

Clinical correlates of raphe serotonergic dysfunction in early Parkinson's disease

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Post-mortem and neuroimaging studies suggest that the serotonergic system, which originates from the brainstem raphe nuclei, is disrupted in Parkinson's disease. This could contribute to the occurrence of non-motor symptoms and tremor, which are only partially explained by dopamine loss. However, the level of involvement of the serotonergic raphe nuclei in early Parkinson's disease is still debated. ¹²³I-FP-CIT single photon emission computed tomography is a marker of dopamine and serotonin transporter availability. While ¹²³I-FP-CIT binds primarily to dopamine transporters in the striatum, its binding in the brainstem raphe nuclei reflects serotonin transporter availability. We interrogated baseline single photon emission computed tomography scans of subjects recruited by the Parkinson's Progression Markers Initiative to determine: (i) the integrity of the brainstem raphe nuclei in early Parkinson's disease; and (ii) whether raphe serotonin transporter levels correlate with severity of tremor and symptoms of fatigue, depression, and sleep disturbance. Three hundred and forty-five patients with early drug-naïve Parkinson's disease, 185 healthy controls, and 56 subjects with possible Parkinson's disease without evidence of dopaminergic deficit were included. In the Parkinson's disease cohort, 37 patients had a tremulous, 106 patients had a pure akinetic-rigid, and 202 had a mixed phenotype. Patients with Parkinson's disease had significantly lower serotonin transporter availability in the brainstem raphe nuclei compared to controls ($P < 0.01$) and subjects without evidence of dopaminergic deficit ($P < 0.05$). However, only 13% of patients with Parkinson's disease individually had reduced signals. Raphe serotonin transporter availability over the entire Parkinson's disease cohort were associated with rest tremor amplitude ($\beta = -0.106$, $P < 0.05$), rest tremor constancy ($\beta = -0.109$, $P < 0.05$), and index of rest tremor severity ($\beta = -0.104$, $P < 0.05$). The tremulous Parkinson's disease subgroup had significantly lower raphe serotonin transporter availability but less severe striatal dopaminergic deficits compared to akinetic-rigid patients with no resting tremor ($P < 0.05$). In tremulous patients, raphe serotonin transporter availability was also associated with rest tremor constancy ($\beta = -0.380$, $P < 0.05$) and index of rest tremor severity ($\beta = -0.322$, $P < 0.05$). There was no association between raphe serotonin transporter availability and fatigue, depression, excessive daytime sleepiness, or rapid eye movement sleep behaviour disorder in early Parkinson's disease. We conclude that the raphe nuclei are affected in a subgroup of early drug-naïve Parkinson's disease patients and that reduced raphe serotonin transporter availability is associated with the severity of resting tremor but not non-motor symptoms.

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Abbreviations: ^{123}I -FP-CIT = 123-ioflupane-fluoropropyl-carbomethoxy-3-beta-4-iodophenyltropane; SPECT = single photon emission computed tomography; SWEDD = subjects without evidence of dopaminergic deficit

Introduction

The pathological hallmark of Parkinson's disease is degeneration of nigrostriatal dopaminergic neurons together with intraneuronal Lewy bodies and Lewy neurites containing aggregated alpha-synuclein (Dauer and Przedborski, 2003). However, in Parkinson's disease, non-dopaminergic neuronal populations also degenerate (Hirsch *et al.*, 2003) and this could contribute to the occurrence of non-motor symptoms and tremor; features that are not fully explained by dopamine loss alone (Fishman, 2008; Barone, 2010).

The serotonergic system, originating from the brainstem raphe nuclei, is known to be disrupted in Parkinson's disease. Post-mortem studies have observed a loss of serotonergic cell bodies with Lewy aggregates in the raphe nuclei (Molliver, 1987; Halliday *et al.*, 1990) and a global deficiency of serotonergic markers in cortical and subcortical structures that receive raphe projections (Kish *et al.*, 2008). Additionally, Braak and colleagues have shown that Lewy pathology in Parkinson's disease occurs in the dorsal raphe nuclei ahead of the substantia nigra (Braak *et al.*, 2003).

Single photon emission computed tomography (SPECT) and PET imaging have been used to measure *in vivo* serotonin transporter levels in the raphe nuclei, an index of serotonergic neuronal integrity, in small cohorts of patients with Parkinson's disease (Haapaniemi *et al.*, 2001; Murai *et al.*, 2001; Kim *et al.*, 2003; Guttman *et al.*, 2007; Albin *et al.*, 2008; Caretti *et al.*, 2008; Politis *et al.*, 2010a; Strecker *et al.*, 2011; Loane *et al.*, 2013). In these studies, in line with post-mortem findings, serotonin transporter binding was significantly reduced in patients with advanced Parkinson's disease compared to healthy controls (Berding *et al.*, 2003; Guttman *et al.*, 2007; Politis *et al.*, 2010a). In some studies, this was associated with severity of tremor and some non-motor symptoms (Doder *et al.*, 2003; Boileau *et al.*, 2008; Caretti *et al.*, 2008; Hesse *et al.*, 2009; Pavese *et al.*, 2010; Politis *et al.*, 2010b; Loane *et al.*, 2013).

