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Increased free water in the substantia nigra in idiopathic REM sleep behaviour disorder

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Imaging markers sensitive to neurodegeneration in the substantia nigra are critically needed for future disease-modifying trials. Previous studies have demonstrated the utility of posterior substantia nigra free water as a marker of progression in Parkinson's disease. In this study, we tested the hypothesis that free water is elevated in the posterior substantia nigra of idiopathic REM sleep behaviour disorder, which is considered a prodromal stage of synucleinopathy. We applied free-water imaging to 32 healthy control subjects, 34 patients with idiopathic REM sleep behaviour disorder and 38 patients with Parkinson's disease. Eighteen healthy control subjects and 22 patients with idiopathic REM sleep behaviour disorder were followed up and completed longitudinal free-water imaging. Free-water values in the substantia nigra were calculated for each individual and compared among groups. We tested the associations between posterior substantia nigra free water and uptake of striatal dopamine transporter in idiopathic REM sleep behaviour disorder. Free-water values in the posterior substantia nigra were significantly higher in the patients with idiopathic REM sleep behaviour disorder patients than in the healthy control subjects, but were significantly lower in patients with idiopathic REM sleep behaviour disorder than in patients with Parkinson's disease. In addition, we observed significantly negative associations between posterior substantia nigra free-water values and dopamine transporter striatal binding ratios in the idiopathic REM sleep behaviour disorder patients. Longitudinal free-water imaging analyses were conducted with a linear mixed-effects model, and showed a significant Group × Time interaction in posterior substantia nigra, identifying increased mean free-water values in posterior substantia nigra of idiopathic REM sleep behaviour disorder over time. These results demonstrate that free water in the posterior substantia nigra is a valid imaging marker of neurodegeneration in idiopathic REM sleep behaviour disorder, which has the potential to be used as an indicator in disease-modifying trials.

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Abbreviations: ^{18}F-FP-CIT = ^{18}F-*N*-(3-fluoropropyl)-2β-carbon ethoxy-3β-(4-iodophenyl) nortropane; DAT = dopamine transporter; HAMD-17 = Hamilton Depression Rating Scale-17; iRBD = idiopathic REM sleep behaviour disorder; RBDSQ = REM Sleep Behaviour Disorder Screening Questionnaire; SBR = striatal binding ratio

Introduction

Parkinson's disease is a progressive neurodegenerative disease that is characterized by the loss of dopaminergic cells in the substantia nigra. Parkinson's disease has a prodromal stage of several years before the manifestation of parkinsonism.[1,2] Idiopathic REM sleep behaviour disorder (iRBD), which is a parasomnia characterized by dream-enacting behaviours and the loss of REM sleep atonia, is now considered a prodromal stage of Parkinson's disease.[1,3] Most patients with iRBD will develop Parkinson's disease.[4] Substantia nigra degeneration occurs in the early stage of Parkinson's disease as well as in iRBD,[5,6] and a pathological study attributed increased dopaminergic dysfunction in the substantia nigra with Parkinson's disease duration.[7] There is a clear need to develop non-invasive markers of substantia nigra degeneration in iRBD to monitor disease progression.

Water molecules that do not experience a directional dependence or other restrictions by the cellular environment are known as free water. Free-water diffusion MRI analysis using a bi-tensor model was developed to explicitly estimate the fractional volume of freely diffusing water molecules within the voxel,[8,9] and this measure is expected to increase with neuroinflammatory and atrophy-based neurodegeneration.[10,11] Previous studies demonstrated that the free-water value in the posterior substantia nigra is elevated in Parkinson's disease patients[12] and increases with Parkinson's disease progression, thereby providing a potential non-invasive progression marker of substantia nigra degeneration.[12-14] Posterior substantia nigra is more sensitive to earlier stages of Parkinson's disease than the anterior substantia nigra;[13,15,16] however, no previous studies have examined free-water content in the substantia nigra of patients with iRBD.

We hypothesized that the free-water volume fraction in the posterior substantia nigra was elevated in iRBD as a probable result of degeneration in the posterior substantia nigra. Moreover, we hypothesized that the mean free-water value in the posterior substantia nigra in patients with iRBD was between the mean free-water value in healthy controls and that of patients with Parkinson's disease, indicating early progressive changes in the posterior substantia nigra. In our study, we selected the anterior and posterior substantia nigra as the region of interest. We compared free-water values in the substantia nigra of iRBD patients with the values in healthy controls and Parkinson's disease patients. Some participants were followed up and completed longitudinal free-water imaging. Furthermore, we used dopamine transporter (DAT) PET/MRI, which is an established biomarker of disease progression to verify that free water in the posterior substantia nigra could be a potential imaging biomarker of neurodegeneration in iRBD.

Materials and methods

Participants

We performed the study at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine between October 2017 and October 2020. The study was approved by the ethics committee at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from each participant.

In the current study, 104 subjects were included comprising 34 patients with iRBD, 38 patients with Parkinson's disease and 32 healthy controls. All recruited participants met the following criteria: (i) age between 45 and 75 years; (ii) no sign of dementia according to the Chinese version of the Mini-Mental State Examination (cMMSE)[17] (the cut-off point 24/25 for participants with more than 6 years of education); (iii) no history of intracranial surgery or traumatic brain injury; (iv) no psychiatric disorders; (v) no alcohol use disorder; and (vi) no history of other neurological disorders.

Parkinson's disease patients were diagnosed according to the current clinical diagnostic criteria of the Movement Disorders Society (MDS)[4] by at least two experienced movement disorder specialists and were evaluated using the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS)[18] and the Hoehn and Yahr stage.[19] All patients with Parkinson's disease were early-stage patients with Hoehn and Yahr scores < 3.

