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Preferential loss of serotonin markers in caudate versus putamen in Parkinson's disease

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Interest in serotonergic involvement in Parkinson's disease (PD) has focussed recently on the possibility that the remaining serotonin neurons innervating striatum (caudate and putamen) might release dopamine as a 'false transmitter'—an action that could have both beneficial and harmful (e.g. promotion of levodopa-induced dyskinesias) consequences. Evidence for a brain serotonergic disturbance in PD is derived in large part from findings of decreased binding of different radioligands to the serotonin transporter (SERT), one 'marker' of serotonin neurons. However, it is not known whether the reported changes in SERT binding reflect actual changes in levels of SERT protein or whether concentrations of all serotonin markers are similarly and markedly decreased in the two striatal subdivisions.

We measured levels of SERT immunoreactivity, and for comparison, protein levels of tryptophan hydroxylase (TPH; the marker synthetic enzyme) using a Western blot procedure, as well as concentrations of serotonin, its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and dopamine by HPLC in post-mortem striatum of patients with PD and normal controls.

Whereas concentrations of dopamine were severely decreased (caudate, −80%; putamen, −98%) and showed little (caudate) or no (putamen) overlap between individual control and patient values, levels of all four serotonin markers were less markedly reduced (−30% to −66%) with some patients having distinctly normal levels. Unlike the preferential loss of dopamine in putamen, the caudate was affected more than putamen by loss of all serotonin markers: serotonin (−66% versus −51%), 5-HIAA (−42% versus −31%), SERT (−56% versus −30%) and TPH (−59% versus −32%). Striatal serotonin concentration was similar in the subset of patients reported to have had dyskinesias versus those not reported to have had this drug complication.

Previous findings of decreased SERT binding are likely explained by loss of SERT protein. Reduced striatal levels of all of the key serotonergic markers (neurotransmitter and metabolite, transporter protein, synthesizing enzyme protein) provide strong evidence for a serotonergic disturbance in PD, but with some patients affected much more than others. The more marked caudate reduction suggests that raphe neurons innervating this area are more susceptible to 'damage' than those innervating putamen and that any functional impairment caused by striatal serotonin loss might primarily involve the caudate. Questions related to the, as yet undetermined, clinical consequences in PD of a striatal serotonin deficiency (caudate: cognitive impairment?) and preservation (putamen: levodopa-induced dyskinesias?) should be addressed in prospective brain imaging and pharmacological studies.

Keywords: Parkinson's disease; serotonin transporter; caudate; putamen; dyskinesia

Abbreviations: 5-HIAA = 5-hydroxyindoleacetic acid; 5-HT = serotonin; HVA = homovanillic acid; NSE = neuron-specific enolase; PD = Parkinson's disease; PMI = post-mortem interval; SERT = serotonin transporter; TPH = tryptophan hydroxylase

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Introduction

Although loss of nigrostriatal dopamine neurons is the fundamental defining feature of idiopathic Parkinson's disease (PD; Ehringer and Hornykiewicz, 1960), much interest has been focussed on the possibility that some clinically significant non-motor problems in PD (e.g. mood, cognitive and sleep disturbances) could be explained by damage to brain serotonin neurons (Meyer *et al.*, 2001; for review see Kish, 2003). It has been suggested that serotonin neurons innervating the striatum (caudate and putamen) might help in the anti-parkinsonian action of levodopa, by converting exogenous dopa to dopamine and subsequently releasing dopamine from the serotonin neurons as a false neurotransmitter (Ng *et al.*, 1970, 1971; Arai *et al.*, 1994, 1995; Tanaka *et al.*, 1999; Maeda *et al.*, 2005). However, new findings indicate that such dopamine might be released from serotonin neurons in a non-physiological manner leading to excessive swings of dopamine release which (as suggested by experimental animal data) could promote levodopa-induced dyskinesias—a situation in which *loss* of serotonin neurons innervating the striatum might actually be helpful in PD (Carta *et al.*, 2007).

There still is not a consensus in the neuropathological literature whether loss of cell bodies in the raphe brainstem area that are considered to provide the serotonergic innervation to the striatum is a characteristic of PD (see later). The evidence for damage to serotonin neurons in PD is in fact inferred, primarily, from biochemical reports of decreased brain levels of serotonin (Bernheimer *et al.*, 1961; Scatton *et al.*, 1983; D'Amato *et al.*, 1987; Wilson *et al.*, 1996; Calon *et al.*, 2003) and binding of a variety of radioligands having some specificity to the serotonin transporter (SERT; Raisman *et al.*, 1986; D'Amato *et al.*, 1987; Chinaglia *et al.*, 1993; Kerenyi *et al.*, 2003; Guttman *et al.*, 2007), one 'marker' of serotonin neurons. However, the generic possibility that conformational changes in SERT might alter protein binding but not expression (see Xie *et al.*, 2006) make this inference uncertain. Furthermore, the question whether a serotonergic disturbance in PD is a robust change is still an open question, with one group recently stating the understanding that the '. . . serotonergic innervation of the striatal complex remains relatively intact in most PD patients. . .' (Carta *et al.*, 2007).

Our aim was to establish systematically the extent to which levels of all available biochemical markers of serotonin neurons (serotonin, its metabolite 5-hydroxyindoleacetic acid [5-HIAA], SERT and tryptophan hydroxylase [TPH, rate-limiting serotonin biosynthetic marker enzyme]) are decreased in striatum of patients with PD, paying special attention to the degree of overlap between the PD and control groups and whether concentrations of the markers are equally decreased in both striatal nuclei. Post-mortem brain was selected for analysis, as this permits, unlike living brain, measurement of all of the serotonin markers. SERT and TPH protein levels were directly determined using a Western blotting procedure.

Patients and methods

Autopsied brains were obtained from a total of 23 patients with PD and 31 normal subjects who died without evidence of neurological or brain neuropathological abnormalities. One half-brain was used for neuropathological examination, whereas the other half was frozen for neurochemical analyses. All patients had received the clinical diagnosis of PD (20 had received levodopa medication) with the exception of two cases described as having 'neurological illnesses' (see Table 1 for patient information). The Hoehn and Yahr scale (Hoehn and Yahr, 1967) was determined retrospectively from the case records for those patients in which sufficient information was provided. Brain neuropathological analyses of all patients showed substantia nigra cell loss (ranging from patchy to severe) and presence of Lewy bodies. Estimated duration of illness was unknown in three cases and ranged from 3 to 29 years for the remaining patients. No information (e.g. by formal testing) could be obtained on the neuropsychiatric status of the patients. Levodopa-induced dyskinesias were documented in seven patients. For 12 patients no levodopa-dyskinesias were reported in the case records that did provide clinical information on the subjects. Of the remaining four patients, two had not received levodopa and no clinical information could be obtained on two other patients. The causes of death for the control subjects were myocardial infarction (11), cardiac failure (5), rupture of atherosclerotic aorta (1), atheroembolization (1), cancer (4), pulmonary embolism (3), bronchial haemorrhage (1), diffuse interstitial pulmonary disease (1), natural death (1) and unknown (3).