However, imaging studies of raphe nuclei integrity in early Parkinson's disease have been less consistent, with reports of serotonin transporter levels being normal (Haapaniemi *et al.*, 2001; Kim *et al.*, 2003; Albin *et al.*, 2008; Strecker *et al.*, 2011) or mildly reduced (Roselli *et al.*, 2010). It should be acknowledged that due to the small number of patients investigated, these studies were probably underpowered to detect subtle changes in serotonergic transporter binding in early disease. Furthermore, imaging studies using ^{18}F -DOPA (^{18}F -fluorodihydroxyphenylalanine), a marker of aromatic acid decarboxylase activity, have demonstrated increased tracer uptake in the raphe nuclei of patients with early Parkinson's disease, suggesting that

a compensatory increase in monoamine turnover rather than a decline initially occurs in these neurons (Moore *et al.*, 2008; Pavese *et al.*, 2011). Therefore, the extent of serotonergic involvement in early Parkinson's disease remains to be fully elucidated.

The Parkinson's Progression Markers Initiative is an international longitudinal study of early, initially untreated, patients with Parkinson's disease, healthy controls, and subjects with possible Parkinson's disease but without evidence of dopaminergic deficit (SWEDD) on imaging (Marek, 2003; Parkinson Progression Marker Initiative, 2011). While SWEDDs may have been incorrectly diagnosed, some could alternatively have an underlying neurodegenerative process that disrupts neuronal systems other than dopamine (Marek *et al.*, 2014).

All participants in the Parkinson's Progression Markers Initiative undergo clinical screening for motor and non-motor symptoms and are scanned with ^{123}I -FP-CIT SPECT. ^{123}I -FP-CIT binds non-selectively to both dopamine and serotonin transporters. However, ^{123}I -FP-CIT binding reflects the availability of dopamine or serotonin transporters depending on the predominant monoamine population in a particular brain region. Hence, ^{123}I -FP-CIT binding in the striatum mainly reflects presynaptic dopamine transporter availability, whereas binding in the raphe nuclei primarily reflects somatodendritic serotonin transporter availability (de Win *et al.*, 2005). ^{123}I -FP-CIT SPECT can, thus, be used to assess the integrity of serotonergic cell bodies in the raphe nuclei of patients with Parkinson's disease and of SWEDDs.

In this current study, we interrogated the baseline ^{123}I -FP-CIT SPECT of subjects recruited by the Parkinson's Progression Markers Initiative to determine the integrity of the serotonergic raphe nuclei in patients with early Parkinson's disease or SWEDD, and its association with tremor and non-motor symptoms of fatigue, depression, and sleep disturbance.

Materials and methods

Subjects

Cohort selection

A total of 422 patients with Parkinson's disease, 215 controls and 83 SWEDD subjects were found on the Parkinson's Progression Markers Initiative online database (<http://www.ppmi-info.org/>). At screening, healthy controls were required to have no neurological dysfunction, normal striatal ^{123}I -FP-CIT, and no first degree relative with Parkinson's disease. Parkinson's disease patients were required to have had their clinical diagnosis for 2 years or less, and be untreated.

SWEDDs were recruited as possible Parkinson's disease patients who were found to have normal striatal ^{123}I -FP-CIT on visual screening. In terms of medication, the Parkinson's Progression Markers Initiative protocol was such that all recruited subjects were not receiving any of the following drugs, within 6 months of the screening visit: neuroleptics, metoclopramide, alpha methyl dopa, methylphenidate, reserpine, or amphetamine derivatives.

The ^{123}I -FP-CIT SPECT scans of seven patients with Parkinson's disease, five controls, and three SWEDDs were not available, so these subjects were excluded. The scans of 19 patients with Parkinson's disease, five controls, and three SWEDD patients aligned poorly along the z-axis with the co-registered magnetic resonance template, and these subjects were also excluded. Twenty healthy controls, 21 SWEDDs, and 51 patients with Parkinson's disease were receiving serotonergic agents at screening. These subjects were excluded as serotonergic medications may interfere with ^{123}I -FP-CIT binding.

Consequently, 345 patients with Parkinson's disease (aged 34–85 years, 231 male), 185 healthy controls (aged 31–84 years, 126 male), and 56 SWEDDs (aged 38–83 years, 40 male) were studied.

Thirty-seven patients with Parkinson's disease (aged 38–82 years, 21 male) had a tremulous phenotype, whereas 106 patients with Parkinson's disease (aged 34–77 years, 78 male) were akinetic-rigid but had no resting tremor; and 202 had a mixed phenotype based on criteria described below. Demographics and clinical data of subjects at screening are displayed in Table 1.

Clinical evaluation

Motor and non-motor ratings conducted with the revised Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) at baseline (within 45 days of screening visit) were downloaded from the Parkinson's Progression Markers Initiative (PPMI) database on 19 March 2014.

Part III of the MDS-UPDRS was used to score patients' rigidity (highest score on item 3.3), bradykinesia (highest score on item 3.14), rest tremor amplitude (highest score on item 3.17), and constancy of rest tremor (item 3.18). An index score of rest tremor severity was calculated using the product

of rest tremor amplitude and rest tremor constancy for each patient.

Patients with Parkinson's disease with a tremulous phenotype had: (i) rest tremor index score ≥ 2 ; (ii) a rigidity score ≤ 1 ; and (iii) a bradykinesia score ≤ 1 . Parkinson's disease patients without resting tremor had a rest tremor index score of zero.

