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Support for dopaminergic hypoactivity in restless legs syndrome: a PET study on D2-receptor binding

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Clinical observations support a central role of the dopamine system in restless legs syndrome (RLS) but previous imaging studies of striatal dopamine D2-receptors have yielded inconclusive results. Extrastriatal dopaminergic function has hitherto not been investigated. Sixteen RLS patients naïve to dopaminergic drugs and sixteen matched control subjects were examined with PET. [^{11}C]Raclopride and [^{11}C]FLB 457 were used to estimate D2-receptor availability in striatum and extrastriatal regions, respectively. Examinations were performed both in the morning (starting between 10:00 and 12:00 h) and evening (starting at 18:00 h). Measures were taken to monitor and control for head movement during data acquisition. In the striatum, patients had significantly higher [^{11}C]raclopride binding potential (BP) values than controls. In extrastriatal regions, [^{11}C]FLB 457 BP was higher in patients than controls, and in the regional analysis the difference was statistically significant in subregions of thalamus and the anterior cingulate cortex. The diurnal variability in BP with [^{11}C]FLB 457 and [^{11}C]raclopride was within the previously reported test–retest reproducibility for both radioligands. The study supports involvement of the dopamine system in both striatal and extrastriatal brain regions in the pathophysiology of RLS. The brain regions where differences in D2-receptor binding were shown are implicated in the regulation of affective and motivational aspects of sensory processing, suggesting a possible pathway for sensory symptoms in RLS. Increased D2-receptor availability in RLS may correspond to higher receptor densities or lower levels of endogenous dopamine. Both interpretations are consistent with the hypothesis of hypoactive dopaminergic neurotransmission in RLS, as increased receptor levels can be owing to receptor upregulation in response to low levels of endogenous dopamine. The results do not support variations in dopamine D2-receptor availability as a correlate to the diurnal rhythm of RLS symptoms.

Keywords: brain; human; positron emission tomography; restless legs syndrome

Abbreviations: ACC = anterior cingulate cortex; AST = associative striatum; BP = binding potential; DVR = distribution volume ratio; LST = limbic striatum; PET = positron emission tomography; RLS = restless legs syndrome; ROI = regions of interest; SPET = single PET; SMST = sensorimotor striatum

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Introduction

Restless legs syndrome (RLS) is a disorder characterized by unpleasant leg sensations together with an irresistible inner urge to move (Ekbom, 1945). Symptoms are worsened at rest and in the evening, leading to insomnia and daytime sleepiness. Using standardized diagnostic criteria (Table 1), the prevalence of RLS has recently been estimated to be

Table 1 Essential diagnostic criteria for restless legs syndrome

(1) An urge to move the legs, usually accompanied or caused by uncomfortable and unpleasant sensations in the legs
(2) The urge to move or unpleasant sensations begin or worsen during periods of rest or inactivity such as lying or sitting
(3) The urge to move or unpleasant sensations are partially or totally relieved by movement, such as walking or stretching, at least as long as the activity continues
(4) The urge to move or unpleasant sensations are worse in the evening or night than during the day or only occur in the evening or night

Established by the International Restless Legs Syndrome Study Group (Allen *et al.*, 2003).

7.2 and 8.5%, respectively, in two large epidemiological studies (Allen *et al.*, 2005; Tison *et al.*, 2005), rendering it one of the most common neurological disorders.

The aetiology and pathophysiology of RLS is poorly understood. Most cases are idiopathic, but RLS has also been shown to occur secondary to other conditions such as iron deficiency, uraemia and pregnancy. Approximately 50% of RLS cases report a positive family history, with a correlation to early disease onset (Winkelmann *et al.*, 2000, 2002; Tison *et al.*, 2005). Three genomic regions have been associated to RLS susceptibility, but no candidate genes have been identified (Mata *et al.*, 2006).

Clinical and pharmacological observations point towards a central role for the dopamine system in the pathophysiology of RLS. Treatment with dopamine agonists shows efficacy as confirmed by controlled trials (Fulda and Wetter, 2005) while dopamine antagonists worsen symptoms or may even elicit RLS (Kraus *et al.*, 1999; Wetter *et al.*, 2002; Pinninti *et al.*, 2005). Indirect support for a role of the dopamine system in restlessness is also given by the observation that akathisia is a common side-effect of antipsychotic drugs that all are dopamine antagonists (Barnes, 1989; Farde, 1992).

Several brain imaging studies of the central dopamine system in RLS have been performed to date. All have focused on the striatum, a brain region receiving dense dopaminergic innervation. On the presynaptic side, two PET studies have shown reduced utilization of [^{18}F]dopa in the striatum (Turjanski *et al.*, 1999; Ruottinen *et al.*, 2000) while one study showed no difference (Trenkwalder *et al.*, 1999*b*). No difference in binding to the dopamine transporter has been found in studies employing single PET (SPET) (Eisensehr *et al.*, 2001; Michaud *et al.*, 2002; Tribl *et al.*, 2002; Linke *et al.*, 2004; Mrowka *et al.*, 2005). Studies on radioligand binding to D2-receptors, a marker primarily for postsynaptic function, have shown discrepant findings. One PET study using [^{11}C]raclopride and one SPET study with [^{123}I]IBZM have shown reduced binding (Turjanski *et al.*, 1999; Michaud *et al.*, 2002; Staedt *et al.*, 1995) while three other SPET [^{123}I]IBZM studies reported no difference (Eisensehr *et al.*, 2001; Tribl *et al.*, 2002, 2004). Taken together, imaging studies so far have not conclusively confirmed the postulated role of dopaminergic neurotransmission in the pathophysiology of RLS.