Brain dissection followed published procedures (Kish *et al.*, 1988) and the caudate and putamen samples were taken from the representative intermediate (along a dorsoventral gradient) subdivision of the middle (along a rostrocaudal gradient) portion of both nuclei, i.e. slices #4 or #5 for caudate and slices #7 or #8 for putamen as described in Kish *et al.* (1988) (see Fig. 1 therein). For the detailed rostrocaudal distribution study of serotonin and dopamine (in a subset of 12 patients and 12 controls), additional samples were taken from the intermediate subdivision of the rostral (slice #2 for caudate and slice #4 for putamen) and caudal (slice #7 for caudate and slice #10 for putamen) portions of both nuclei. Levels of dopamine, serotonin and their metabolites homovanillic acid (HVA) and 5-HIAA, respectively, were measured by HPLC-electrochemical detection (Wilson *et al.*, 1994) in all of the 23 PD patients and in 23 control subjects, which were matched for age (76 ± 2 and 73 ± 2 years, respectively; mean ± SEM) and post-mortem interval (PMI; 12 ± 1 and 12 ± 1 h, respectively). Concentrations of dopamine, HVA, serotonin and 5-HIAA have previously been reported in ten of the 23 patients with PD (Wilson *et al.*, 1996). Levels of SERT, TPH and the 'control' proteins α-tubulin and neuron-specific enolase (NSE) were determined in 10 PD patients and in 14 control subjects who were also matched for age (77 ± 3 and 73 ± 2 years, respectively) and post-mortem interval (14 ± 2 and 12 ± 2 h, respectively).

Levels of SERT and TPH immunoreactivity were determined in tissue homogenates (5–60 μg protein for SERT; 50 μg for TPH) by

Table 1 Patient information and striatal levels of serotonin and the serotonin transporter

Case No./	PMI	Disease	H-Y	Pathology[a]		Levodopa exposure			Cause of death	Serotonin[c]		SERT[d]	
Sex/age, years	h	Duration, years	Stage	SN	LC	Dose, mg/d[b]	Duration, years	Dyskinesias		CN	PUT	CN	PUT
1/F/75	22	10	?	++	++	250	8	Yes	Unknown	0.13	0.09	NE	NE
2/F/87	10	10	5	+++	+++	500	3	No	Bronchopneumonia	0.03	0.06	NE	NE
3/M/71	16	5.5	5	+++	+++	1500	2.5	No	Unknown	0.09	0.06	NE	NE
4/M/61	5	6	5	++	++	400	6	No	Pulmonary embolism	0.02	0.10	NE	NE
5/F/71	18	7	5	++	–	400	5	No	Bronchopneumonia	0.09	0.10	NE	NE
6/M/78	12	5	2	++	++	400	0.5	No	Ruptured MI	0.08	0.20	NE	NE
7/M/73	18	7	3	+++	+	?	7	Yes	Bronchopneumonia	0.17	0.20	NE	NE
8/F/80	3	?	1	++	+	No levodopa			MI	0.19	0.34	NE	NE
9/M/84	12	5.5	5	+++	+++	400	5.5	No	Unknown	0.15	0.15	NE	NE
10/M/78	12	15	4	+++	?	400	8	No	Pneumonia	0.09	0.27	NE	NE
11/M/67	12	27	?	+++	–	1000	15	Yes	Pneumonia	0.04	0.06	NE	NE
12/M/74	2	28	?	+++	+	800	?	?	Septic shock	0.11	0.17	NE	NE
13/M/72	3	10	5	+++	+++	?	?	Yes	Bronchopneumonia	0.17	0.11	NE	NE
14/F/79	15	19	5	+/++	+	600	8	Yes	Pneumonia	0.09	0.12	0.21	0.40
15/F/77	6	16	3	+/++	+	1000	12	Yes	Unknown	0.28	0.14	0.62	0.81
16/F/66	18	3	2	+/++	+	?	3	No	Aspiration of gastric content	0.16	0.16	0.33	0.61
17/F/81	19	29	5	++	–	500	19	Yes	MI	0.02	0.16	0.18	0.29
18/M/69	18	?	?	+++	+++	No levodopa			Bronchopneumonia	0.09	0.13	0.29	0.44
19/F/96	18	6	4	+++	+++	?	6	No	Unknown	0.16	0.15	0.23	1.33
20/M/79	10	>10	?	++/+++	+/++	?	?	?	Pneumonia	0.13	0.18	0.26	0.43
21/M/70	11	25	4	+++	+	800	23	No	Unknown	0.14	0.16	0.53	0.99
22/F/71	3.5	25	5	++	+	?	>10	No	Pneumonia	0.07	0.06	0.26	0.54
23/M/83	19	17	5	++/+++	+/++	200	>10	No	Unknown	0.14	0.13	0.15	0.38

H-Y = Hoehn-Yahr; CN = caudate; LC = locus coeruleus; MI = myocardial infarction; NE = not examined; PMI = postmortem interval; PUT = putamen; SERT = serotonin transporter; SN = substantia nigra. All patients had a clinical diagnosis of Parkinson's disease except for patients 8 and 18, who were diagnosed as having a neurological illness and degenerative neurological disorder, respectively. The presence of Lewy bodies in substantia nigra was confirmed in all cases.
[a] – unremarkable; + mild; ++ moderate; +++ severe. [b]most recent before death. [c]Serotonin levels in ng/mg of wet tissue. [d]serotonin transporter levels in μg tissue standard protein/μg tissue sample protein.

quantitative immunoblotting as described in detail (Kish *et al.*, 2005). This procedure employed five concentrations of tissue standard (0.5–5 μg of protein), consisting of a pooled human caudate sample, running on each gel together with the samples. SDS–PAGE samples for SERT protein measurement were incubated at room temperature for 30 min (i.e. not subjected to any elevated temperature per Qian *et al.*, 1995; see also Wilson *et al.*, 1996). The method for TPH protein determination was similar with the exception of 5-min boiling of the homogenate sample prior to assay and the use of the following primary (RBI, mouse monoclonal anti-tryptophan hydroxylase, clone WH-3, cat#T0678 from Sigma, St Louis, Missouri) and secondary (Goat anti-mouse IgG3, cat#1100-05 from Southern Biotechnology Associates, Inc., Birmingham, AL) antibodies. The concentration of SERT or TPH protein immunoreactivity in each lane was determined by interpolation from the linear standard curve and expressed as microgram tissue standard protein per microgram sample protein. The antibody raised against SERT was immunoreactive for a predominant broad band that centered at approximately 77 kDa (see details of SERT method and assay reliability in Kish *et al.*, 2005). As expected, the antibody raised against TPH was immunoreactive for a predominant band that centred at ~53 kDa and which is considered, in human brain, to be predominantly the TPH2 isoform (Sakowski *et al.*, 2006). The coefficients of variation were 11.8 and 9.3% for TPH immunoreactivity within and between blots. To address issues of protein loading and loss of cellular elements, levels of α-tubulin and NSE were also measured by quantitative immunoblotting procedure in the same tissue homogenates as described (Tong *et al.*, 2007). For simplicity only, SERT or TPH 'immunoreactivity' will be referred to as 'protein'.