Fatigue ratings were obtained from item 1.10 of Part I of the MDS-UPDRS. Excessive daytime sleepiness scores were obtained using the Epworth Sleepiness Scale, an eight item self-rating questionnaire (Johns, 1991). Severity of rapid eye movement sleep behaviour disorder was rated with the Rapid Eye Movement Sleep Disorder Screening Questionnaire, a 13-item yes/no self-rating questionnaire (Stiasny-Kolster *et al.*, 2007). Levels of depressive features were scored with the Geriatric Depression Scale, a 15-item self-rating yes/no questionnaire (Weintraub *et al.*, 2006).

Imaging

^{123}I -FP-CIT SPECT protocol

^{123}I -FP-CIT SPECT was performed during the screening visit for all subjects at their respective Parkinson's Progression Markers Initiative imaging centres where standardized imaging protocols were used. Each imaging centre was visited for a technical site evaluation and set up which involved obtaining an anthropomorphic striatal phantom filled with ^{123}I -Ioflupane and acquired on the same camera with the same parameters and collimators as the subsequent patients imaged in the study.

SPECT images were obtained 4 ± 0.5 h after injection with a dose range of 111–185 MBq of ^{123}I -FP-CIT. Subjects were pretreated with iodine (10 drops in water) solution or perchlorate (1000 mg) prior to injection. Raw SPECT data were acquired into a 128×128 matrix stepping each 3° for a total of 120 (or 4° for a total of 90) projections in a window centred on $159 \pm 10\%$ KeV with a total scan duration of 30–45 min.

Raw SPECT data for all subjects were transferred back to the core imaging at the Institute for Neurodegenerative Disorders, New Haven, Connecticut for processing and interpretation. Imaging data were imported to a HERMES (Hermes Medical Solutions) system for iterative (HOSEM) reconstruction and subsequent processing was performed using PMOD (PMOD Technologies). A Chang 0 attenuation correction was applied using a customized mu determined empirically from

Table 1 Subjects' demographics and clinical data

Characteristics	Control	SWEDD	PD	PD-WT	PD-T
<i>n</i>	185	56	345	106	37
Sex, M/F	126/59	40/16	231/114	78/28	21/16
Age, years, mean \pm SD (range)	61 ± 11 (31–84)	60 ± 10 (38–83)	62 ± 10 (34–85)	59 ± 9 (34–77)	64 ± 11 (38–82) [†]
Family members with PD, <i>n</i>	8	21*	87*	30	11
Disease duration, months, mean \pm SD (range)		6.5 ± 6.6 (1–24)	6.3 ± 6.0 (0–35)	5 ± 4.1 (1–23)	5.9 ± 5.7 (1–25)
MDS UPDRS total, mean \pm SD (range)		24.6 ± 13.8 (6–59)	30.4 ± 13.2 (6–76) [#]	29.7 ± 13.4 (6–73)	23.0 ± 10.5 (12–52) [†]
MDS UPDRS-III motor, mean \pm SD (range)		12.5 ± 6.8 (2–36)	19.6 ± 8.9 (3–57) [#]	18.3 ± 8.8 (3–41)	14.7 ± 6.1 (6–33)
HY stage, mean \pm SD (range)		1.4 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)	1.3 ± 0.5 (1–2)

PD = Parkinson's disease; PD-WT = Parkinson's disease patients without resting tremor; PD-T = Parkinson's disease patients with a tremulous phenotype; MDS-UPDRS = Movement Disorder Society Unified Parkinson's Disease Rating Scale; HY = Hoehn and Yahr stage.

Significant differences indicate differences compared to controls (* $P < 0.05$), compared to SWEDD ([#] $P < 0.05$), and compared to PD-WT ([†] $P < 0.05$).

the anthropomorphic brain phantom acquired at each site. A standard Gaussian 3D 6.0 mm filter was applied to each image volume and then normalized to standard MNI space. Two independent readers who were blinded to the subjects' demographics and characteristics interpreted each scan. In the event of a disagreement there was a consensus review process to determine the final scan interpretation.

Region of interest analysis

For this study, normalized SPECT images for all subjects were downloaded from the Parkinson's Progression Markers Initiative website (<http://www.ppmi-info.org/>) on 19 March 2014. Available images were loaded together with a single subject MRI template in Montreal Neurological Institute space available in Statistical Parametric Mapping using Analyze 11.0 software (Mayo Clinic). Transverse image slices for each subject were visually inspected in the x -, y -, and z -axes to ensure the images were adequately aligned to the MRI. Misaligned images were excluded as detailed above.

Specific ^{123}I -FP-CIT binding in the striatum and the raphe nuclei was calculated using a region of interest analysis. Region of interest sampling of the putamen, rostral raphe nuclei, caudal raphe nuclei, and occipital cortex were manually drawn on the MRI template using Analyze 11.0, before being loaded onto the SPECT images. Once transferred, regions of interest were manually adjusted on the SPECT image to account for individual variation without changing the shape or size of the template.

A region of interest was traced by hand according to the anatomical border of the right and left putamen on 10 consecutive slices, respectively. Average region of interest volumes for the right putamen and left putamen were 530 and 478 mm³, respectively.

Region of interest sampling of the raphe complex were drawn according to the anatomical location of the rostral and caudal raphe nuclei, as described by Hornung (2003). A region of interest was drawn to encompass the nuclei of the rostral group in the midbrain and rostral pons on nine consecutive slices. A region of interest was drawn along the caudal pons and medulla corresponding to the caudal raphe nuclei on eight consecutive slices. Average region of interest volumes for the rostral and caudal raphe were 591 and 264 mm³, respectively.