We recruited iRBD patients with typical RBD histories (dream-enacting behaviours) and performed video-polysomnography to confirm the RBD diagnosis using a Compumedics E-Series EEG/PSG Recording System (Compumedics Ltd). A diagnosis of iRBD

was based on clinical assessment and polysomnographic evidence according to the International Classification of Sleep Disorders-3 criteria.[20] All iRBD patients had no motor symptom complaints and were examined by two experienced neurologists to exclude motor signs of parkinsonism. We excluded iRBD patients with secondary causes and other coexistent neurological diseases (e.g. withdrawal of medication or substances and obstructive sleep aponea-hypopnoea syndrome).

We recruited healthy control participants from several communities in Shanghai and among social workers in Ruijin Hospital. The healthy participants had no RBD symptom complaints and were checked by two neurologists to confirm the absence of any signs of other neurological illnesses.

We assessed all participants' cognitive function with the cMMSE and Montreal Cognitive Assessment (MoCA)[21] and their depression state with the 17-item Hamilton Depression Rating Scale (HAMD-17).[22] All participants underwent MRI (free-water imaging) scan, 18 patients with iRBD also underwent DAT-PET/MRI scan. Participant demographics and patient clinical characteristics are listed in Table 1. In addition, during the research period, all iRBD patients and healthy controls who were followed up over 18 months and agreed to participate in the longitudinal study, were invited to undergo longitudinal MRI (free-water imaging) scans, which yielded follow-up groups of 18 healthy controls and 22 iRBD patients [follow-up periods ranged from 20 to 37 months, mean ± standard deviation (SD): 29 ± 6 months]. Participant demographics of the follow-up group are listed in Supplementary Table 1.

MRI and PET acquisition

MRI data acquisition was performed at the Functional Imaging Centre of the Institute of Neuroscience of the Chinese Academy of Science using a Siemens Trio 3 T MRI scanner equipped with a 12-channel head coil.

T_1-weighted images were obtained using a 3D magnetization-prepared rapid acquisition gradient-echo (MPRAGE) sequence (176 axial slices, flip angle of 9°, $1 \times 1 \times 1$ mm^3 voxel size, echo time/repetition time/inversion time = 3.0/2300/1000 ms) for volumetric and registration purposes.

Diffusion tensor imaging (DTI) data were acquired with the following parameters: field of view = 220 mm^2, 42 slices, slice thickness = 3 mm, voxel size = $1.7 \times 1.7 \times 3.0$ mm^3, echo time = 94 ms, repetition time = 6000 ms, b-values = 0 and 1000 s/mm^2, and diffusion gradient directions of 30.

All the ^{18}F-*N*-(3-fluoropropyl)-2β-carbon ethoxy-3β-(4-iodophenyl) nortropane (^{18}F-FP-CIT) hybrid PET/MRI examinations were performed on Siemens Biograph mMR scanner (Siemens Healthcare) using an 8-channel phase-array head coil. Patients were intravenously injected with ^{18}F-FP-CIT at a mean dose of 3.7 MBq/kg body weight. ^{18}F-FP-CIT was supplied by Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. Static ^{18}F-FP-CIT PET data were acquired in sinogram mode for 15 min with the following parameters: 128 slices per slab, gap 0.5 mm; matrix size 344 × 344, reconstructed with ordered subsets expectation maximization (OSEM) iterative reconstruction with subsets 21, iterations four and post-filtered with an isotropic full-width at half-maximum Gaussian kernel of 2 mm. Attenuation correction (AC) was performed using advanced PET attenuation correction with unique 5-compartment model including bones. MRI was performed simultaneously to PET data acquisition. We performed 3D T_1-MPRAGE sequences (as above) followed by PET sequences.

Free-water mapping analysis

Free-water maps were calculated by fitting a bi-tensor model based on the diffusion measurements.[12,13,23] The bi-tensor model predicts the signal attenuation of the water molecules contributed by intracellular and extracellular water, respectively. Free water mostly occupies the extracellular space. The free-water maps reflect the volume fraction of free-water content within each voxel.

Figure 1 illustrates the procedure used to analyse and draw regions of interest in the substantia nigra. Iron-rich substantia nigra segmentation was defined using an age-specific (60–70 years of age) quantitative susceptibility mapping (QSM) template defined in the MNI space owing to its high contrast between the substantia nigra and its surroundings.[24] To avoid

Table 1 Demographic and clinical characteristics of the three participant groups

	iRBD (*n* = 34)	PD (*n* = 38)	Healthy control (*n* = 32)	*P*-value
Age, years	64.7 ± 7.5	64.4 ± 5.0	63.2 ± 5.4	0.55*
Sex (%)				
Male	22 (65)	21 (55)	14 (44)	0.23**
Female	12 (35)	17 (45)	18 (56)	
Time since medical diagnosis, years	1.2 ± 1.2	3.4 ± 3.2	–	–
Time since onset of symptoms, years	6.0 ± 4.5	4.8 ± 3.6	–	–
MDS-UPDRS III score	–	20.2 ± 10.5	–	–
Hoehn and Yahr stage	–	1.5 ± 0.5	–	–
LEDD		304 ± 227		
RBDSQ	8.7 ± 2.2	5.2 ± 3.5	0.5 ± 0.8	< 0.001***
cMMSE	28.5 ± 1.2	28.4 ± 1.3	28.8 ± 1.1	0.372***
MoCA	25.2 ± 2.6	25.1 ± 2.2	25.8 ± 2.4	0.295***
HAMD-17	3.5 ± 3.3	3.6 ± 3.1	1.2 ± 1.3	< 0.001***

Data are expressed as the mean ± SD. cMMSE = Chinese version of the Mini-Mental State Examination; LEDD = L-DOPA equivalent daily dose; MDS-UPDRS III = Movement Disorder Society Unified Parkinson's Disease Rating Scale part III; MoCA = Montreal Cognitive Assessment; PD = Parkinson's disease.
*ANOVA.
**chi-square test.
***Kruskal-Wallis test.