Our pre-planned hypothesis was that levels of the four major outcome measures, serotonin, 5-HIAA, SERT and TPH, would be decreased in striatum of the patients with PD.

Statistical analyses were performed by using StatSoft STATISTICA 7.1 (Tulsa, Oklahoma, USA). Differences in levels of the outcome measures between PD patients and control subjects and between the striatal subdivisions were analysed by two-way ANOVA followed by *post hoc* Newman–Keuls tests. Differences in percentage changes in PD patients as compared to the control subjects between the striatal subdivisions were analysed by paired two-tailed Student's t-tests or by repeated measures ANOVA followed by *post hoc* Newman–Keuls tests. Possible correlations between the outcome measures and the clinical indices of the patients were examined by Pearson product-moment correlation or by Spearman rank-order correlation as indicated in the text. No statistically significant correlation (Pearson) was observed between age or PMI of the subjects and any of the outcome measures.

Table 2 Changes in dopamine and serotonin markers in the striatum in Parkinson's disease (PD)

	Control	PD	*P*	%change
Caudate				
Dopamine	4.00 ± 0.39 (23)	0.79 ± 0.17 (23)**	<0.0001	−80†
HVA	6.00 ± 0.54 (23)#	2.75 ± 0.31 (23)**	<0.0001	−54†
HVA/DA (mol/mol)	1.4 ± 0.1 (23)	10.0 ± 2.7 (23) #	0.003	+600†
Serotonin	0.34 ± 0.04 (23)	0.11 ± 0.01 (23)**	<0.0001	−66†
5-HIAA	0.77 ± 0.08 (23)#	0.45 ± 0.05 (23)*#	0.0013	−42†
5-HIAA/5-HT (mol/mol)	2.4 ± 0.2 (23)	4.9 ± 1.1 (23)*	0.033	+108
5-HT/DA (mol/mol)	0.08 ± 0.01 (23)	0.45 ± 0.12 (23)#	0.005	+450†
SERT	0.69 ± 0.10 (14)	0.31 ± 0.05 (10)*#	0.005	−56†
TPH	0.28 ± 0.04 (14)	0.11 ± 0.03 (10)	0.011	−59‡
NSE	0.72 ± 0.03 (14)	0.82 ± 0.03 (10)	0.04	+14
α-tubulin	0.88 ± 0.08 (14)	0.93 ± 0.08 (10)	0.67	+8
Putamen				
Dopamine	4.83 ± 0.45 (23)	0.11 ± 0.03 (23)**	<0.0001	−98
HVA	8.25 ± 0.72 (23)	2.71 ± 0.28 (23)**	<0.0001	−67
HVA/DA (mol/mol)	1.5 ± 0.1 (23)	43.5 ± 7.2 (23)**	<0.0001	+2800
Serotonin	0.29 ± 0.03 (23)	0.14 ± 0.01 (23)**	<0.0001	−51
5-HIAA	1.12 ± 0.13 (23)	0.77 ± 0.08 (23)*	0.023	−31
5-HIAA/5-HT (mol/mol)	3.9 ± 0.4 (23)	5.9 ± 0.9 (23)	0.041	+53
5-HT/DA (mol/mol)	0.06 ± 0.01 (23)	2.74 ± 0.61 (23)**	<0.0001	+4600
SERT	0.89 ± 0.11 (14)	0.62 ± 0.10 (10)	0.11	−30
TPH	0.56 ± 0.12 (14)	0.38 ± 0.10 (10)	0.28	−32
NSE	0.79 ± 0.03 (14)	0.91 ± 0.03 (10)	0.02	+15
α-tubulin	1.03 ± 0.11 (14)	1.07 ± 0.08 (10)	0.77	+5

Caudate and putamen samples were taken from the intermediate subdivision of the middle section of both nuclei (see Methods). Data are given as mean ± SEM (n) in ng/mg of wet tissue for dopamine, homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) and in μg tissue standard protein/μg tissue sample protein for serotonin transporter (SERT), tryptophan hydroxylase (TPH), neuron specific enolase (NSE), and α-tubulin. The *P* values given are results of 2-tailed Student's *t*-tests. *$P < 0.05$, **$P < 0.0005$, PD versus control; #$P < 0.05$, caudate versus putamen (two-way ANOVA followed by *post hoc* Newman-Keuls tests). †$P < 0.05$, ‡$P = 0.05$, caudate versus putamen (paired, 2-tailed Student's *t*-tests).

Results

Striatal monoamine neurotransmitter markers

Table 2 summarizes the mean levels of dopamine and serotonin markers in striatum of all the patients with PD and matched controls. Mean levels of dopamine were severely and significantly decreased (caudate, −80%; putamen, −98%) in the striatum (representative 'middle' slice of both nuclei) of the patients with PD. In contradistinction, striatal concentrations of the four serotonin markers (serotonin, 5-HIAA, SERT, TPH) were on average less markedly decreased (range: −30 to –66%) and with serotonin itself being the most affected by loss. Striatal concentrations of the two 'control' proteins α-tubulin and NSE were normal (α-tubulin) or slightly (by 15%) increased (NSE). SERT immunoreactivity was also decreased by 34 to 55% in extra-striatal brain areas (frontal, occipital, parietal cortices; hippocampus; medial dorsal and pulvinar thalamus) but with significant changes limited to frontal cortex (−55%) and medial dorsal thalamus (−39%) (data not shown).

Caudate versus putamen differences

Within the striatum, mean levels of dopamine and HVA were, as expected (Kish *et al.*, 1988), decreased more in putamen than in caudate (Table 2). Similarly, analysis of individual subject values for the striatum (Fig. 1A) revealed that 22 of the 23 patients with PD had dopamine concentrations decreased more in putamen than in caudate. [The one exception was a patient having a very long duration (27 years) of illness with severe (−98% in the putamen and –99% in the caudate) dopamine loss in both striatal subdivisions.] In contrast, mean levels of the four serotonin markers were decreased more in caudate than in putamen (serotonin: caudate, −66%, putamen, −51%; 5-HIAA: caudate, −42%, putamen, −31%; SERT: caudate, −56%, putamen, −30%; TPH: caudate, −59%, putamen, −32%). Similarly, in individual patients, the caudate was typically affected more than the putamen by loss of the serotonin marker (Fig. 1A, serotonin and 5-HIAA, 18 of 23 patients; TPH, 9 of 10 patients; SERT, all 10 patients; see also Fig. 1B for representative blots of SERT, TPH, and control proteins in caudate versus putamen in controls and patients with PD). The molar ratio of 5-HIAA to 5-HT, an index of serotonin metabolism/turnover, was increased in striatum, with the differences more marked in caudate (+108%) than in putamen (+53%). These differences between controls and PD patients were statistically significant for serotonin, 5-HIAA, the 5-HIAA/5-HT molar ratio and SERT in the caudate, whereas the changes