The occipital cortex was chosen as a reference region for non-specific signal due to its low levels of monoaminergic transporters (Olivier *et al.*, 2000). A region of interest was drawn encompassing the right and left occipital cortex on 10 consecutive slices. The average volume of the right and left occipital region of interest was 2648 mm³.

From regional count densities, specific binding ratios were calculated using the following formula. This is also referred to as non-displaceable binding potential for tracers at equilibrium binding (Innis *et al.*, 2007).

Specific binding ratio

$$= \frac{\left(\frac{\text{mean region of interest counts}}{\text{pixel}}\right) - \left(\frac{\text{mean occipital counts}}{\text{pixel}}\right)}{\left(\frac{\text{mean occipital counts}}{\text{pixel}}\right)} \quad (1)$$

The mean specific binding ratio values for the right and left putamen were averaged to give the binding ratio for the

average putamen. The specific binding ratio of the rostral raphe and caudal raphe was summed to give the specific binding ratio for the total raphe nuclei.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences version 22. Between-subject comparisons of Parkinson's disease, control and SWEDD findings were evaluated with ANOVA followed by a *post hoc* Bonferroni correction. Comparisons of subject characteristics between patients with tremulous Parkinson's disease and patients without resting tremor were performed using the Student's *t*-test.

The Kolmogorov-Smirnov normality test was used to assess for normal distribution of the data. As the specific binding ratio data was not normally distributed, a natural logarithm transformation, $\ln(x + \text{constant})$, was used to successfully normalize the data. The transformed specific binding ratio data were used in all subsequent statistical analysis.

For intergroup comparisons of specific binding ratios, an age-adjusted general linear model was used for pair-wise comparisons and a Bonferroni correction for multiple comparisons was applied. A linear regression analysis was used to investigate the association between raphe specific binding ratios, resting tremor scores, and levels of non-motor symptoms in patients with Parkinson's disease.

Results

Serotonin transporter availability

^{123}I -FP-CIT raphe binding did not correlate with subjects' age ($\beta = -0.02$, $P = 0.619$). Specific binding ratios of the raphe nuclei, reflecting serotonin transporter availability, were compared between the three cohorts (Controls, SWEDDs, and Parkinson's disease). The mean raphe serotonin transporter availability for the Parkinson's disease cohort was significantly lower than both healthy controls ($P < 0.01$) and SWEDD subjects ($P < 0.05$). Healthy controls and SWEDDs showed similar raphe binding (Fig. 1).

To further evaluate raphe function in this large Parkinson's disease cohort compared to controls, we identified individual Parkinson's disease patients with raphe binding ratios 1.5 and 2 standard deviations (SD) above and below the control's mean raphe binding ratio (Fig. 2). Two hundred and eighty-seven Parkinson's disease patients (83.2% of cohort) had normal raphe binding ratios lying within 1.5 SD above and below the control mean. Fifteen patients with Parkinson's disease (4.3% of cohort) had a raphe binding ratio > 1.5 SD above the control's mean. Twenty-two patients with Parkinson's disease (6.4% of cohort) had a raphe binding ratio between 1.5 and 2 SDs below the control's mean. Twenty-one patients (6.1% of cohort) had an abnormal raphe binding ratio < 2 SD below the control's mean. Figure 3 shows an example of a patient with Parkinson's disease with normal raphe binding compared to a patient with reduced binding.

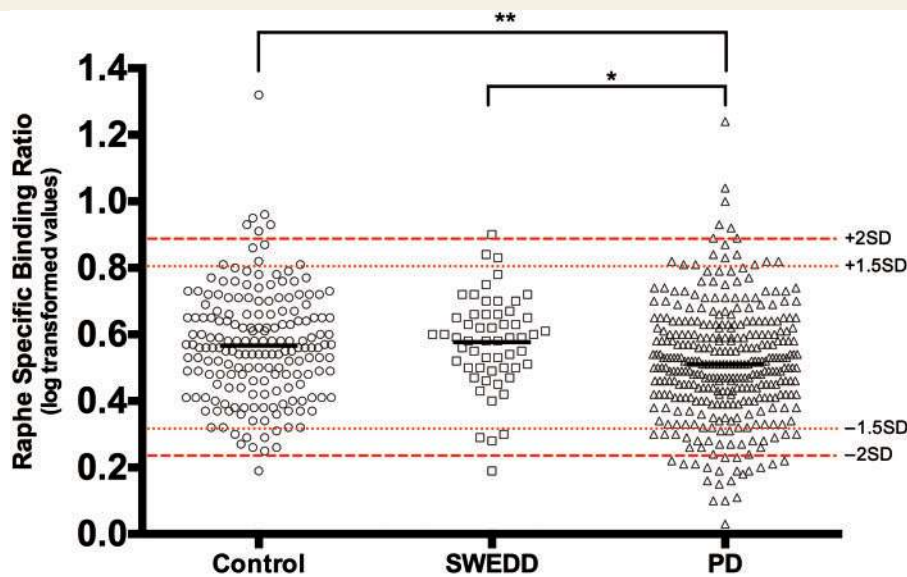


Figure 1 Raphe binding in control, SWEDD, and Parkinson's disease cohort. Raphe values represent mean of the natural log transformation, $\ln(\text{Raphe specific binding ratio} + \text{constant})$. Error bars represent 95% confidence intervals. Sample size: Controls ($n = 185$), SWEDD ($n = 56$) and Parkinson's disease ($n = 345$). Significant differences: $**P < 0.01$, $*P\text{-value} < 0.05$. PD = Parkinson's disease.