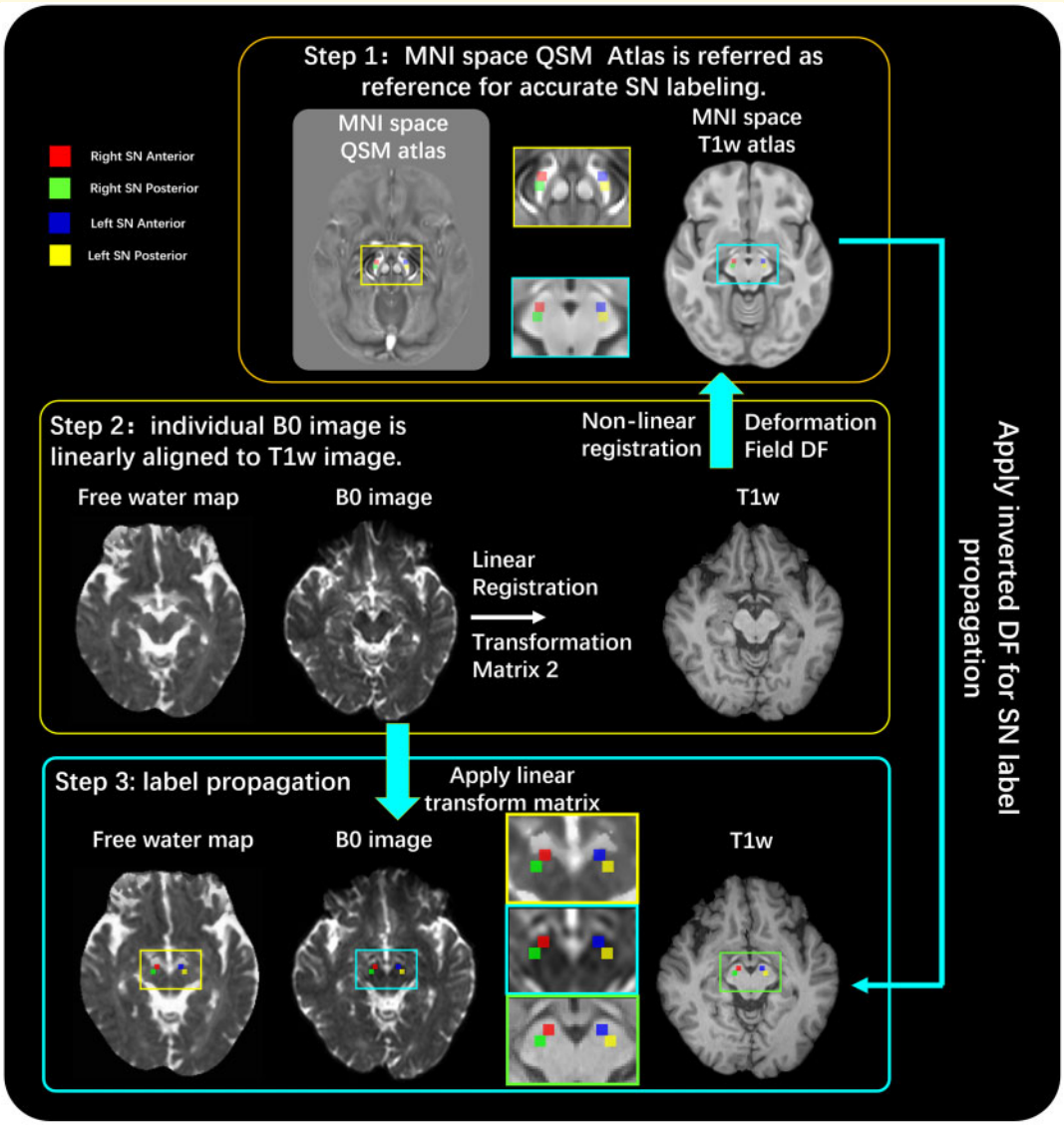

Figure 1 Illustration of processing pipeline and regions of interest in the substantia nigra. DF = deformation field; SN = substantia nigra. T1w = T_1-weighted.

subjective bias by manual segmentation of the substantia nigra, the free-water maps were registered to the standard MNI space to facilitate the automatic quantification of nigral free-water content. Specifically, we use the T_1-weighted image as the intermedium for unifying the multi-contrast magnetic resonance images for each subject, and to lead region of interest annotations from standard MNI space to individual image space. As demonstrated in Fig. 1, step 1, the anterior and posterior subregions of bilateral substantia nigra and the bilateral subthalamic nucleus (STN) are manually annotated based on the QSM template in standard MNI space. The bilateral STN are labelled using 2 × 2 voxels squares on a single axial slice (z = −10). The average centre of coordinates for the STN were x = −10, y = 12, z = −10 for the right and x = 10, y = 12, z = −10 for the left. Similarly, bilateral substantia nigra were labelled using 2 × 2 voxels on each axial slice for anterior and posterior, respectively. In total, 32 voxels were selected spanned on two axial slices (z = −12 and −14). The average centre of coordinates for the anterior substantia nigra was x = −9, y = 13, z = −13 for the right and x = 9, y = 13, z = −13 for the left; for the posterior substantia nigra were x = −11, y = 17, z = −13 for the right and x = 11, y = 17, z = −13 for the left. Figure 1, step 2, illustrates the process to (i) linearly align individual free-water maps with the T_1-weighted image; and (ii) non-linearly register individual T_1-weighted images to the MNI T_1-weighted template. The registration process is a combination of affine (DOF = 12) transformation (FLIRT),[25] and diffeomorphic registration (DEMONS).[26] Then, as shown in Fig. 1, step 3, the deformation fields from individual T_1-weighted to MNI T_1-weighted template were inverted to warp substantia nigra and STN labels into the individual T_1-weighted image space. Thus, the regions of interest are simultaneously propagated to the b0 image and free-water maps. Next, the substantia nigra region was extracted, and visual examinations were performed to identify