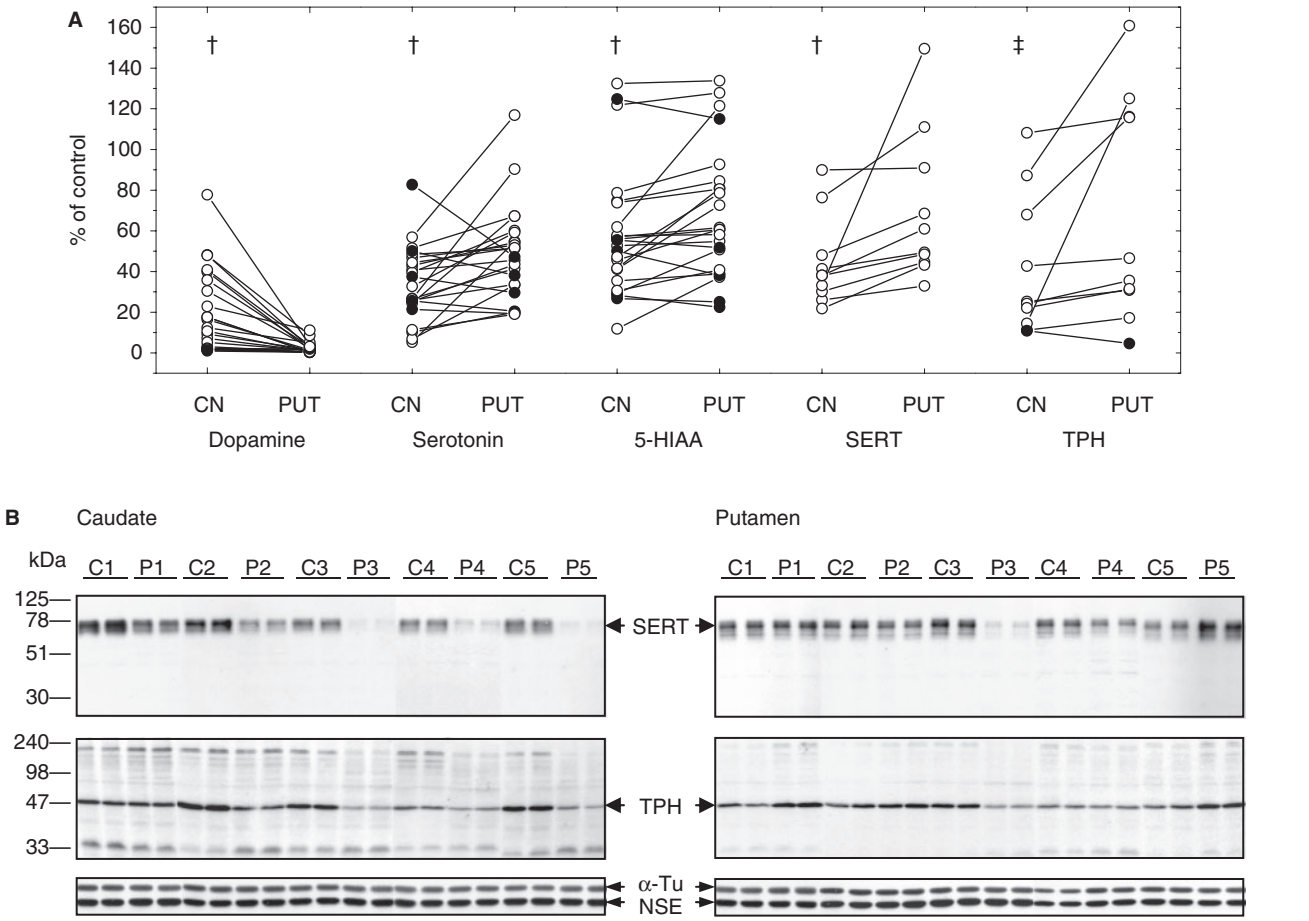

Fig. 1 Preferential loss of the serotonergic markers in the caudate nucleus versus putamen in Parkinson's disease. (**A**) Levels of dopamine and the serotonergic markers, including serotonin, 5-hydroxyindoleacetic acid (5-HIAA), serotonin transporter (SERT) and tryptophan hydroxylase (TPH) expressed as percentages of the mean of the control subjects, in the caudate nucleus (CN) and putamen (PUT) in Parkinson's disease ($n = 23$ for dopamine, serotonin and 5-HIAA; $n = 10$ for SERT and TPH). The solid circles identify cases where the caudate level is higher than that in the putamen. $^{\dagger}P < 0.05$, $^{\ddagger}P = 0.05$, caudate versus putamen (paired, 2-tailed Student's *t*-tests). (**B**) Representative immunoblots of SERT and TPH in the caudate nucleus and putamen in five cases each of the control subjects (C1-C5) and the PD patients (P1-P5). Duplicate samples were run for each subjects and the blots were stripped and reprobed for control proteins α-tubulin (α-Tu) and neuron-specific enolase (NSE).

were significant for only serotonin and 5-HIAA in the putamen.

To establish whether the caudate versus putamen differences for serotonin would be observed along the entire rostrocaudal gradient of the striatum, we also examined the intra-striatal anterior–posterior distribution of serotonin in caudate and putamen in a subset ($n = 12$ each) of the PD patients and matched control subjects. As shown in Table 3, the caudate was more affected by serotonin loss than the putamen in the rostral and middle slices (−67% versus −50%; −64% versus −42%, respectively) whereas the extent of loss was similar in the very posterior portions of the nuclei (caudal caudate, −43%; caudal putamen, −45%). For dopamine, the situation was reversed: the putamen was affected more than the caudate by dopamine loss in the middle and caudal slices (putamen, −97% and −99%; caudate, −80% and −64%, respectively), whereas the extent of reduction was similar in the rostral portions of both nuclei (putamen, −89%; caudate, −91%).