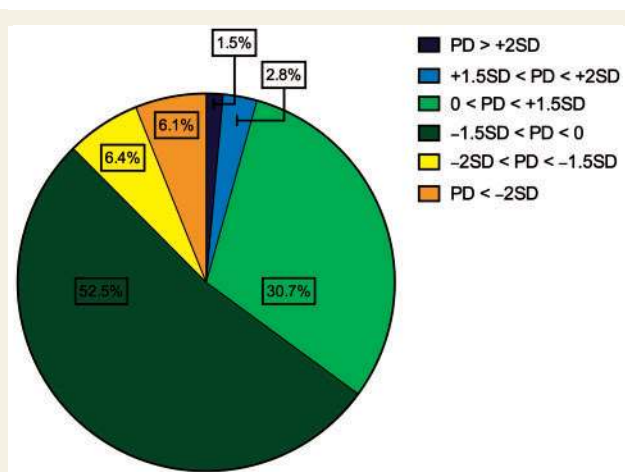


Figure 2 Raphe binding in the Parkinson's disease cohort in relation to healthy controls. Patients with Parkinson's disease (PD, $n = 345$) were sorted into six groups based on their raphe specific binding ratio relative to controls' mean and standard deviation: (i) binding > 2 SD above controls' mean; (ii) binding lies between 2 SD and 1.5 SD above controls' mean; (iii) raphe binding lies between 1.5 SD above mean of control and controls' mean; (iv) raphe binding lies between control's mean and 1.5 SD below controls' mean; (v) raphe binding between 1.5 and 2 SD below controls' mean; and (vi) raphe binding lower than 2 SD below control's mean. The percentage of the Parkinson's disease cohort in each group is displayed on the corresponding pie slice.

Raphe serotonin transporter availability was also compared between Parkinson's disease patients with a tremulous phenotype and patients without resting tremor. Tremulous patients had a significantly lower mean raphe specific binding ratio compared to patients without tremor

($P < 0.05$). Interestingly, the tremulous group had a significantly higher specific binding ratio in the putamen, reflecting dopamine transporter availability, than patients without tremor ($P < 0.05$) (Fig. 4).

Clinical correlations

A linear regression analysis was used to determine the association between raphe specific binding ratios and scores of resting tremor including amplitude, constancy, and index of tremor severity. In the entire Parkinson's disease cohort, raphe serotonin transporter binding was associated with all of these parameters including rest tremor amplitude ($\beta = -0.106$, $P < 0.05$), rest tremor constancy ($\beta = -0.109$, $P < 0.05$), and the index of rest tremor severity ($\beta = -0.104$, $P < 0.05$). The correlation between raphe serotonin transporter binding and index of rest tremor severity in the Parkinson's disease cohort is shown in Fig. 5. In the subgroup of Parkinson's disease patients with a tremulous phenotype, raphe binding ratios were significantly associated with rest tremor constancy ($\beta = -0.380$, $P < 0.05$) and the index of rest tremor severity ($\beta = -0.322$, $P < 0.05$). The correlation with index of rest tremor severity in the tremulous Parkinson's disease group is shown in Fig. 6. However, there was no correlation with rest tremor amplitude in the tremulous Parkinson's disease group.

A similar regression analysis was used to investigate the association between raphe binding and non-motor symptoms of fatigue, depression, excessive daytime sleepiness, and rapid eye movement sleep behaviour disorder. Levels of raphe binding did not correlate with severity of any of these non-motor symptoms in the Parkinson's disease cohort (Table 2).

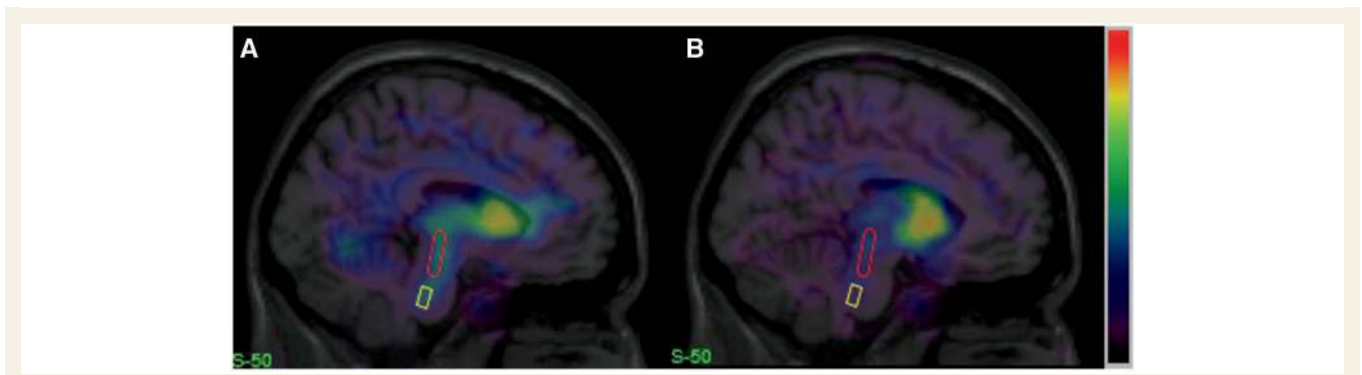


Figure 3 SPECT and MRI scans of Parkinson's disease patients with preserved raphe binding and decreased binding. Fusion scans of SPECT and MRI are demonstrated showing the placement of region of interests (rostral group in red, caudal group in gold) in the midbrain-pons. The colour scale of the SPECT is normalized to each slice. (A) Parkinson's disease patient with preserved binding in the raphe brainstem complex. (B) Parkinson's disease patient with decreased raphe binding.