any co-registration bias. The averaged bilateral free-water value was calculated from the anterior and posterior substantia nigra. We also included a control region of interest in the STN, a region where structural changes in Parkinson's disease are not expected.[13]

PET region of interest analysis

Briefly, ^{18}F-FP-CIT PET was used to assess dopaminergic transporter availability in the putamen and caudate. A region of interest analysis was performed and regional ^{18}F-FP-CIT PET striatal binding ratios (SBRs) were measured according to previous studies.[12,27] Regions of interest were drawn by two board certified nuclear medicine experts according to their anatomical borders. The MRI template incorporating these regions of interest was loaded onto the PET scan of each patient. Regions of interest were then manually adjusted to account for individual variation, without changing their size and shape. All discrepancies were resolved through discussion. Putamen and caudate were set as target regions of interest. A region of interest of the left and right occipital cortices was used as reference region. SBRs were calculated as [(target region/reference region) – 1]. The raters quantified striatal and occipital SBRs blinded to free-water map. All separate images were analysed using ITK-SNAP (version 3.8.0, http://www.itksnap.org/pmwiki/pmwiki.php) and the MR Oncology package (Syngovia, version VB10, Siemens Healthcare). The average of right and left putamen, and left and right caudate were taken as the total putamen SBR and total caudate SBR, respectively.

Statistical analysis

We analysed participant demographic and clinical characteristics using chi-square tests, ANOVAs or Kruskal-Wallis H tests, depending on the type and distribution of dependent variables. We analysed the free-water value among the three participant groups using ANOVA, and Tukey's *post hoc* tests were applied. The discriminative power of the free-water value in the posterior substantia nigra was evaluated by receiver operating characteristic curve (ROC) analysis. The relationships between free-water values in the posterior substantia nigra and DAT SBRs in the iRBD were investigated using Spearman correlation analyses. The relationships between free-water values in the posterior substantia nigra and clinical assessments (disease duration and RBDSQ, MDS-UPDRS III and HAMD-17 scores) in iRBD and Parkinson's disease patients were investigated using Spearman correlation analyses (according to the type of variable and distribution of the data). These statistical analyses were performed with IBM SPSS version 22 (SPSS Inc., Chicago, IL, USA). For longitudinal data, a linear mixed-model method fitted with restricted maximum likelihood estimators (RMLE) was performed to evaluate Time, Group, and Time × Group interaction effects. The AR(1) covariance structure was selected based on the best goodness-of-fit (as evaluated by Akaike information criterion). The Group, Time, and Group × Time interaction were modelled as fixed effects. Standardized age and sex were included as covariates. When investigating group effects, healthy controls were set as the reference group (i.e. 0). Longitudinal data were analysed using the MIXED procedure of SAS 9.4 (Cary, NC, USA). $P < 0.05$ was considered statistically significant for all analyses.

Data availability

All data included in this study will be shared by request from any qualified investigator.

Results

Demographic and clinical information

The demographic and clinical characteristics of the iRBD, Parkinson's disease and healthy control participants are shown in Table 1. There were no significant differences in age, sex, cMMSE score or MoCA score ($P > 0.23$). There were significant differences in the RBDSQ scores and HAMD-17 scores among the three groups ($P < 0.001$). The iRBD group had a higher RBDSQ score than the healthy control group ($P < 0.001$) and the Parkinson's disease group ($P = 0.002$). The iRBD group had a higher HAMD-17 score than the healthy control group ($P = 0.002$). The Parkinson's disease group had higher RBDSQ scores and higher HAMD-17 scores than the healthy control group ($P < 0.001$). There were no significant differences in HAMD-17 scores between the iRBD group and the Parkinson's disease group ($P = 1.000$).

Free-water content in the substantia nigra

There were significant differences in the mean free-water values in the posterior substantia nigra [$F(2,101) = 36.4$, $P < 0.001$, partial $\eta^2 = 0.42$] among the three participant groups. Tukey's *post hoc* tests showed that the mean free-water value in the posterior substantia nigra was significantly higher in the iRBD group (mean ± SD: 0.181 ± 0.022) than in the healthy control group (0.162 ± 0.015, $P < 0.001$). The mean free-water value in the posterior substantia nigra was significantly higher in the Parkinson's disease group (0.201 ± 0.020, $P < 0.001$) than in the healthy control group. Meanwhile, the mean free-water value in the posterior substantia nigra was significantly larger in the Parkinson's disease group than in the iRBD group ($P < 0.001$) (Fig. 2A). There were no significant differences in the mean free-water values in the anterior substantia nigra [$F(2,101) = 1.58$, $P = 0.211$, partial $\eta^2 = 0.03$] among the three participant groups (Fig. 2B). There were no significant differences in the mean free-water values in the STN [$F(2,101) = 1.32$, $P = 0.271$, partial $\eta^2 = 0.03$] among the three participant groups.