Analysis of individual control versus PD values

As shown in Fig. 2, there was no overlap between the ranges of the individual control and PD values for dopamine in the putamen while three PD subjects had caudate dopamine values falling just within the lower limit of the control range. In contrast, there was a substantial overlap between control and PD striatal serotonin marker values with many patients having values falling within the range of the controls (Fig. 3). However, for most of the serotonin markers, a greater proportion of PD patients had values below the lower limit of the control range in the caudate than in the putamen (see Fig. 3; serotonin: caudate,

Table 3 Rostrocaudal distribution of serotonin and dopamine in the striatum in Parkinson's disease (PD)

		Control (n = 12)	PD (n = 12)	%loss
Serotonin				
	Slice #2, rostral	0.29 ± 0.03	0.09 ± 0.02**	−67†
Caudate	Slice #4, middle	0.27 ± 0.03	0.10 ± 0.02**	−64†
	Slice #7, caudal	0.17 ± 0.02#	0.10 ± 0.01	−43
	Slice #4, rostral	0.35 ± 0.04	0.17 ± 0.04**	−50
Putamen	Slice #7, middle	0.26 ± 0.03	0.15 ± 0.03	−42
	Slice #10, caudal	0.20 ± 0.03#	0.11 ± 0.02	−45
Dopamine				
	Slice #2, rostral	2.82 ± 0.28	0.25 ± 0.06**	−91
Caudate	Slice #4, middle	3.76 ± 0.48	0.73 ± 0.19**	−80‡
	Slice #7, caudal	3.28 ± 0.29	1.19 ± 0.30**	−64‡
	Slice #4, rostral	4.79 ± 0.48	0.51 ± 0.30**	−89
Putamen	Slice #7, middle	4.47 ± 0.38	0.13 ± 0.05**	−97
	Slice #10, caudal	5.22 ± 0.66	0.06 ± 0.03**	−99

Data are given as mean ± SEM in ng/mg of wet tissue. **$P < 0.0005$, PD versus control; #$P < 0.05$, the caudal *versus* rostral portion in levels of serotonin (two-way ANOVA followed by post hoc Newman–Keuls tests). †$P < 0.05$, rostral/middle caudate versus caudal caudate and middle/caudal putamen in % loss of serotonin; ‡$P < 0.05$, caudal caudate versus all other striatal subdivisions or middle caudate versus middle/caudal putamen in % loss of dopamine (repeated measures ANOVA followed by post hoc Newman-Keuls tests).

48%, putamen, 30%; 5-HIAA: caudate, 26%, putamen 22%; SERT: caudate, 60%, putamen, 10%; TPH: caudate, 60%, putamen, 10%).

Correlations

Statistically significant positive correlations (Pearson) were consistently observed between striatal levels of SERT and TPH in both control and PD groups (r: 0.68–0.85, $P < 0.05$). In addition, there was consistent, significant positive correlation (Pearson) between levels of 5-HIAA and SERT or TPH in both caudate and putamen of the PD patients (r: 0.64–0.97, $P < 0.05$). However, there was no significant correlation (Pearson) between striatal levels of dopamine and the serotonergic markers in PD ($P > 0.05$).

Statistically significant correlations or trends for negative correlations (Spearman) were observed between the disease stage (Hoehn–Yahr) for the PD patients for which this information was available and striatal levels of serotonin (caudate, $r = -0.56$, $P = 0.02$; putamen, $r = -0.74$, $P = 0.0005$; $n = 18$), 5-HIAA (caudate, $r = -0.52$, $P = 0.03$; putamen, $r = -0.45$, $P = 0.06$; $n = 18$), SERT (caudate, $r = -0.77$, $P = 0.03$; putamen, $r = -0.70$, $P = 0.05$; $n = 8$), and TPH (caudate, $r = -0.74$, $P = 0.04$; putamen, $r = -0.70$, $P = 0.05$; $n = 8$). No significant correlation was observed between disease duration of the patients (3–29 years) and levels of the serotonin markers examined. Caudate dopamine levels were significantly negatively correlated with disease duration ($r = -0.53$, $P = 0.02$; $n = 20$).

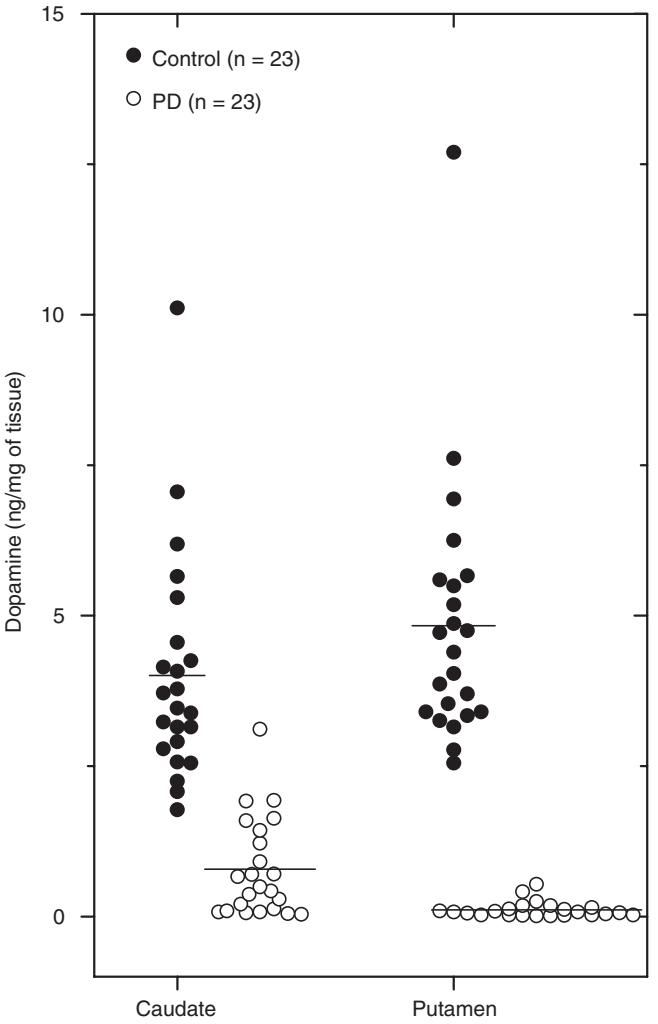

Fig. 2 Individual caudate and putamen levels of dopamine in the control subjects and patients with Parkinson's disease (PD).

Serotonin markers versus levodopa-dyskinesias

Table 4 shows levels of the biochemical outcome measures in a subgroup of seven patients who had levodopa dyskinesias and in six levodopa-treated patients (more than 6 years drug treatment) who were not reported in the case records to have had dyskinesias. For these six patients, we could not rule out absolutely the possibility that the patients developed levodopa-dyskinesias shortly before death. However, the last neurological assessment was within one year of their death for all of the patients, with the three patients who had more than 10 years of levodopa treatment (Table 1) last assessed within 3 months of their death. The mean duration of illness and levodopa treatment was similar in both groups. Statistically significant differences between the two groups were limited to increased dopamine in the caudate nucleus (+325%) and decreased molar ratios of HVA/dopamine (−61%) and 5-HT/dopamine (−77%) in the dyskinetic versus non-dyskinetic group.

