase type B (McDonald *et al.*, 2010). Increased monoamine oxidase type B activity has also been linked with astrogliosis (Oreland and Gottfries, 1986; Ekblom *et al.*, 1993). Specifically, upon brain insult monoamine oxidase type B levels increase through reactive astroglia inducing the expression of several cytokines, including IL-1, IL-6, and TNF-a (Ekblom *et al.*, 1994; Quintana *et al.*, 2005). Taken together, these data suggest that I_2BS are also linked with neuroinflammatory response through their high affinity with increased monoamine oxidase type B activation and reactive astroglia response. Therefore, it could be argued that changes in I_2BS levels measured here may reflect changes in monoamine oxidase type B. However, a series of blocking studies *in vivo* have confirmed the selectivity and specificity of the [^{11}C]BU99008 radioligand for I_2BS on astroglia (Parker *et al.*, 2014; Tyacke *et al.*, 2018). To validate the selectivity of [^{11}C]BU99008 for I_2BS, a blocking study in rhesus monkeys showed that blocking with monoamine oxidase inhibitors had no significant effects on [^{11}C]BU99008 signal while blocking I_2BS inhibitor exhibited a dose-dependent decrease in signal (Parker *et al.*, 2014). A recent blocking study in healthy controls, demonstrated no signal reduction following combined monoamine oxidase type A and B inhibition while treatment with an I_2BS inhibitor resulted in loss of [^{11}C]BU99008 signal (Tyacke *et al.*, 2018). Combined

these studies support the use of [^{11}C]BU99008 as a suitable radioligand for the quantification of I_2BS as a marker of astroglia activation *in vivo* with high specificity and selectivity for I_2BS.

Age, gender, depression, pain and dopaminergic medication are potential confounding factors which could influence I_2BS levels. Therefore, to control for these we included as co-variates age, gender, LED_{DA}, $LED_{L\text{-}DOPA}$, depression and pain in all statistical analysis. To address potential issues of bias from volume loss, we applied partial volume correction to [^{11}C]BU99008 V_T values. While, we found volumetric loss of approximately 7% in the brainstem in early and moderate/advanced Parkinson's patients, this was much less than the 52% increase and 48% decrease in [^{11}C]BU99008 V_T in early and moderate/advanced Parkinson's disease patients, respectively. Given the percentage change of [^{11}C]BU99008 V_T in the brainstem is greater than the degree of brainstem atrophy, it is unlikely that volumetric loss was driving the change in [^{11}C]BU99008 V_T. Furthermore, the high levels of activated astroglia before pronounced atrophy becomes evident may suggest that reactive astrogliosis occurs prior to manifested neuronal loss. To our knowledge, this is the first report demonstrating reactive astrogliosis is present in early Parkinson's disease with loss of astroglia function as the disease advances and disease burden increases in moderate to advanced disease stages, using [^{11}C]BU99008 PET *in vivo*. Therefore, further studies are warranted to confirm these findings and to further explore the association between loss of I_2BS and cognitive decline in a larger cohort of Parkinson's patients with cognitive impairment.

In conclusion, our findings provide novel knowledge and evidence *in vivo* in patients for the role of astroglia pathology in Parkinson's disease. The early phenomenon of increased levels of I_2BS reflecting cortical and brainstem reactive astrogliosis is intriguing and warrants further investigation in cohorts at risk for development of Parkinson's disease including those with

idiopathic rapid eye movement (REM) behavioural disorder and asymptomatic/premotor carriers of parkinsonism related mutations. Longitudinal data would also be of great value in order to assess the potential of [^{11}C]BU99008 PET as a marker of disease progression.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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Data availability: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

FIGURE LEGENDS

Figure 1 Bar graphs showing regional total distribution volume (V_T) in [^{11}C]BU99008 in early and moderate/advanced Parkinson's disease patients compared with healthy controls in cortical regions (A) and subcortical regions and the brainstem (B). Increased [^{11}C]BU99008 V_T uptake is observed in early Parkinson's disease, with greatest increase in the brainstem, while in advanced disease stages there is a global loss of [^{11}C]BU99008 V_T. Partial volume corrected

[^{11}C]BU99008 V_T expressed in cm^3/mL. *P<0.05 Bonferroni corrected for multiple-comparisons compared with healthy controls.

Figure 2 [^{11}C]BU99008 standardized uptake value (SUV) images showing increased I_2BS in early Parkinson's disease patients while moderate/advanced Parkinson's disease patients show global loss of I_2BS compared with healthy controls. Axial, sagittal and coronal (MNI co-ordinates: X= 43; Y = 46; Z = 39) [^{11}C]BU99008 SUV images from a representative healthy control (A; 65 year old male), early Parkinson's' disease patient (B; 60 year old female; disease duration 2 years; MDS-UPDRS-III=11; MoCA=28) and moderate/advanced Parkinson's disease patient (C; 63 year old male; disease duration 16 years; MDS-UPDRS-III=69; MoCA=24).

Figure 3 Loss of [^{11}C]BU99008 V_T correlated with longer disease duration (A) and high total disease burden (MDS-UPDRS Total; B) in Parkinson's disease patients. In the subgroup of moderate/advanced Parkinson's patients, loss of [^{11}C]BU99008 V_T correlated with global cognitive impairment, as measured by lower Montreal Cognitive Assessment (MoCA) scores (C).

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