The discriminative power of the free-water value

The discriminative power of free-water was evaluated with ROC analysis. We found that for the mean free-water value in the posterior substantia nigra, the area under the curve

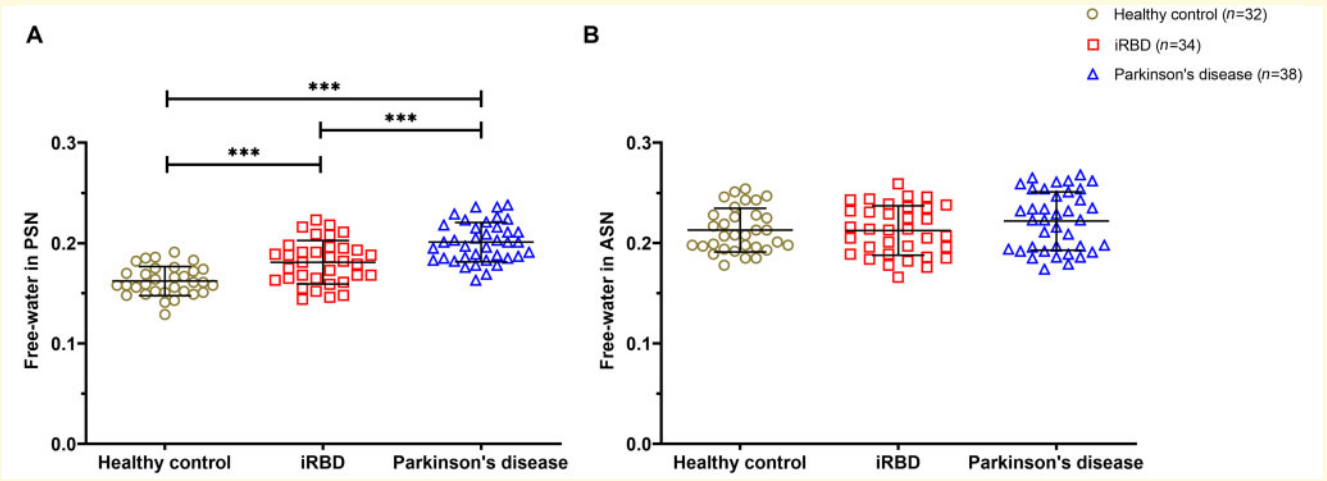

Figure 2 Free-water content in the substantia nigra among the three groups. The mean free-water content of the posterior (**A**) and anterior (**B**) substantia nigra for healthy controls, iRBD patients and Parkinson's disease patients. Error bars represent the standard error. ***$P < 0.001$. ASN = anterior substantia nigra; PSN = posterior substantia nigra.

(AUC) of the ROC for Parkinson's disease patients versus healthy controls was 0.949 (sensitivity, 0.921; specificity, 0.844; Fig. 3A); for iRBD patients versus healthy controls, AUC, sensitivity, and specificity were 0.751, 0.618 and 0.750, respectively (Fig. 3B); whereas for Parkinson's disease versus iRBD patients, AUC, sensitivity, and specificity were 0.743, 0.842 and 0.529, respectively (Fig. 3C).

Correlations between the free-water value and dopamine transporter uptake

Spearman correlation analysis showed that there was a significant negative correlation between DAT SBR in the putamen and mean free-water values in the posterior substantia nigra in the iRBD group ($\rho = -0.60$, $P = 0.009$; Fig. 4A). And there was a significant correlation between DAT SBR in the caudate and mean free-water values in the posterior substantia nigra in the iRBD group ($\rho = -0.54$, $P = 0.021$; Fig. 4B). No significant correlations between the disease duration, MDS-UPDRS III, RBDSQ scores or HAMD-17 scores and the mean free-water values in the posterior substantia nigra were found in the Parkinson's disease group ($P > 0.08$). No significant correlations between disease duration, RBDSQ scores or HAMD-17 scores and the mean free-water values in the posterior substantia nigra were found in the iRBD group ($P > 0.09$).

Longitudinal free-water mapping analysis

The Group, Time, and Group × Time interaction of mean free-water value in the substantia nigra were illustrated for healthy controls and iRBD patients. The group effect resulted in greater mean free-water values in the posterior substantia nigra for iRBD patients compared with healthy controls (estimates = 0.02064, $t = 2.97$, $P = 0.005$; Table 2 and Fig. 5A). The Group × Time interaction (estimates = 0.00069, $t = 2.11$, $P = 0.041$; Table 2 and Fig. 5A) identified increased mean free-water values in the posterior substantia nigra for iRBD patients over time, whereas there were no significant differences between baseline and follow-up values for healthy controls. Furthermore, there were no significant effects for free water in the anterior substantia nigra across Group, Time, or Group × Time interactions ($P > 0.05$; Table 2 and Fig. 5B).

Discussion

In this study, we applied free-water imaging to investigate microstructural changes in the substantia nigra of patients with iRBD and compared findings to those in healthy controls and patients with Parkinson's disease. We found that free-water values in the posterior substantia nigra were significantly higher in patients with iRBD than in healthy controls, but lower than in pateints with Parkinson's disease. In addition, we observed significantly negative associations between posterior substantia nigra free-water values and DAT SBRs in patients with iRBD. Moreover, we demonstrated longitudinal increases in the posterior substantia nigra free-water value in patients with iRBD compared to healthy controls. Taken together, these results indicated the potential of free water as an imaging biomarker for substantia nigra integrity in patients with iRBD.