The phenomenology of RLS indicates that sensory symptoms precede motor symptoms. Accordingly, RLS can be viewed as a disorder of sensory perception (Trenkwalder and Paulus, 2004), and it has previously been proposed that the pathophysiology is related to an impairment of central somatosensory processing (Schattschneider *et al.*, 2004). This disease mechanism suggests involvement of extrastriatal brain regions implicated in the processing of sensory stimuli. [^{11}C]Raclopride is an established PET radioligand for examining striatum but not for imaging of the minute dopamine D2-receptor concentrations in extrastriatal regions. The advent of high-affinity radioligands such as [^{11}C]FLB 457 has now made it feasible to quantitatively examine low-density D2-receptor populations using PET (Olsson *et al.*, 1999, 2004).

The aim of the present PET study was to examine dopamine D2-receptors in 16 RLS patients and 16 control subjects. The radioligands [^{11}C]raclopride and [^{11}C]FLB 457 were used to allow for examination of both striatal and extrastriatal brain regions. The circadian component of the disorder was addressed by performing measurements both in the morning and in the evening.

Material and methods

Subjects

Sixteen patients with idiopathic RLS (8 males, 8 females) and sixteen age- and sex-matched control subjects were recruited by advertising in daily newspapers. The study was approved by the Ethics and Radiation Safety committees of the Karolinska Institute. Written informed consent was obtained from all subjects. The age was 55 ± 7 years for patients and 56 ± 8 years for controls (these and all subsequent values represented as mean ± SD). The subjects had no history of significant psychiatric or somatic illness as assessed by medical interview, physical examination, routine blood tests and brain MRI. None of the subjects were nicotine users.

RLS patients were examined by a clinical neurologist who confirmed the diagnosis according to the IRLSSG diagnostic criteria. All patients were naïve to dopaminergic drugs as well as oipiod agonists. Other medication for RLS or sleep was confined to the occasional use of zolpidem by one of the patients and this was discontinued >5 half-lives prior to the first PET examination. The symptom duration was 27 ± 12 years and heritability for RLS, defined as at least one first-order relative being affected, was observed in 9 of the 16 cases. In order to confirm study eligibility, RLS symptoms in patients were rated on the day of the first PET examination using the IRLSSG severity rating scale (Walters *et al.*, 2003). The average rating was 18.5 ± 3.9. Individual demographic data for RLS patients are presented in Table 2.

For all subjects, medication taken during the PET study days was oestrogen substitution (9 female subjects), inhalation treatment for asthma (2), loratadin (1) and paracetamol (1). None of these drugs were judged to interfere with the study. Subjects were required to abstain from products containing caffeine during days of PET examinations. On each day of PET measurements, a urine

Table 2 Demographic data of RLS patients

Patient	Sex	Age (years)	Duration of symptoms (years)	Family history*	IRLS score†
1	F	61	20	+	17
2	M	58	10	–	12
3	F	57	20	–	24
4	F	52	5	–	27
5	M	60	40	+	18
6	F	61	40	+	23
7	M	60	30	+	16
8	M	60	40	–	19
9	M	41	20	–	17
10	M	55	25	+	16
11	F	54	25	–	21
12	M	42	30	+	21
13	F	62	35	+	18
14	M	42	10	–	17
15	F	56	45	+	17
16	F	62	40	+	13

*'+' indicates positive family history and '–' indicates no family history; †IRLS rating on day of first PET examination.

toxicology screening, a pregnancy test and an electrocardiogram was done.

Objectives and study design

The primary objective in this PET study was to examine differences in radioligand binding to dopamine D2-receptors between control subjects and RLS patients. D2-receptor availability was determined in striatum with the radioligand [^{11}C]raclopride and in extrastriatal brain regions with the radioligand [^{11}C]FLB 457. All subjects underwent PET measurements with each of the two radioligands in the evening (starting at 18:00 h) on two separate days. The examination days were <16 days apart (for one patient and one control subject this was extended to 34 and 19 days, respectively, due to technical reasons).

The secondary objective was to examine group differences in the diurnal variation of radioligand binding. In eight RLS patients and eight control subjects a morning examination (starting between 10:00 and 12:00 h) was made with [^{11}C]raclopride on the same day as the evening [^{11}C]raclopride was performed. In the remaining eight RLS patients and eight control subjects a morning measurement with [^{11}C]FLB 457 was made on the same day as the evening [^{11}C]FLB 457 examination. To avoid any order effects, subject pairs were randomized into four different scanning regimes (Fig. 1).

MRI and the head fixation system

To allow for the same head positioning in all measurements and to minimize head movement, a plaster helmet was made for each subject individually and used during both MRI and PET examinations (Bergstrom *et al.*, 1981). MR images were acquired using a 1.5T GE Signa system (Milwaukee, WI) with a T1 and T2-weighted protocol. T2 images were examined for structural pathology at subject inclusion. T1 images were reconstructed using a 256 × 256 × 156 matrix with an original resolution of 1.02 × 1.02 × 1 mm^3 and were used for the subsequent data analysis. For three subjects the original *z*-axis resolution was 1.2 mm and in one case 1.5 mm, which was owing to temporary problems with the MRI camera.

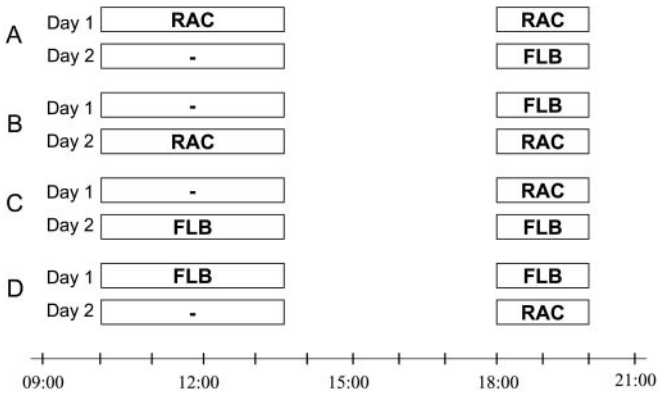

Fig. 1 16 RLS patients and 16 control subjects were randomized in pairs to four different PET examination sequences shown as A, B, C and D. Rectangles show the time frames for the PET measurements. RAC = [^{11}C]raclopride; FLB = [^{11}C]FLB 457.