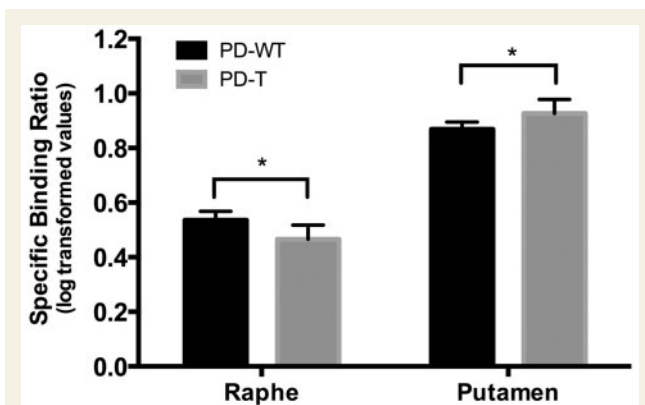


Figure 4 Raphe and striatal binding in Parkinson's disease patients with a tremulous phenotype and patients without resting tremor. Specific binding ratio values represent mean of the natural log transformation, $\ln(\text{specific binding ratio} + \text{constant})$. Error bars represent 95% CI. Significant differences: $*P < 0.05$. Patients with a tremulous phenotype ($n = 37$) were defined as having a rest tremor index score (product of rest tremor amplitude and constancy) ≥ 2 , a bradykinesia score ≤ 1 and rigidity score ≤ 1 . Patients without resting tremor ($n = 106$) were identified from the Parkinson's disease cohort with a rest tremor index score of zero. PD-WT = Parkinson's disease patients without resting tremor; PD-T = Parkinson's disease patients with a tremulous phenotype.

Discussion

To our knowledge, this is the largest study to assess the integrity of the brainstem raphe nuclei in patients with Parkinson's disease and the first involving SWEDD subjects who have possible Parkinson's disease but no evidence of dopaminergic deficit.

In this cohort of 345 patients with early stage Parkinson's disease, we found a significant reduction of ^{123}I -FP-CIT binding in the brainstem raphe nuclei compared to both healthy controls and SWEDD patients, suggesting reduced levels of serotonin transporters located on the somatodendritic compartment of serotonergic neurons

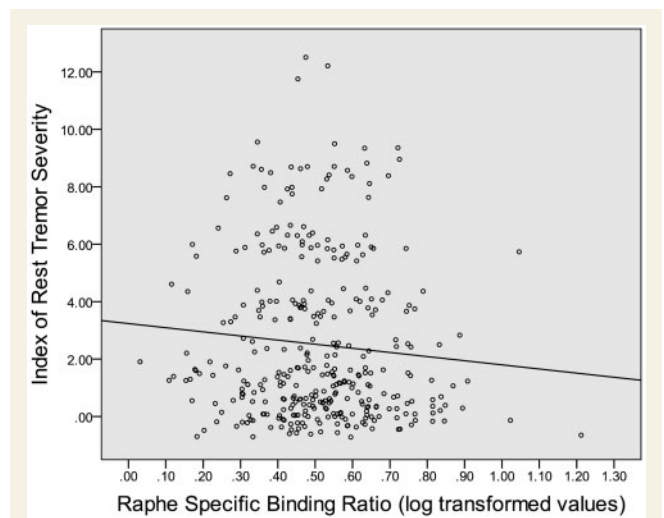


Figure 5 Raphe association with the index of rest tremor severity in Parkinson's disease cohort. Scatter plot showing natural log transformation of raphe binding ratio for patients with Parkinson's disease ($n = 345$) plotted against their rest tremor index scores. Line of best fit is shown. Raphe binding is associated with the index of rest tremor severity ($\beta = -0.104$, $P < 0.05$).

of the raphe complex. However, analysis of individual cases showed that serotonin transporter binding in the raphe nuclei was decreased in 12.5% of the Parkinson's disease cohort (severely in 6.1% and moderately in 6.4%) whereas the majority of Parkinson's disease patients had raphe serotonin transporter availability comparable to that of healthy controls. Interestingly, 4.3% of patients showed increased serotonin transporter availability in the raphe nuclei relative to controls. However, similar high values were also seen in some controls. These findings suggest that, in contrast to the dopaminergic degeneration seen in all Parkinson's disease cases, the brainstem raphe complex is affected only in a subgroup of early drug-naïve Parkinson's disease patients.

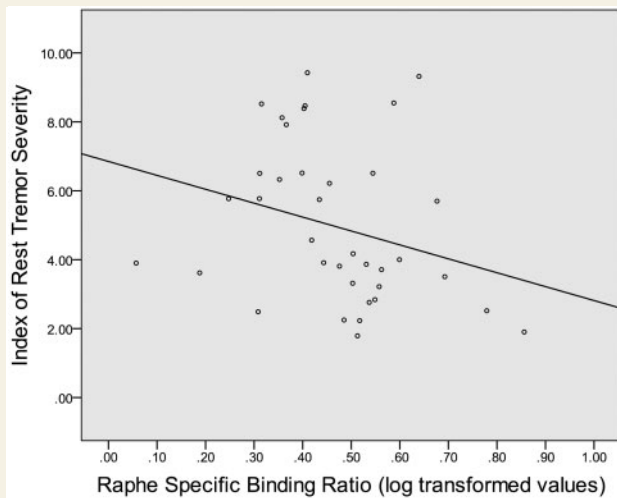


Figure 6 Raphe association with the index of rest tremor severity in Parkinson's disease patients with a tremulous phenotype. Scatter plot showing natural log transformation of raphe binding ratio for Parkinson's disease patients with tremulous phenotype ($n = 37$) plotted against their rest tremor index scores. Line of best fit is shown. Raphe binding is associated with the index of rest tremor severity ($\beta = -0.322$, P -value < 0.05).