IRBD is currently the strongest known predictor of the development of neurodegenerative diseases, as longitudinal studies have demonstrated that most iRBD patients were ultimately diagnosed with a synucleinopathy with increasing follow-up times.[4,28-31] Patients with iRBD are ideal candidates for neuroprotective trials because they are in the

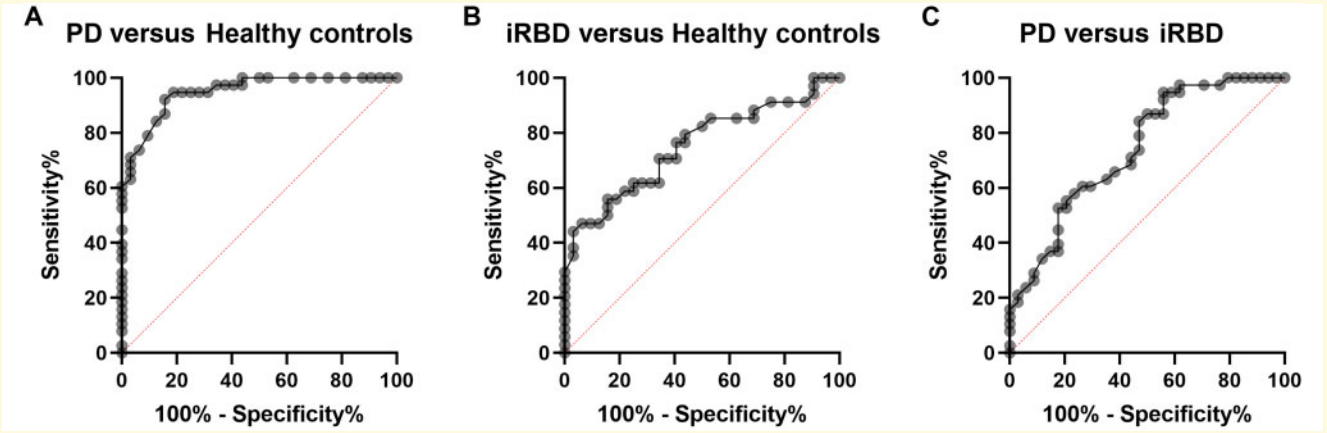

Figure 3 ROC curves showing comparisons of the free-water values among the three groups.

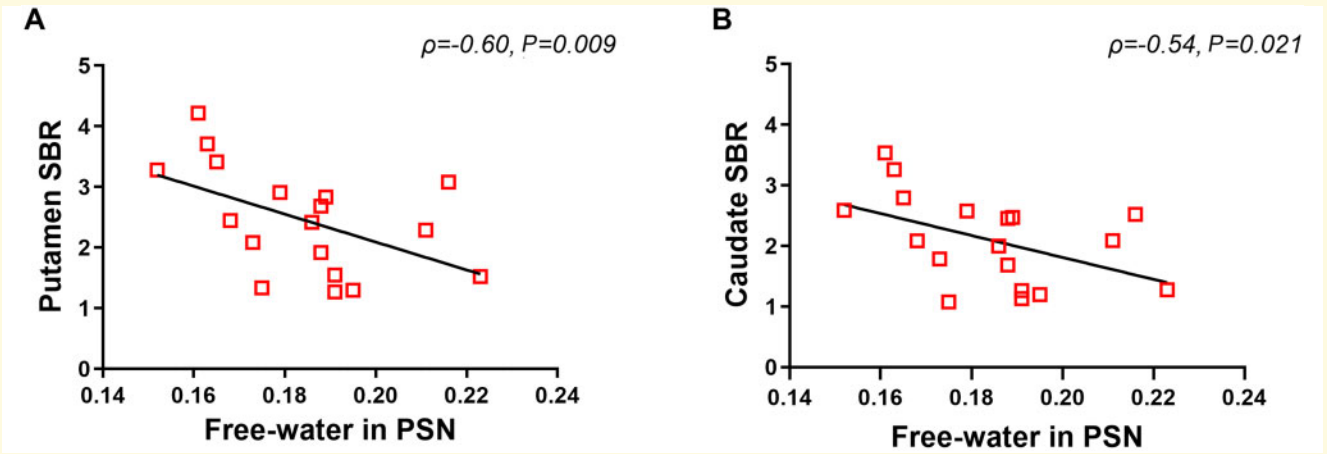

Figure 4 Correlations between the free-water values and DAT striatal binding ratio. There was a significant negative correlation between mean free-water values in the posterior substantia nigra and (**A**) DAT SBR in the putamen (ρ = –0.60, P = 0.009), and (**B**) DAT SBR in the caudate (ρ = –0.54, P = 0.021) in the iRBD group. PSN = posterior substantia nigra.

Table 2 Longitudinal mean free-water values changes in iRBD

	Posterior SN			Anterior SN		
Effect	**Estimates**	***t*-value**	***P*-value**	**Estimates**	***t*-value**	***P*-value**
Intercept	0.15630	6.66	<0.001	0.23290	7.29	<0.001
Group	0.02064	2.97	0.005	–0.00060	–0.07	0.941
Time	–0.00021	–0.87	0.387	–0.00003	–0.15	0.881
Time × Group	0.00069	2.11	0.041	0.00014	0.47	0.639
Age	0.00002	0.07	0.945	–0.00030	–0.63	0.530
Sex	0.00336	0.65	0.522	–0.00236	–0.33	0.742

SN = substantia nigra.

prodromal stage of neurodegeneration, which is early enough for meaningful intervention.[32,33] Furthermore, patients with iRBD are not taking antiparkinsonian medications, suggesting that a major confounding factor in the disease-modifying intervention is removed.[32,33] Although iRBD provides a window of opportunity in which disease-modifying interventions could be most beneficial, there remain new issues regarding selecting suitable end points in