PET experimental procedure

PET studies were performed on an ECAT Exact HR system (CTI/Siemens, Knoxville, TN) run in 3D mode (Wienhard *et al.*, 1994). The transaxial resolution of the system is 3.8 mm full width at half-maximum (FWHM) at the centre of the field of view and 4.5 mm FWHM tangentially and 7.4 mm radially at 20 cm from the centre. Axial resolution is 4 mm FWHM at the centre and 6.8 mm at 20 cm from the centre. Prior to each emission scan a transmission scan of 10 min was performed using three rotating ^{68}Ge–^{68}Ga sources. The information was used for attenuation correction.

[^{11}C]Raclopride and [^{11}C]FLB 457 were prepared from [^{11}C]methyl triflate as described previously (Langer *et al.*, 1999; Sandell *et al.*, 2000). The radioligands were given intravenously as a rapid bolus and the cannula was flushed with saline. Radioactivity in brain was measured during 51 min for [^{11}C]raclopride and 87 min for [^{11}C]FLB 457 by a consecutive sequence of frames (3 × 1, 4 × 3, 6 × 6 and 3 × 1, 4 × 3, 12 × 6 min, respectively). After correction for attenuation, random and scattered events, images were reconstructed using a Hann filter (2 mm FWHM). The reconstructed volume was displayed as 47 horizontal sections with a centre-to-centre distance of 3.125 mm and a pixel size of 2.02 × 2.02 mm^2 (Fig. 2D–H).

Symptom ratings and control of movement during PET examinations

During all PET measurements, a video capture of head movement was collected and subsequently reviewed. The information was categorized using an operational scale as follows: 0 (no or indecisive head movement); 1 (discrete—at least once <3 mm); and 2 (pronounced—at least once >3 mm). Observed leg movement during examinations was recorded by the investigator (S.C.) and similarly categorized as follows: 0 (no or occasional discrete body movement); 1 (discrete—e.g. repeated toe and ankle movement, occasional leg movement); and 2 (pronounced—e.g. repeated movement of legs or arms). After each evening examination, all subjects were asked to rate uncomfortable leg sensations and the urge to move using a 5-point scale ranging from none (0), mild (1), moderate (2), severe (3) to very severe (4) symptoms.

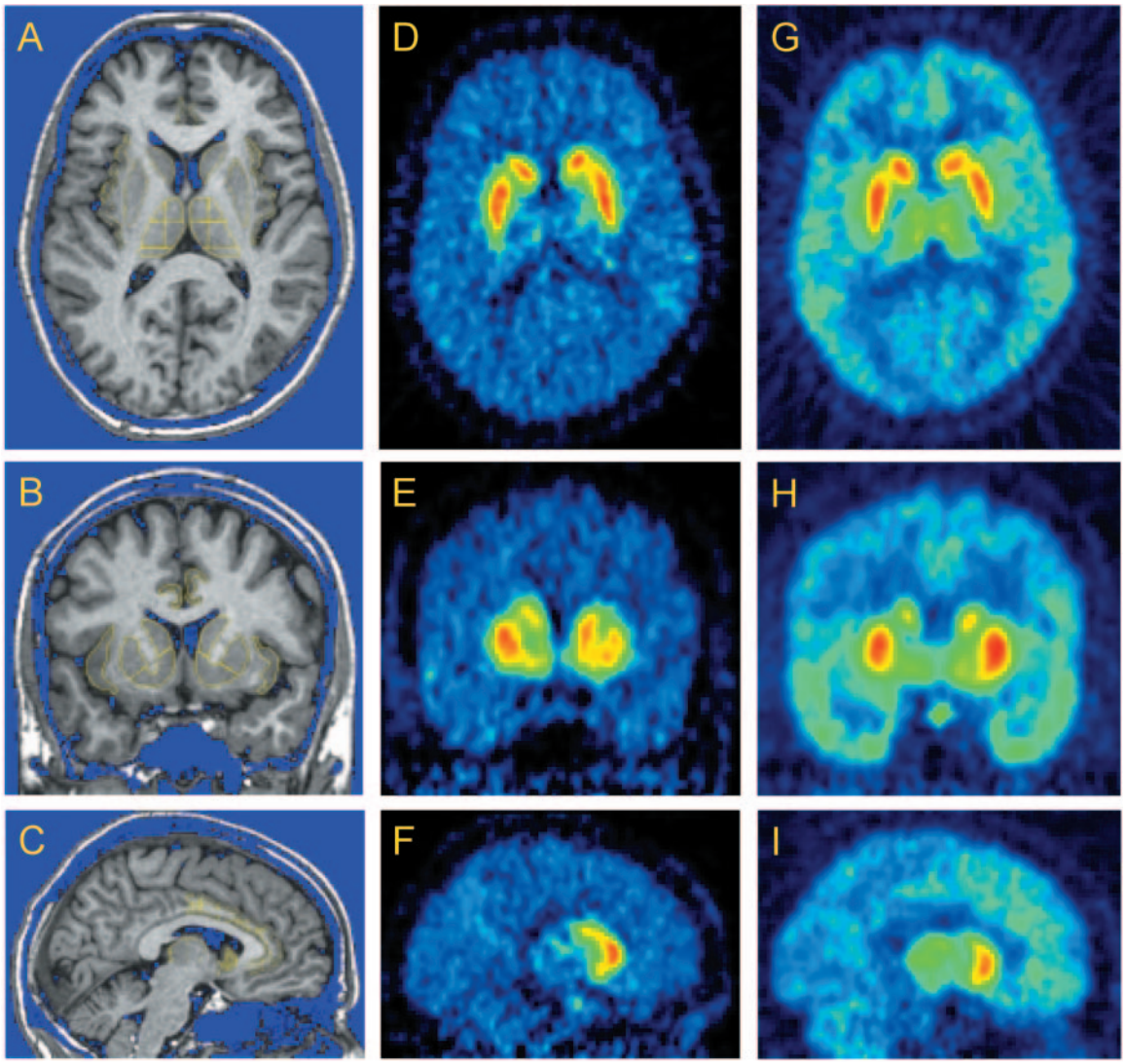

Fig. 2 MR images showing ROIs (**A**–**C**) and PET images showing regional radioactivity after i.v. injections of [^{11}C]raclopride (**D**–**F**) and [^{11}C]FLB 457 (**G**–**I**). The projections are from top to bottom: horizontal, coronal and sagittal. PET images shown are based on summed data from 9 min to the end of the examination. All images are from the same male control subject.