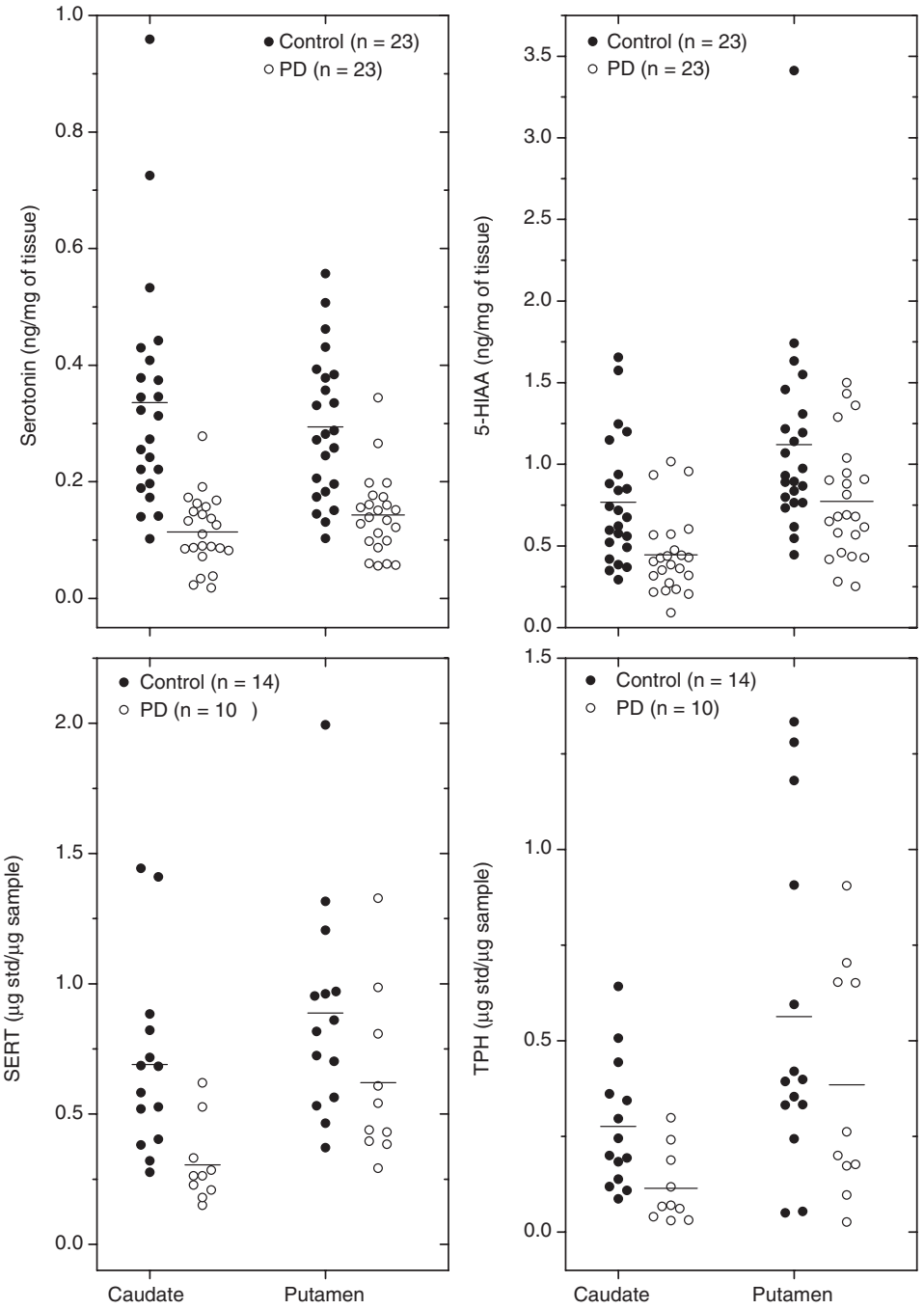

Fig. 3 Individual caudate and putamen levels of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), serotonin transporter (SERT) and tryptophan hydroxylase (TPH) in the control subjects and patients with Parkinson's disease (PD).

Discussion

To our knowledge, this is the first systematic neurochemical examination in a single study of all of the key presynaptic serotonin markers in brain of patients with PD as well as the first measurement of brain SERT and TPH proteins in this disorder.

Because of the possible intra-regional heterogeneity in biochemical outcome measures, special attention was devoted to ensure that the tissue sampling was taken in a reproducible manner. We found that striatal concentrations of all serotonin markers were, on average, decreased, but with the magnitude of the reduction highly variable amongst the individual patients, and with the caudate subdivision, in particular the rostral and middle portions, more affected than the putamen.

Potential problems associated with postmortem brain investigation in PD include, for example, the possibility that dopaminergic medication (see later) or other factors might

Table 4 Levels of dopamine and serotonin markers in Parkinson's disease (PD) with ($n = 7$) and without levodopa-dyskinesias ($n = 6$)

	PD (-dyskinesias)	PD (+dyskinesias)
Age, years	77 ± 5 (6)	75 ± 2 (7)
Postmortem interval, h	11 ± 3 (6)	14 ± 3 (7)
Duration of disease, years	16 ± 4 (6)	17 ± 3 (7)
Duration of levodopa exposure, years	11 ± 3 (6)[a]	12 ± 2 (6)[b]
Caudate		
Dopamine	0.24 ± 0.14 (6)	1.02 ± 0.31 (7)*#
HVA	2.09 ± 0.39 (6)	2.96 ± 0.53 (7)
HVA/DA (mol/mol)	21.4 ± 7.9 (6)	8.1 ± 3.9 (7)
Serotonin	0.10 ± 0.02 (6)	0.13 ± 0.03 (7)
5-HIAA	0.41 ± 0.11 (6)	0.42 ± 0.11 (7)
5-HIAA/5-HT (mol/mol)	5.0 ± 1.7 (6)	3.5 ± 0.5 (7)
5-HT/DA (mol/mol)	1.1 ± 0.3 (6)	0.2 ± 0.1 (7)
SERT	0.29 ± 0.08 (4)	0.34 ± 0.14 (3)
TPH	0.09 ± 0.05 (4)	0.10 ± 0.05 (3)
Putamen		
Dopamine	0.11 ± 0.09 (6)	0.11 ± 0.02 (7)
HVA	1.91 ± 0.41 (6)	2.80 ± 0.27 (7)
HVA/DA (mol/mol)	58.7 ± 17.0 (6)*#	22.6 ± 2.5 (7)
Serotonin	0.14 ± 0.03 (6)	0.13 ± 0.02 (7)
5-HIAA	0.84 ± 0.21 (6)	0.72 ± 0.12 (7)
5-HIAA/5-HT (mol/mol)	5.5 ± 1.1 (6)	5.7 ± 0.8 (7)
5-HT/DA (mol/mol)	4.3 ± 1.3 (6)*#	1.0 ± 0.2 (7)
SERT	0.81 ± 0.21 (4)	0.50 ± 0.16 (3)
TPH	0.48 ± 0.19 (4)	0.28 ± 0.19 (3)

Data are given as mean ± SEM (n) in ng/mg of wet tissue for dopamine, homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) and in μg tissue standard protein/μg tissue sample protein for serotonin transporter (SERT), tryptophan hydroxylase (TPH). [a]For the two patients with known levodopa use more than ten years but without dyskinesias, 10 years were used for the calculation of average duration of levodopa exposure. [b]The exact duration of levodopa use was not reported for one patient with dyskinesias. *$P < 0.05$, PD with versus without dyskinesias; #$P < 0.05$, caudate versus putamen (two-way ANOVA followed by post hoc Newman–Keuls tests).

have affected the biochemical outcome measures. In addition, one cannot rule out the possibility that some of the patients might have had a brain serotonergic disturbance associated with an undisclosed depressive illness, given the difficulty in establishing retrospectively mood state in a post-mortem study. However, recent data suggest that brain levels of one of the indices measured in our study, namely, SERT, are either normal or increased in patients with major depressive illness (Meyer *et al.*, 2004; see Meyer, 2007 for review). One important advantage, on the other hand, of the autopsied brain approach over living brain studies is that it permits measurement of key serotonin markers that cannot be directly examined in living brain (serotonin and metabolite, SERT and TPH proteins) and simultaneous estimation in individual patients of all serotonin markers such that the changes in the different markers can be compared.