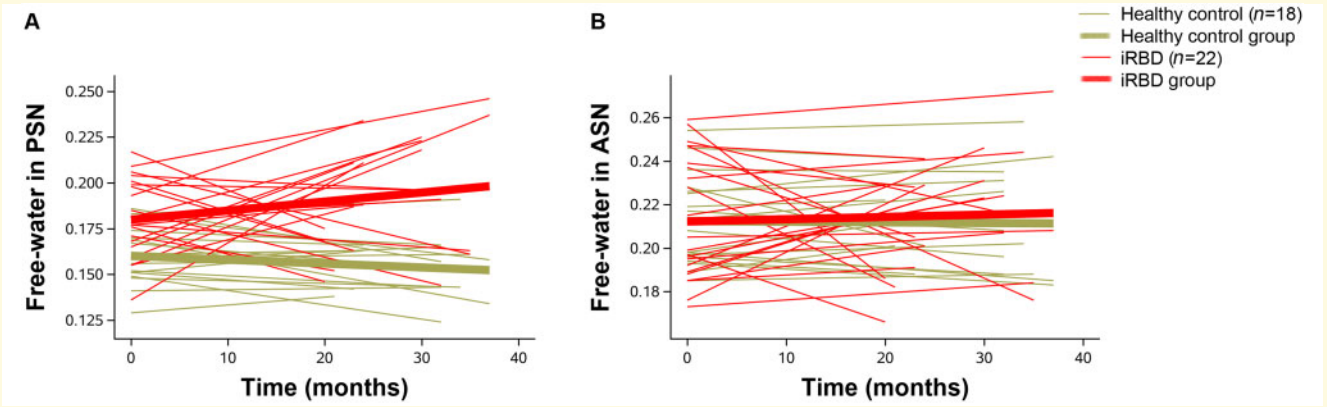

Figure 5 Longitudinal free-water content in the substantia nigra between healthy controls and iRBD patients. The mean free-water content of the posterior (**A**) and anterior (**B**) substantia nigra for healthy controls and iRBD patients at baseline and follow-up. ASN = anterior substantia nigra; PSN = posterior substantia nigra.

clinical trials. The use of phenoconversion to synucleinopathies as the end point in clinical trials could be difficult and would require long follow-up times,[33] as the interval between iRBD diagnosis and the development of a defined neurodegenerative disease is heterogeneous.[29,30] Furthermore, longitudinal studies showed that although most iRBD patients converted to overt neurodegenerative symptoms after 12 years of follow-up, more than 20% of iRBD patients still had preserved motility and cognition.[29,30] Because of the limitations of clinical markers, imaging biomarkers of the nigrostriatal dopaminergic system have been proposed as promising end points because disease-modifying strategies focus on halting or preventing dopaminergic neuron loss.[32,33] For example, a study using serial ^{123}I-FP-CIT single-photon emission computed tomography (SPECT) revealed a progressive decline in striatal tracer uptake in iRBD, suggesting that functional imaging markers could be helpful to monitor the progression of nigrostriatal dysfunction in disease-modifying trials in these patients.[34] Although functional imaging end points such as SPECT and PET are more objective and sensitive than clinical markers, they also have several limitations, such as high expense, low availability and potential radioactive damage. While some other non-invasive biomarkers may have other problems. Such as echogenicity of the substantia nigra, in our previous study,[35] we found that transcranial sonography (TCS) failed to visualize mesencephalon in 127 patients (30.24%) due to poor penetration of ultrasound through the bony window, which could be caused by different temporal bone structure in Asians.[36] Also, in a large Japanese cohort study, the poor penetration rate reached above 60% in older females over 60 years old.[37] To prepare for future disease-modifying trials in iRBD patients, it is necessary to develop advanced imaging biomarkers of substantia nigra to monitor the process of neurodegeneration.

Free-water mapping is a novel DTI analysis technique obtained from a bi-tensor model. This bi-tensor model can provide more microstructural information than the conventional single-tensor model because it can separate the diffusion properties of water in brain tissue from those of water in extracellular space (i.e. free water).[9] A recent study demonstrated that free-water measures had a higher sensitivity than conventional DTI measures to detect within- and between-group differences in healthy adults.[38] Free water has been shown to reflect pathological changes, including neuroinflammation, gliosis, axonal damage and cell loss,[39,40] all of which are known to occur in the process of neurodegeneration. Moreover, free water in white matter structures, midline cortical areas and medial thalamic areas increased significantly with elevated brain interferon-gamma (IFN-γ), suggesting the free-water signal is sensitive to neurodegenerative and inflammatory patterns specific to IFN-γ.[10] Thus, changes in free-water values may be closely associated with neurodegeneration. A previous study revealed that free-water values in the posterior substantia nigra were elevated in patients with Parkinson's disease compared with healthy controls at a single site and across a multisite study.[12] Moreover, several longitudinal studies demonstrated that free-water values in the posterior substantia nigra increased over time in Parkinson's disease, and baseline free water in the posterior substantia nigra could be used to predict changes in clinical symptoms in Parkinson's disease patients.[12,13] These findings indicated that free-water imaging could provide an indirect measure of dopaminergic degeneration within the substantia nigra and monitor the progression of neurodegeneration.