Image processing

PET and MR images were stripped of any information identifying the subjects and the file names were coded, rendering the investigator (S.C.) blind to the diagnostic status of the subjects during the image analysis. The MRI images were realigned to the AC–PC plane using SPM2 software and resliced to a resolution of $2 \times 2 \times 2$ mm^3. For each subject, all three PET images were coregistered to the resliced MR image using the normalized mutual information method implemented in SPM2 (Maes *et al.*, 1997). During the process of reslicing, the PET images were resampled to MR resolution in order to minimize loss of information. For determination of regional radioligand binding, regions of interest (ROIs) were manually delineated on each individual MR image using the Human Brain Atlas software (Roland *et al.*, 1994).

Regions of interest

For [^{11}C]raclopride examinations, ROIs for striatum included both putamen and the caudate nucleus. For a detailed subregional analysis, ROIs were defined according to a method described in the literature (Mawlawi *et al.*, 2001; Martinez *et al.*, 2003) in which striatum is divided into limbic (LST), associative (AST) and sensorimotor (SMST) subregions based on the differential connectivity of the striatum (Joel and Weiner, 2000).

[^{11}C]FLB 457 provides a signal for a series of cortical and subcortical extrastriatal regions. In the present study, the selection of ROIs for [^{11}C]FLB 457 examinations was guided by the literature on neuronal pathways for processing of sensory information. The brain regions included were thalamus, insula and the anterior cingulate cortex (ACC). All regions were defined according to the published guidelines as described briefly in the following sections.

ROIs for thalamus were defined using a procedure described previously (Buchsbaum *et al.*, 1996; Gilbert *et al.*, 2001; Yasuno *et al.*, 2004) with some modifications. In order to avoid inclusion of mesencephalic or hypothalamic areas within the ROI, the rostral end of the superior colliculi rather than the inferior border of the

third ventricle was defined as the inferior boundary of the thalamus. Furthermore, the anterior and central portions of both the medial and lateral thalamus can be viewed as homogeneous with regard to D2-receptor density (Hall *et al.*, 1996; Rieck *et al.*, 2004) and, therefore, in order to reduce statistical noise only three thalamic subdivisions were defined—medial, lateral and posterior. ROIs for insula were delineated according to guidelines described by Crespo-Facorro *et al.* (2000*a*, *b*). For ACC, ROIs were drawn for both rostral and caudal subregions with the most anterior point of the inner surface of genu corporis callosi defining the vertical dividing plane (Crespo-Facorro *et al.*, 2000*b*; Ballmaier *et al.*, 2004). Finally, a ROI for cerebellum was drawn below the appearance of the petrosal bone in five slices, corresponding to a thickness of 10 mm. For an example of ROI delineation, see Fig. 2A–C.

Quantitative analysis

The ROIs were transferred to the series of PET images to generate time–activity curves (TACs). This was done for each subregional ROI individually, on both right and left side. Spatially weighted averages of the original curves were then calculated to create TACs for larger regions.

Regional binding potential (BP) values were calculated using the simplified reference tissue model with the cerebellar TAC as input function (Lammertsma and Hume, 1996). This approach has previously been found suitable for both [^{11}C]raclopride and [^{11}C]FLB 457 (Lammertsma and Hume, 1996; Olsson *et al.*, 1999, 2004). Cerebellum is a region where D2-receptor density is negligible (Hall *et al.*, 1996; Olsson *et al.*, 1999) and serves as an indirect approximation of free and non-specifically bound radioligand concentration, though a recent study with [^{11}C]FLB 457 in rodents has shown that it cannot be excluded that a few per cent of the activity in cerebellum may represent specific binding to dopamine D2-receptors (Ahmad *et al.*, 2006).

The parameter BP in this context represents the ratio of receptor density (B_{max}) and apparent affinity (K_d) (Mintun *et al.*, 1984). The affinity is termed 'apparent' when non-specific binding in the brain ($f2$) is not corrected for. No correction of partial volume effects was done.

Statistical parametric mapping

For [^{11}C]FLB 457 examinations, a parametric mapping analysis was made in order to investigate changes in brain independent of ROI definition, and also to examine homogeneity within regions. The wavelet approach was used, the background and procedure of which has been described in detail previously (Turkheimer *et al.*, 2000; Cselenyi *et al.*, 2002). Briefly, the original images were transformed frame-by-frame to the wavelet space using a 3D stationary wavelet transform (Cselenyi *et al.*, 2006). The depth of decomposition was set at 2 and the kernel length was 16. The resulting coefficients were analysed quantitatively using the reference region version of Logan's linear graphical estimation (Logan *et al.*, 1996), yielding a parametric wavelet transform describing the distribution volume ratio (DVR). The same cerebellar reference regions as in the ROI-based analysis were used. In the next step a wavelet reconstruction was applied, resulting in 3D DVR images in normal space. The images were then normalized to the MNI template in SPM2 and smoothed using a Gaussian filter (FWHM 10 mm) before statistical calculations.