Decreased striatal serotonin markers in PD

The specificity of our finding of decreased serotonin markers in striatum of patients with PD is indicated by the different inter-regional pattern of dopamine versus serotonin marker loss (see later) and by the demonstration that levels of the two control proteins (NSE, α-tubulin) in the same samples were not decreased.

Our data confirm and extend, by employing a much larger sample size, the original observation of Bernheimer and colleagues (Bernheimer *et al.*, 1961) and others (for review see Kish, 2003) that levels of serotonin and its metabolite 5-HIAA are decreased in striatum of patients with PD. Because measurement of striatal SERT in post-mortem brain studies (Raisman *et al.*, 1986; D'Amato *et al.*, 1987; Chinaglia *et al.*, 1993) and in neuroimaging investigations (Kerenyi *et al.*, 2003; Guttman *et al.*, 2007) in PD was only inferred from binding data employing a low (submaximal) concentration of the radioligand, it is possible that the observed decrease in SERT binding could have been due to a conformational change in SERT affecting affinity rather than a change in SERT concentration. Our finding of decreased striatal SERT immunoreactivity provides direct evidence in PD for an actual reduction in striatal SERT protein itself, addressing concerns that changes in binding might not reflect actual differences in protein amount. The magnitude of the decreased striatal SERT immunoreactivity in our post-mortem study (caudate, −56%; putamen, −30%) was within or close to the range of the SERT binding reduction observed in two recent imaging investigations in PD (caudate, −50%, −30%; putamen, −35%, −26%; Kerenyi *et al.*, 2003; Guttman *et al.*, 2007, respectively). Previous examination of striatal TPH in PD has been limited to measurement of activity of this unstable enzyme (Carkaci-Salli *et al.*, 2006) in a sample size too low to allow meaningful interpretation of the data (Sawada *et al.*, 1985). The observation of decreased striatal TPH protein indicates that the striatal serotonergic abnormality in this disorder extends to an actual loss of the rate-limiting biosynthetic enzyme.

Reduced brain levels of all of the key markers for the serotonin neurotransmitter system (neurotransmitter and metabolite; transporter and rate-limiting biosynthetic enzyme proteins) provide compelling evidence for a striatal serotonergic abnormality in PD.

Given that PD is a neurodegenerative brain disorder, the low serotonin marker levels might involve either down-regulation of macromolecules within intact serotonin neurons or, perhaps more likely, actual loss of serotonin neurons originating in the dorsal raphe area (considered to provide the innervation to mammalian striatum) and innervating the striatum (see also later). The neuropathological literature on the status of the dorsal raphe in PD is not entirely clear: Although one group has reported loss of cell bodies in this region in PD (Jellinger, 1986; Paulus and

Jellinger, 1991), this has not been consistently confirmed by other investigators (Mann and Yates, 1983; Sawada *et al.*, 1985; Zweig *et al.*, 1989; Halliday *et al.*, 1990). In addition, the possibility has to be considered that any structural damage to raphe-striatal neurons in PD might be limited to the nerve terminal region. This is in fact suggested by one autoradiographic report of distinctly normal SERT binding in the dorsal raphe of patients with PD who show the expected decreased SERT binding in striatum (Chinaglia *et al.*, 1993).

Caudate versus putamen differences

Our post-mortem findings, by employing all major presynaptic serotonin markers as well as (for serotonin itself) a detailed subregional analysis, extend the results of previous investigations that examined serotonin or SERT binding in both striatal nuclei (Bernheimer *et al.*, 1961; Chinaglia *et al.*, 1993; Wilson *et al.*, 1996; Kerenyi *et al.*, 2003; Guttman *et al.*, 2007) and strengthen the, as yet unappreciated, 'consensus' that the intra-striatal pattern of loss of serotonin markers (caudate $\geqslant$ putamen) in PD is substantially different than that for the dopamine system (putamen $>>$ caudate). In this regard, examination of the original report of Bernheimer and colleagues (Bernheimer *et al.*, 1961) disclosed that the loss of serotonin was slightly (64% versus 56%, respectively) more marked in the caudate than in the putamen. Our new data show that the preferential decrease of serotonin in caudate encompasses the bulk (head) of the nucleus.

The explanation for the caudate–putamen difference is unknown but might involve differential susceptibility to damage of the raphe serotonergic neurons innervating the two striatal nuclei. The literature is unclear as to the location of the source of serotonergic innervation to the human caudate versus putamen. However, non-human primate data (Parent *et al.*, 1983) suggest that the source within the dorsal raphe area of serotonergic innervation to the two striatal nuclei might be different (putamen, dorsolateral portion of raphe; caudate, more ventral portion), allowing for the possibility of differential susceptibility to a toxic impact at the cell body level. In principle, the as yet unknown toxic process in PD affecting striatal serotonergic status might also involve a primary insult limited to the nerve terminal regions. Given the different intra- and inter-regional patterns of striatal loss of serotonin and dopamine, the toxic processes, if limited to the nerve terminal regions, might be different for the two monoamine neuronal systems.

Variable loss of serotonin markers

As mentioned in the 'Introduction' section, it has been assumed that striatal serotonergic innervation is relatively intact in most patients with PD (Carta *et al.*, 2007). The results of our post-mortem study, employing all of the key serotonin markers, suggest that this assumption is perhaps correct for the putamen subdivision, in which we found a near complete overlap (in the low control range) between patient and control values, but might not be true for the caudate nucleus, in which a substantial proportion of patients had values below the lower limit of the control range. The explanation for the wide variability in levels of serotonin marker values in PD is not known, and our analyses did not disclose any significant correlations between marker levels and estimated disease duration or age. However, the negative correlations between the disease stage (Hoehn–Yahr) and levels of the serotonin markers do suggest that serotonergic disturbance in PD is to some extent related to the neurodegeneration.