Table 2 Linear regression analysis of raphe specific binding ratio and non-motor symptoms

Non-motor symptom	Beta coefficient	P-value
Fatigue	-0.069	0.203 (n.s)
Depression	0.040	0.463 (n.s)
Excessive daytime sleepiness	0.035	0.515 (n.s)
Rapid eye movement sleep behaviour disorder	0.050	0.359 (n.s)

n.s = not significant.

Post-mortem studies have shown a loss of serotonergic neurons and the presence of Lewy bodies in the raphe nuclei of patients with Parkinson's disease (Halliday *et al.*, 1990). Therefore, the reduction in the level of somatodendritic serotonin transporters observed in a subgroup of our patients is likely to reflect a loss of serotonergic cell bodies in the raphe nuclei.

Our finding that the raphe complex is preserved in a majority of early Parkinson's disease cases does not argue against Braak staging, which is based exclusively on the presence of immunoreactive Lewy neurites and Lewy bodies (Braak *et al.*, 2003). Rather, our findings suggest that alpha synuclein pathology in the raphe nuclei observed by Braak and colleagues is unlikely to reflect profound neuronal loss in this brain region. However, it is important to recognize that normal raphe serotonin transporter binding, seen in the majority of studied patients, does not imply normal serotonergic function in these patients. In fact, the degenerative process in the raphe-striatal neurons in Parkinson's disease could target the nerve terminals rather than the cell bodies (Chinaglia *et al.*, 1993). Given the

non-selective nature of the ligand, ^{123}I -FP-CIT, it is not possible to use this method to assess serotonergic terminal field integrity in brain structures where other monoaminergic neuronal populations predominate.

Our findings parallel previous SPECT and PET imaging studies which have shown that serotonin transporter availability was significantly reduced in the brainstem raphe nuclei in patients with advanced Parkinson's disease (Berding *et al.*, 2003; Guttman *et al.*, 2007; Politis *et al.*, 2010a) but normal or only mildly reduced in early stages of the disease. Two ^{123}I - β -CIT (^{123}I -ioflupane-beta-carbomethoxy-3-beta-4-iodophenyltropane) SPECT studies of 27 and 45 patients with early Parkinson's disease showed that their midbrain serotonin transporter availability was similar to controls (Haapaniemi *et al.*, 2001; Kim *et al.*, 2003) whereas a ^{123}I -FP-CIT SPECT study of 15 patients with early Parkinson's disease showed mild reductions in midbrain serotonin transporter availability (Roselli *et al.*, 2010). PET studies of 10 patients or less have also shown raphe serotonin transporter availability to be normal in early stage Parkinson's disease (Albin *et al.*, 2008; Politis *et al.*, 2010a; Strecker *et al.*, 2011). However, these studies did not include a subgroup of tremulous cases and were underpowered to detect subtle yet significant changes in early stages of the disease. Our finding that the brainstem raphe complex is affected primarily in tremulous Parkinson's disease provides an explanation for the discrepancy between our findings and some previous smaller imaging studies.

Raphe serotonin transporter availability in patients with Parkinson's disease was associated with rest tremor amplitude, constancy, and the index of rest tremor severity. Resting tremor appears to have an independent pathophysiology to the rigidity and bradykinesia seen in Parkinson's disease and may be more resistant to conventional dopaminergic treatments (Fishman, 2008). This suggests that non-dopaminergic systems could be implicated in the aetiology of parkinsonian tremor. Our finding that patients with lower serotonin transporter availability have more severe resting tremor is in keeping with a previous ^{11}C -WAY100635 PET study. This showed that patients with Parkinson's disease have significantly lower levels of 5-HT_{1A} (serotonin receptor subtype 1A) binding in the midbrain raphe compared to healthy controls and this correlated with their severity of resting tremor (Doder *et al.*, 2003).

Furthermore, our patients with a tremulous motor phenotype, defined as having prominent resting tremor but mild bradykinesia and rigidity, had significantly lower serotonin transporter availability in the brainstem raphe nuclei compared to patients with no resting tremor. Serotonin transporter availability was significantly associated with the constancy and severity index of resting tremor although not with amplitude in these tremulous patients. The lack of association with rest tremor amplitude in the tremulous group may simply be due to a lack of a broader spread of amplitude scores. The majority of

patients in this tremulous group had milder rest tremor amplitude scores of two or less, while only five patients had a score of three and none had the maximum possible score of four. Interestingly, this group of patients had less dopaminergic deficits in the putamen compared to patients with non-tremulous Parkinson's disease, as indicated by higher ^{123}I -FP-CIT binding. These findings are in line with post-mortem studies that have shown that patients with tremor-predominant Parkinson's disease have less dopaminergic cell loss in the substantia nigra compared to non-tremor patients, reinforcing the concept that resting tremor is unlikely to occur as a result of dopaminergic degeneration *per se* (Paulus and Jellinger, 1991; Selikhova *et al.*, 2009).