Statistical analysis

In the ROI-based analysis, average differences in BP between RLS patients and control subjects were calculated as ([patient − control]/control) × 100%. Statistical differences between groups were investigated using a repeated-measures analysis of variance (ANOVA, general linear model module, Statistica 7.1, StatSoft, Tulsa, OK). Group and region were set as within-factors, thus taking advantage of the matched design of the study. In the first part of the analysis, group effects were determined using BP values for whole striatum, thalamus, insula and ACC. In the second part of the analysis, subregional as well as side differences were taken into account. BP values from all individual subregional ROIs, right and left sides separately, were entered in the ANOVA. This gave six levels for the region factor for [^{11}C]raclopride and 12 levels for [^{11}C]FLB 457 examinations. For comparison of individual regions and subregions, a contrast analysis was performed. A two-tailed probability value of $P < 0.05$ uncorrected was selected as significant.

In PET studies, the measurement of dopamine-receptor binding in different regions of the brain in the same individual cannot be regarded as independent observations. This view is based on the biological condition that cell populations throughout the brain are innervated almost exclusively by efferents originating from a restricted area in the midbrain. In accordance, correction for multiple comparisons is not a prerequisite in the present analysis. However, as part of a conservative statistical approach, the results were also evaluated using Bonferroni correction.

Group differences in diurnal variation were examined with a repeated-measures ANOVA, with group, region and time as within-factors. Subregional ROIs were not included in this part of the analysis.

In the parametric analysis, a voxel-based paired *t*-test was performed in SPM2. For this exploratory analysis, an unrestricted search volume was used. The significance threshold was set to $P < 0.005$ uncorrected with a minimal cluster size of >50 voxels.

Group differences in categorical variables were estimated using Wilcoxon's matched pairs test and correlations between BP and categorical variables were tested using Spearman rank order correlation. The significance level in these tests was set to $P < 0.05$.

Results

Pet examination variables

For timing of radioligand injections, injected radioactivity and injected radioligand mass see Table 3. There was no systematic group difference in either variable as determined with a two-tailed *t*-test for both morning and evening examinations.

Movement and subjective RLS symptoms during PET

RLS patients displayed more body movement than control subjects in evening [^{11}C]raclopride examinations (mean score 1.0 and 0.4, respectively, $P = 0.028$ using Wilcoxon's matched pairs test) and evening [^{11}C]FLB 457 measurements (1.7 and 0.8, $P < 0.005$). However, there was no systematic difference between groups in scoring for head movement for either scan ($P = 0.46$, 0.89). Body movement scores were higher in the evening compared with the morning for RLS

Table 3 Radioligand injection statistics

	Injection time (± min)	Injected radioactivity (MBq)	Specific radioactivity (GBq/μmol)	Injected radioligand mass (μg)
[^{11}C]Raclopride				
RLS (a.m.)	11.02 ± 39	192 ± 12	98 ± 52	1.07 ± 1.00
Controls (a.m.)	10.45 ± 13	194 ± 04	138 ± 119	0.86 ± 0.64
RLS (p.m.)	18.04 ± 03	194 ± 06	104 ± 72	1.67 ± 2.39
Controls (p.m.)	18.11 ± 26	196 ± 04	173 ± 126	0.62 ± 0.40
[^{11}C]FLB 457				
RLS (a.m.)	10.35 ± 54	199 ± 19	191 ± 80	0.45 ± 0.19
Controls (a.m.)	10.31 ± 39	195 ± 32	153 ± 140	0.68 ± 0.32
RLS (p.m.)	18.03 ± 03	204 ± 18	210 ± 90	0.44 ± 0.23
Controls (p.m.)	18.04 ± 05	201 ± 37	213 ± 162	0.58 ± 0.43

All values are mean ± SD.