In this study, we found significantly increased free-water values in the posterior substantia nigra in iRBD patients compared with healthy controls, indicating dopaminergic neuron loss within the substantia nigra in patients with iRBD. Furthermore, free-water levels in the posterior substantia nigra were elevated in Parkinson's disease patients compared to iRBD patients, suggesting that there may be a progressive increase in free-water levels in the posterior substantia nigra from the prodromal to the clinical stage of Parkinson's disease. These findings are consistent with

previous pathological and imaging studies, which showed that the loss of dopaminergic cells within the posterior substantia nigra actually begins in the prodromal stage of Parkinson's disease and that nigrostriatal degeneration can be detected ~5–10 years prior to the emergence of typical motor symptoms.[28,34,41,42] The free-water values in the posterior substantia nigra showed high accuracy to discriminate Parkinson's disease patients from healthy controls and moderate accuracy to discriminate iRBD patients from healthy controls (Fig. 3). As the posterior substantia nigra free-water values in patients with iRBD were between those in Parkinson's disease patients and healthy controls (Fig. 2), it is not surprising that the posterior substantia nigra free-water values were more accurate in distinguishing Parkinson's disease patients from healthy controls than they were for distinguishing iRBD patients from healthy controls.

Consistent with prior studies,[12,13,23] we did not find any significant differences among three groups in anterior substantia nigra free water. This is not surprising, since dopaminergic cell loss in Parkinson's disease occurs mostly in the posterior substantia nigra.[7,23] Further, a previous study[40] has pointed out that anterior substantia nigra is more likely to become affected while the disease spreads and becomes more severe. Our study including iRBD and early stage Parkinson's disease patients demonstrated that posterior substantia nigra was affected rather than anterior substantia nigra. Similar to previous reports, we found that the anterior substantia nigra had elevated free-water values compared with the posterior substantia nigra in all groups, which could be caused by excessive partial volume effects near CSF.[12] In addition, the free-water values calculated from the diffusion-weighted images might still vary between differences in the spatial resolution, signal to noise ratio, and the disease duration of the patients.

In the present study, we found a significantly negative association between posterior substantia nigra free-water values and SBRs in the iRBD group (Fig. 4), indirectly suggesting the potential of posterior substantia nigra free water as a progression biomarker of iRBD, since longitudinal studies have demonstrated that DAT PET/SPECT in iRBD is helpful to monitor disease progression and identify individuals at high risk of phenoconversion.[34,43,44] Consistent with a prior multisite study,[12] we did not observe any significant correlation between posterior substantia nigra free-water and UPDRS-III or disease duration in Parkinson's disease. Some studies[23,45] indicated that age was a significant confounder of free water in the substantia nigra, and free-water values increased with age. In this cohort, no significant correlations were found between substantia nigra free-water and age ($P > 0.106$) in either the Parkinson's disease group, iRBD group or healthy control group, or all groups. The result is consistent with the previous study,[12] which showed that the correlation for age and substantia nigra free water was not significant. The reason was likely due to discrepancies in image acquisition parameters, patient characteristics or study methods.

Longitudinally, we provided some evidence that the posterior substantia nigra free water had the potential to monitor the progression of dopaminergic degeneration in iRBD patients. First, there was Group × Time interaction for the posterior substantia nigra free-water trajectories (Fig. 5). Patients with iRBD showed a significantly higher rate of increase in the posterior substantia nigra free water over time compared to controls. The divergent longitudinal evolution of the posterior substantia nigra free-water between the groups make it suitable for detecting the progressing substantia nigra degeneration in iRBD, though a clear overlap of free-water values was found between the groups cross-sectionally. In the present study, the anterior substantia nigra free water was not different between the groups for healthy controls and iRBD patients in the longitudinal study. Similar results have been reported in Parkinson's disease patients,[12,13] and are consistent with pathology studies showing that Parkinson's disease-related dopaminergic cell loss occurs mainly in the posterior substantia nigra.[46] These findings suggest that the posterior substantia nigra free water is a more sensitive measure of the progression of dopaminergic degeneration than the anterior substantia nigra.

The posterior substantia nigra free water is a promising MRI biomarker in disease-modifying trials, because it has many clinical advantages in quantifying prodromal neurodegeneration and predicting future Parkinson's disease. First, from the perspective of clinical practice, MRI biomarkers are more cost-efficient and widely available than PET and SPECT. Second, without intravenous radionuclide agents, MRI scanning are more friendly to patients with insufficient renal function. Third, MRI biomarkers are free of ionizing radiation, which is essential in future disease-modifying trials where repeated scans are required.[47] Finally, from the perspective of an analytical method, substantia nigra free water is more consistent, which the algorithm in different studies is from Dr Ofer Pasternak.[9,12,13,23]

Several limitations of the study should be acknowledged. First, the sample size in this study was relatively small. However, the present study could be considered as a valid preliminary research, and our results deserve to be replicated with a larger study cohort in the future. Second, because of the small sample size, we were unable to conduct a group comparison between converters and individuals who remained disease-free. Thus, a longer follow-up period is warranted to determine the predictive value of posterior substantia nigra free water in the conversion of iRBD. Additionally, most previous free-water studies used a 2-mm slice thickness and 64 gradient directions for the DTI sequence,[12,13,23,40] while we used a 3-mm slice thickness and only 30 gradient directions in this study. The lower image and angular resolutions may have decreased our sensitivity to detect between-group differences. Finally, we only compared iRBD with Parkinson's disease in this study, while iRBD is widely recognized as a prodromal stage of synucleinopathies, including Parkinson's disease, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA).[29,30,48] Though previous studies have reported

increased substantia nigra free water in MSA compared with healthy controls,[40,45] it is necessary to compare substantia nigra free water of iRBD with those of MSA and DLB directly in the future study.

In summary, the present study is the first study to show that free water in the posterior substantia nigra was elevated cross-sectionally and longitudinally in iRBD patients compared with healthy controls. This extends previous studies using the posterior substantia nigra free-water as a marker of neurodegeneration in Parkinson's disease.[12,13,23] Our findings suggest that free-water imaging could be used to monitor the severity of substantia nigra degeneration in patients with iRBD and thus has the potential to serve as an end point in future disease-modifying trials.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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