A previous PET study from our Unit reported that tremor-predominant patients had lower serotonin transporter availability in the caudate and putamen compared to akinetic-rigid patients. However, this reduction was associated with severity of action-postural tremor rather than resting tremor in this group (Loane *et al.*, 2013). This study used the selective ligand, ^{11}C -DASB, to assess serotonin transporters in patients with well-established Parkinson's disease. In contrast, we did not find an association between raphe serotonin transporter availability and action-postural tremor in our early Parkinson's disease population using the non-selective ligand, ^{123}I -FP-CIT. It will be interesting to see whether such an association will be present at follow-up studies of our patients when the disease progresses further.

The observed serotonergic changes in the brainstem raphe nuclei do not necessarily mean a serotonin deficiency *per se* causes tremor. It could reflect local parkinsonian pathology in the raphe nuclei interrupting serotonergic output to cerebellar, thalamic, and basal ganglia structures implicated in the aetiology of tremor (Dovzhenok and Rubchinsky, 2012). Further studies should aim at identifying the specific serotonergic circuits associated with different components of tremor which may have important therapeutic implications.

Rape serotonin transporter availability in patients with Parkinson's disease was not associated with non-motor symptoms of fatigue, depression, or sleep disturbance. In a recent study, fatigued patients with Parkinson's disease had lower serotonin transporter availability in striatal and limbic structures but not in the raphe nuclei compared to non-fatigued patients (Pavese *et al.*, 2010). In keeping with these findings, we did not find that serotonin transporter availability in the raphe complex predicts severity of fatigue suggesting that it may be terminal serotonergic disruption in limbic structures that is associated with the occurrence of fatigue.

Previous neuroimaging studies have investigated the role of the serotonergic raphe complex in the pathophysiology of depression in Parkinson's disease. However, the results of these studies have been inconsistent. A ^{123}I - β -CIT SPECT study showed that midbrain raphe serotonin transporter availability was similar in depressed and

non-depressed Parkinson's disease patients with no correlation between radiotracer uptake and depression scores (Kim *et al.*, 2003). In contrast, a ^{123}I -FP-CIT SPECT study found that depressed patients with Parkinson's disease had lower serotonin transporter availability in the midbrain compared to non-depressed patients (Hesse *et al.*, 2009). PET studies have suggested that depression and depressive symptoms are associated with higher serotonin transporter availability (Boileau *et al.*, 2008; Politis *et al.*, 2010b). The reason for the discrepancy between these SPECT and PET findings is unclear and again could be related to small sample sizes in these studies. In the large Parkinson's disease cohort in this study, we report that serotonin transporter availability in the raphe does not predict depressive symptoms.

Preclinical studies suggest the brainstem raphe nuclei contribute to an ascending arousal system that promotes wakefulness and prevents excessive daytime sleepiness (Schwartz and Roth, 2008). In our study, raphe serotonin transporter availability in early Parkinson's disease was not associated with excessive daytime sleepiness though this symptom was not an issue for most cases. In a recent study, patients with excessive daytime sleepiness showed significant decreases in both ^{18}F -DOPA and ^{11}C -DASB binding in the main sleep regulatory centres compared to age-matched healthy volunteers without sleep disorders, suggesting that excessive daytime sleepiness in Parkinson's disease is associated with a complex monoaminergic dysfunction in the neuronal networks responsible for the establishment and maintenance of wakefulness (Pavese *et al.*, 2012). Interestingly, sleep disordered-breathing, which may contribute to daytime somnolence, was not found to be associated with caudal brainstem changes in a PET study of patients with Parkinson's disease (Lelieveld *et al.*, 2012).

A recent imaging study has suggested that cholinergic denervation in cortical, thalamic and limbic structures, rather than dopaminergic or serotonergic denervation, is associated with the occurrence of rapid eye movement sleep behaviour disorder (Kotagal *et al.*, 2012). In line with these findings, we did not find any association between raphe serotonin transporter availability and rapid eye movement sleep behaviour disorder.

While serotonin transporter availability in the raphe nuclei was significantly reduced in patients with Parkinson's disease, SWEDD subjects showed similar serotonin transporter binding to that of healthy controls. SWEDD subjects in the Parkinson's Progression Markers Initiative were patients with a clinical diagnosis of Parkinson's disease but with ^{123}I -FP-CIT imaging showing no evidence of a striatal dopaminergic deficit (Marek, 2003). The PRECEPT study, which was a longitudinal study of 91 SWEDDs suggests that these patients are unlikely to have idiopathic Parkinson's disease. After 22 months of follow-up there was little change in their clinical status and 44% of SWEDDs were diagnosed with an alternative disorder (Marek *et al.*, 2014). Here, we have shown that SWEDD subjects, along with no evidence of a

dopaminergic deficit, have no significant disruption in the raphe serotonergic system that could account for their clinical symptoms.

In conclusion, ^{123}I -FP-CIT SPECT can be used to assess the integrity of the brainstem raphe nuclei in patients with Parkinson's disease. In early stages of the disease, the serotonergic raphe nuclei are affected in about 13% of patients. Longitudinal follow-up studies of these patients with early raphe involvement are required to investigate whether they have a different progression of clinical features and imaging biomarkers. It is possible that over time these patients might develop a different motor and non-motor phenotype and might require a different type of treatment. Raphe complex serotonin transporter availability is associated with the severity of resting tremor but it does not appear to be associated with non-motor symptoms of fatigue, depression, and sleep disturbance in these early stages of the disease.

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