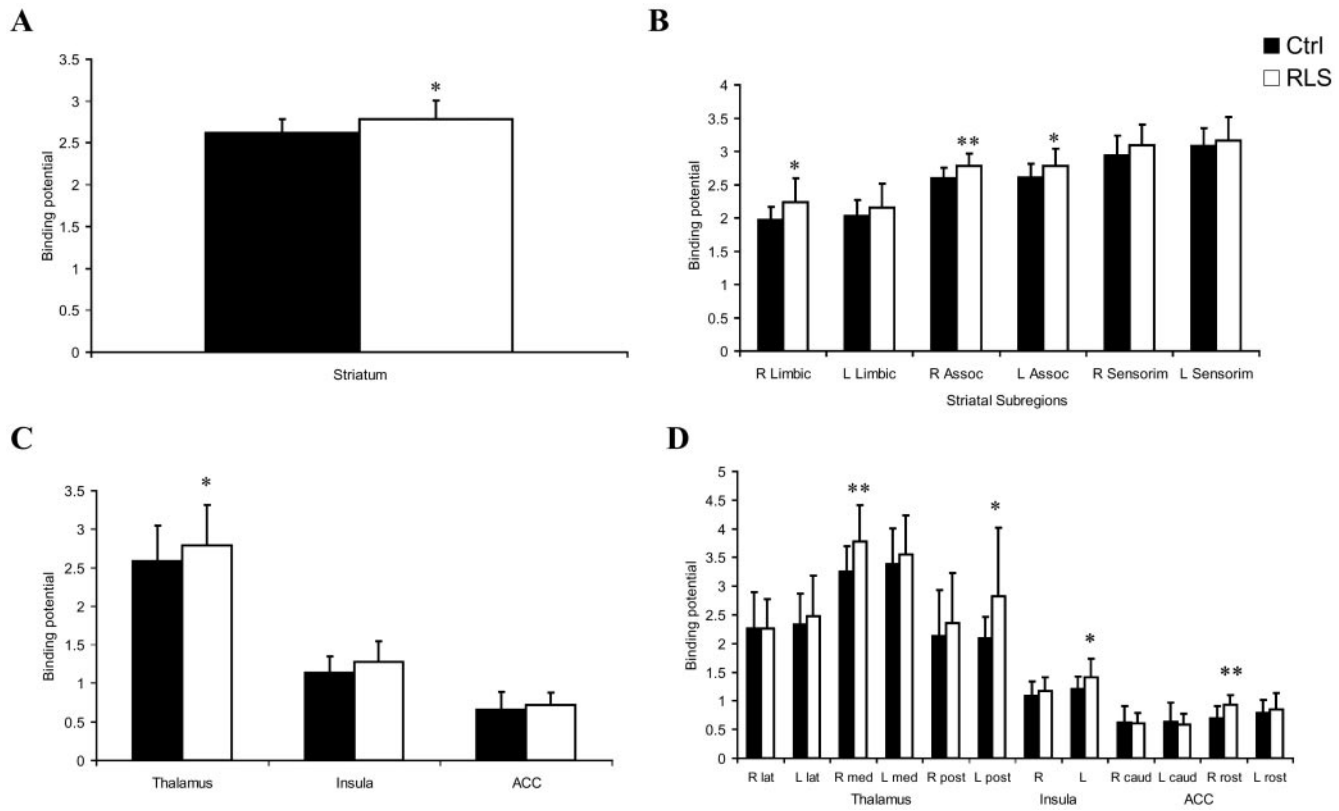

Fig. 3 Histograms showing mean values and standard deviations of the binding potential (BP) in RLS patients and control subject from evening examinations with [^{11}C]raclopride (**A** and **B**) and [^{11}C]FLB 457 (**C** and **D**). In graph **B**, the regions are limbic striatum, associative striatum (assoc) and sensorimotor striatum (sensorim). In graph **C**, ACC denotes anterior cingulate cortex. In **D**, the regions are lateral (lat), medial (med) and posterior (post) thalamus; insula; caudal (caud) and rostral (rost) ACC. R = right; L = left. *$P < 0.05$; **$P < 0.01$ uncorrected.

patients (2.7 and 1.1, $P < 0.001$) but not for control subjects (1.25 and 0.75, $P = 0.086$). Subjective symptoms as assessed after evening examinations were higher for RLS patient than control subjects both for [^{11}C]raclopride (3.2 versus 0.6, $P < 0.005$) and [^{11}C]FLB 457 (4.8 versus 0.9, $P < 0.001$).

Group comparisons of evening examinations (n = 16 + 16)

BP values in striatum as measured with [^{11}C]raclopride were higher for RLS patients than for control subjects (2.79 ± 0.22 versus 2.61 ± 0.17, $P = 0.029$) (Fig. 3A). In the ensuing subregional analysis, values were numerically higher for RLS patients in all subregions, with the average difference ranging from 3% in the left SMST to 15% in the right LST (Fig. 3B). The group effect was statistically significant ($F = 4.72$, $P = 0.046$; repeated-measures ANOVA), but not the group and region interaction effect ($F = 1.22$, $P = 0.31$). When performing the analysis of individual subregions, the elevation was statistically significant in right LST and AST bilaterally. After applying Bonferroni correction ($k = 6$), the difference in right AST was still significant.