Regarding the less severe loss of striatal serotonin versus dopamine markers, one interesting possibility is that the degeneration of nigrostriatal dopamine neurons may lead to a 'reactive' hyperinnervation of serotonergic neurons, a notion supported by some (Zhou *et al.*, 1991; Gaspar *et al.*, 1993; Rozas *et al.*, 1998; Maeda *et al.*, 2003), but not all (Iwamoto *et al.*, 1976; Stachowiak *et al.*, 1984; Breese *et al.*, 1984; Erinoff and Snodgrass, 1986; Snyder *et al.*, 1986; Takeuchi *et al.*, 1991; Karstaedt *et al.*, 1994) animal data employing dopamine neuron toxins. This might 'mask' the true extent of the serotonin loss in PD. While we can provide no evidence that would directly bear on this question, this notion receives some circumstantial support by our finding that, of the two striatal subdivisions, the putamen, which had the most severe dopamine loss, was least affected by loss of serotonin markers. However comparison of dopamine and serotonin marker levels in the different striatal subdivisions of the patients with PD did not disclose any statistically significant correlations.

Animal data suggest that dopamine derived from levodopa, which almost all of the patients with PD had received, might lower striatal serotonin levels by competing with 5-hydroxytryptophan for the decarboxylase and displacing endogenous serotonin from its neuronal storage sites in serotonin neurons (Carta *et al.*, 2007 and references therein). Thus, it is possible that the magnitude of striatal serotonin reduction (which was slightly more than that of the other serotonin markers) could have been overestimated should this drug effect occur in patients with PD. However, the post-mortem brain finding that striatal (caudate) serotonin levels in PD patients who had received levodopa within 24 h of death were not lower than those who had discontinued taking the drug for at least 4 days before death (Scatton *et al.*, 1983) suggests that the last levodopa dose in chronically drug treated patients with PD might not have lowered tissue levels of serotonin.

Functional considerations: caudate

The striatal components of the basal ganglia represent the primary input stations of the segregated (but interacting) cortical-basal ganglia-thalamocortical circuits (Alexander *et al.*, 1986). Whereas the putamen has been identified

from animal studies as the main input station of the 'motor' circuit, the caudate is considered to be part of a 'complex' cortico-subcortical association loop system, subserving higher-level ('cognitive') functions (Alexander *et al.*, 1986). Animal data have yet to be provided disclosing the critical threshold of loss of serotonin or serotonergic innervation to the striatum necessary for dysfunction. However, experimental findings in the rat show that a striatal serotonin loss of 20–50%, a magnitude of reduction observed in our patients with PD, is associated with abnormal behaviour (increased spontaneous locomotor activity; Carter and Pycock, 1979; Schwarting and Carey, 1985). This suggests that only a moderate loss of striatal serotonin might result in some functional difficulty. The loss of serotonin markers in caudate might suggest a possible role for a caudate serotonin deficiency in some associative-cognitive problems in PD. In this regard, improved function of the caudate nucleus (including cognitive aspects of motor control) could be considered as a possible beneficial consequence of pharmacologic measures aimed at correcting the serotonin deficiency in PD.

Functional considerations: putamen

With respect to the putamen, the finding that most of the patients had serotonin marker levels in this striatal subdivision falling within the normal range suggests that any motor dysfunction due to a putamen serotonin *deficiency* might not be a common feature of PD. Experimental findings in dopamine neuron-lesioned animals indicate that dopamine can be stored and released by serotonin neurons (Ng *et al.*, 1970, 1971; Arai *et al.*, 1994, 1995; Tanaka *et al.*, 1999; Maeda *et al.*, 2005). Thus, the remaining serotonin neurons in putamen could participate in a therapeutic (anti-parkinsonian) effect of levodopa by releasing dopamine derived from the drug. Furthermore, given imaging data in PD suggesting that levodopa-induced dyskinesias might be caused in part by a drug-induced exaggerated increase in striatal synaptic dopamine level (de la Fuente-Fernández *et al.*, 2004; Pavese *et al.*, 2006), serotonin nerve terminals, which can take up dopamine from the extracellular space (Berger and Glowinski, 1978), could be of some benefit by helping to normalize extracellular dopamine concentration. Assuming that the anti-dyskinetic effect of 3,4-methylenedioxymethamphetamine (MDMA) in a monkey model of PD (Iravani *et al.*, 2003) is related to the ability of the amphetamine derivative to release serotonin, serotonin itself might also help to alleviate dyskinesias in the human.

One group (Carta *et al.*, 2007), however, has recently suggested that the remaining striatal serotonin neurons in PD might actually contribute to a detrimental, rather than beneficial process with respect to drug-induced dyskinesias. Thus, experimental data, obtained in the rat having lesion of nigrostriatal dopamine neurons, show that *decreasing* striatal serotonergic activity by either lesion or pharmacological treatment can actually block levodopa-induced dyskinesias in the animals (Carta *et al.*, 2007). In this context, the imaging findings in human PD suggesting an association between dyskinesias and excessively increased synaptic dopamine (as inferred from changes in striatal ^{11}C-raclopride binding; de la Fuente-Fernández *et al.*, 2004; Pavese *et al.*, 2006) could be explained by dysregulated swings in levodopa-derived extracellular dopamine, released from the remaining striatal serotonin neurons that are unable to regulate normally the dopamine release. In such a scenario, the relative *preservation* of serotonergic function (inferred from marker levels) in putamen (the 'motor' component of the striatum) would be detrimental to the patient with PD with respect to this adverse drug effect.

The results of our post-mortem study, however, provide no support for either a beneficial or detrimental influence of serotonin on dyskinesias as levels of serotonin were similar in putamen (and caudate) of those seven patients described, in the case records, as having levodopa-dyskinesias versus those for which this drug complication was not reported. We caution, however, that these data must be considered tentative in view of the small sample size and the difficulty in establishing accurately the presence of specific clinical signs in a retrospective post-mortem brain study and in matching the patients with respect to the severity of the PD and levodopa exposure. Nevertheless, our data are in agreement with the post-mortem observation of Calon and colleagues (Calon *et al.*, 2003), who, employing a similar sample size of patients, also found no difference in putamen serotonin in dyskinetic versus non-dyskinetic patients with PD. Using data from our recent PET imaging study of SERT binding of living patients with clinically advanced PD (Guttman *et al.*, 2007), most of whom had drug-induced dyskinesias, SERT binding was similar in putamen of those patients having more versus less severe dyskinesias (Guttman and Kish, unpublished observations); however, the small sample size ($n = 3$–4 per group) precludes any definitive conclusion regarding SERT and dyskinesias.

The above considerations regarding the possible functional significance (e.g. cognitive problems; levodopa dyskinesias) of loss (or preservation) of serotonergic innervation to the two striatal regions clearly deserve further study. Although clinical significance will be difficult to 'prove', this could be addressed by future prospective pharmacological studies and *in vivo* imaging investigations using SERT as a biomarker. It is well recognized today that cognitive dysfunction and severe dyskinetic behaviour rank high on the list of the major domains of long-term disability especially difficult to treat, being, in some patients with advanced PD, the main factors responsible for reduced quality of life (Schrag *et al.*, 2000).

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