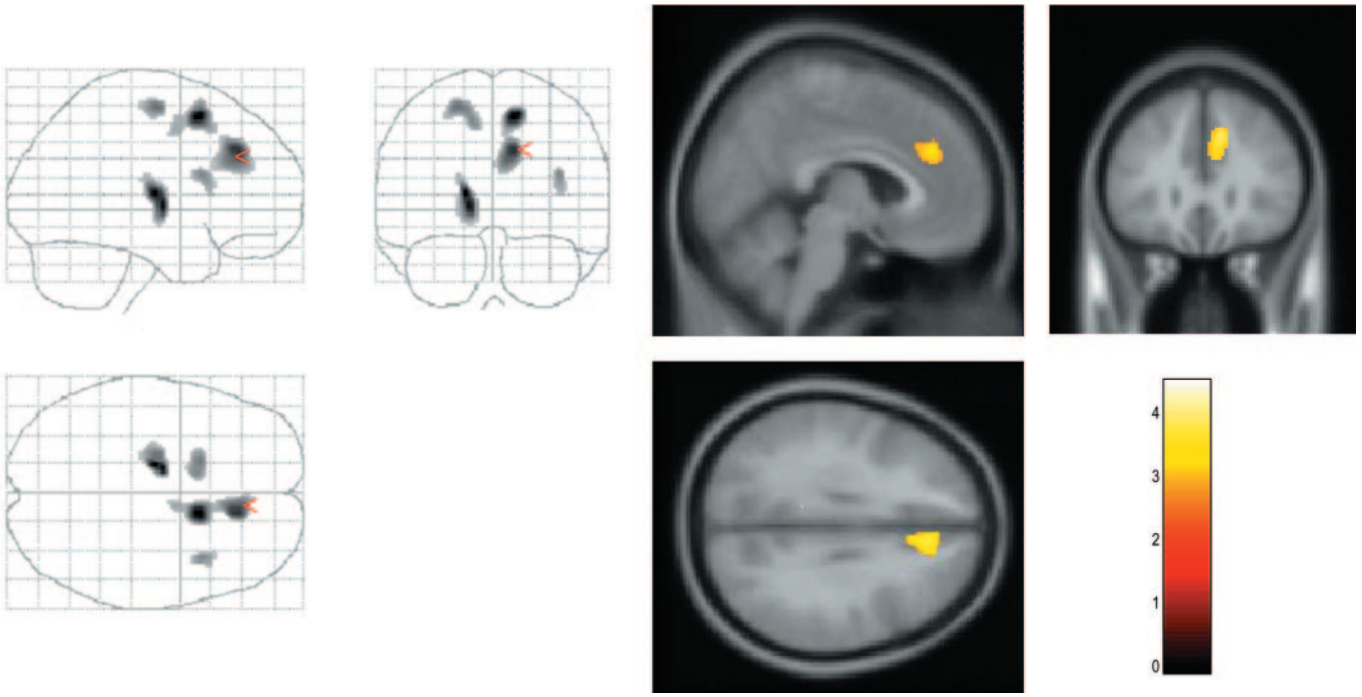

Fig. 4 Data from evening [^{11}C]FLB 457 examinations were transformed into binding parameter maps using the wavelet approach (see references in text). The maps were normalized to a template and statistically analysed using SPM2. Images show clusters of individual voxels where DVR values were significantly higher in RLS patients compared to control subjects using a paired *t*-test. The threshold for significance was set at $P < 0.005$ and the minimal cluster size was 50 voxels. Images are presented in the neurological convention (right is right). In the *left panel*, all clusters throughout the whole brain are demonstrated in the 'glass brain' in SPM2. The red arrow indicates a significant cluster of 363 voxels in the right ACC. In the *right panel*, this cluster is overlain on the Montreal Neurological Institute template brain from SPM2. Colour scale shows *T*-score.

For [^{11}C]FLB 457 examinations, BP values were numerically higher in RLS patients compared to control subjects in thalamus, insula and ACC (Fig. 3C). The difference was statistically significant in thalamus (2.79 ± 0.52 versus 2.58 ± 0.46, $P = 0.029$). When values from these regions were entered into the repeated-measures ANOVA, the group effect was close to significant ($F = 4.53$, $P = 0.0502$), while the group and region interaction effect was not significant ($F = 2.14$, $P = 0.14$). When entering data from all subregional ROIs, the repeated-measures ANOVA showed a significant group effect ($F = 7.63$, $P = 0.015$). The group and region interaction effect was also significant ($F = 3.25$, $P < 0.001$), and in the analysis of individual subregions, the group difference reached statistical significance in right medial thalamus, left posterior thalamus, left insula and the rostral ACC on the right side (Fig. 3D). In these regions, the average difference ranged from 17% in right medial thalamus to 47% in the right rostral ACC. The significance values in right medial thalamus and right rostral ACC survived Bonferroni correction (k = 12).

The parametric analysis of [^{11}C]FLB 457 images showed results partly in congruence with the ROI-based analysis. Significantly higher DVR values for RLS patients were seen in right ACC, left thalamus and the anterior tip of right insula. Clusters were also found in prefrontal cortex bilaterally (Fig. 4). The only region where the group difference reached statistical significance on a cluster level was the right ACC ($P = 0.03$).

Group comparisons of morning examinations (n = 8 + 8)

Eight RLS patients and eight control subjects underwent morning examinations with each radioligand. Morning examinations with [^{11}C]raclopride showed elevated BP values in RLS patients compared to control subjects in striatum (2.81 ± 0.10 versus 2.59 ± 0.22; $P = 0.0052$). The group difference remained when taking subregional and side differences into account in a repeated-measures ANOVA ($F = 9.72$, $P = 0.017$), while the group and region interaction effect was not significant. For [^{11}C]FLB 457 studies, the group difference was not statistically significant as estimated by repeated-measures ANOVA, either when examining whole regions or subregions ($F = 0.056$, $P = 0.82$; $F = 1.0$, $P = 0.45$).

Group comparisons of diurnal variation (n = 8 + 8)

The average change between morning and evening examinations was small for both groups in all regions, from <1% for striatum and thalamus up to 6% for the ACC (Table 4). No systematic difference was seen between groups, with

Table 4 Intraindividual change in BP between morning and evening (n = 8 + 8)

	Controls			RLS		
	A.M.	P.M.	Average difference (%)	A.M.	P.M.	Average difference (%)
Striatum	2.59 ± 0.22	2.61 ± 0.22	0.8	2.81 ± 0.10	2.78 ± 0.16	−0.9
Thalamus	2.60 ± 0.53	2.56 ± 0.39	−0.6	2.65 ± 0.34	2.64 ± 0.37	−0.1
Insula	1.22 ± 0.36	1.18 ± 0.23	1.6	1.18 ± 0.28	1.22 ± 0.30	3.2
ACC	0.65 ± 0.26	0.65 ± 0.24	1.4	0.73 ± 0.15	0.68 ± 0.17	−5.9

Average difference = [(P.M. − A.M.)/A.M.] × 100.

repeated-measures ANOVA showing no significance for the time and group interaction effect in [^{11}C]raclopride (F = 0.24, P = 0.64) or [^{11}C]FLB 457 (F = 0.086, P = 0.78) examinations. The interaction term for time, group and region for [^{11}C]FLB 457 studies was also not significant (F = 0.89, P = 0.43). Furthermore, when analysing groups separately no significant difference was seen between morning and evening examinations for any region.

Correlations

No significant correlation was seen between symptom levels during evening examinations and regional radioligand binding in striatum, thalamus, insula or the ACC using the Spearman rank order correlation. Similarly, no correlation was found between BP values and RLS symptom ratings performed on the day of the first PET examination.

Discussion

Previous molecular imaging studies of striatal dopamine D2-receptors in RLS patients have shown reduced radioligand binding or no difference when compared with control subjects. In the present study, D2-receptors were examined in 16 drug-naïve RLS patients and 16 matched controls. To our knowledge, this is the first study in RLS patients where D2-receptor binding has been examined also in extrastriatal brain regions.

Previous findings of reduced BP in striatum could not be confirmed. Striatal [^{11}C]raclopride binding was significantly higher in RLS patients, as estimated both in the morning and in the evening. A subsequent detailed anatomical analysis showed that this difference was not driven by a specific striatal subregion, but instead may reflect a more general engagement of the striatal complex.

BP values were higher in RLS patients also in extrastriatal brain regions as measured with [^{11}C]FLB 457. The group effect when analysing thalamus, ACC and insula bordered on the significant, which could be interpreted in terms of a wider engagement of dopaminergic neurotransmission in the pathophysiology of RLS. However, both the regional and voxel-based analysis provided some support for involvement of specific subregions in thalamus and the ACC.

The selection of extrastriatal ROIs was partly based on the literature on neuronal pathways involved in processing of nociceptive sensory information. A background is that the sensory symptoms of RLS are consistently described as uncomfortable and as many as 45% of the patients report the sensations as being painful (Winkelmann *et al.*, 2000). The thalamus, insula and ACC are all part of the medial nociceptive system which is thought to regulate the affective-motivational component of pain (for a review, see Price, 2000). This component of pain includes the desire to terminate and reduce a negative sensation, and it is tempting to view this as similar to the urge for movement in RLS symptoms. In the present study, regions within the ACC showed the most consistent group difference in both the subregional and the voxel-based analysis. Activity in the ACC has previously been shown to correlate to the unpleasant component of pain (Rainville *et al.*, 1997; Tolle *et al.*, 1999). The regional group differences in D2-receptor availability demonstrated in this study are consistent with the hypothesis of RLS as a disorder of somatosensory processing.

BP was the most important parameter used for comparisons in this study. BP is a ratio between the concentration of available receptor sites (B_{max}) and the apparent *in vivo* affinity (K_d) of the PET radioligand. An elevation in BP may thus theoretically correspond to a difference in either of these two binding parameters. These two interpretations of the present results will be discussed separately in the following in relation to the pertinent literature on dopaminergic neurotransmission.

In healthy volunteers, interindividual variance in BP has been suggested to be due mainly to variability in B_{max} rather than K_d (Farde *et al.*, 1995). In clinical PET studies, BP is thus commonly used as an index of receptor density (Laruelle, 2003). According to this view, the present results may indicate increased D2-receptor density levels in RLS patients.

Receptor density levels in adult brain may be a consequence of genetic factors (Jonsson *et al.*, 1999) but may also be subject to adaptive mechanisms during life as supported by data from animal research. For instance, D2-receptor expression in rodents can be upregulated by D2-receptor antagonists (Hurley *et al.*, 1996; Huang *et al.*, 1997; Joyce, 2001; Andersson *et al.*, 2005) or by depletion of synaptic dopamine concentrations (Stanwood *et al.*, 2000) while chronic stimulation with D2 agonists can lead to a decrease in receptor concentration (Stanwood *et al.*, 2000). In non-human primates, chronic treatment with

amphetamine has been shown to result in decreased striatal D2 density (Ginovart *et al.*, 1999). According to this line of observations, an extended interpretation of the present results is that the increased D2-receptor density in RLS patients could be owing to receptor upregulation in response to hypoactivity in dopaminergic neurotransmission.

Apart from influencing binding parameters by a secondary effect on receptor density, endogenous dopamine has been shown to exert a direct effect on D2-receptor BP estimates. Compounds increasing synaptic dopamine concentrations can lead to a decrease in [^{11}C]raclopride binding (for review, see Laruelle, 2000) while pharmacological depletion of dopamine levels can increase binding (Ginovart *et al.*, 1997; Verhoeff *et al.*, 2001). The results from similar experiments with [^{11}C]FLB 457 are not conclusive though there is evidence that this radioligand is also sensitive to endogenous dopamine levels (Chou *et al.*, 2000). Consequently, it cannot be excluded that the present findings may also reflect lower synaptic levels of endogenous dopamine in RLS patients. Importantly, both interpretations presented above are consistent with the hypothesis of hypoactive dopaminergic neurotransmission in RLS.

In relation to the symptomatology of RLS, it is of particular interest that the dopamine system is involved in the modulation of nociceptive signals, as has been demonstrated in PET studies in man. It has been shown that dopamine D2-receptor BP in the striatum of healthy subjects inversely correlated to pain threshold (Hagelberg *et al.*, 2002*b*; Martikainen *et al.*, 2005) while in other studies patients with chronic orofacial pain exhibited higher dopamine D2-receptor BP (Hagelberg *et al.*, 2003*a*, *b*). These data suggest an association between hypoactivity of the dopaminergic system and a reduced pain threshold in these models. A similar mechanism could be involved in the perception of sensory symptoms in RLS.

Recently, von Spiczak *et al.* (2005) demonstrated a negative correlation between RLS severity and opioid binding in areas involved in the medial pain system as measured with the non-selective opioid-receptor radioligand [^{11}C]diprenorphine. This observation was interpreted as elevated release of endogenous opioids. Previously, alfentanil, a mu-opioid agonist, has been shown to increase [^{11}C]raclopride binding in the striatum (Hagelberg *et al.*, 2002*a*) and also [^{11}C]FLB 457 binding in several extrastriatal regions, most significantly in medial thalamus and anterior cingulate cortex (Hagelberg *et al.*, 2004). Taken together, it can thus not be excluded that our findings in the dopamine system are secondary to changes in oipiod neurotransmission.

The secondary aim of this study was to examine diurnal variability in dopaminergic parameters, with respect to the circadian pattern of RLS symptoms. The results failed to show any significant differences between patients and control subjects. In both groups, the average change between examinations performed in the morning and evening during the same day was within the previously reported test–retest reproducibility for both radioligands (Hietala *et al.*, 1999; Vilkman *et al.*, 2000; Mawlawi *et al.*, 2001; Sudo *et al.*, 2001; Hirvonen *et al.*, 2003). A possible explanation could be that the evening PET examinations were not performed sufficiently late to coincide with RLS symptoms, which are reported to be most prominent between midnight and 4 a.m. (Hening *et al.*, 1999; Trenkwalder *et al.*, 1999*a*; Michaud *et al.*, 2004). However, the validity of the present protocol is supported by the observation that patients had significantly more pronounced leg movement in the evening as compared with the morning examinations and the higher rating of immobilization symptoms for patients compared to controls. The absence of diurnal variation of D2-receptor availability could imply that events downstream or parallel to D2-receptor signalling are responsible for the circadian pattern of RLS symptoms.

As stated above, the finding of increased striatal D2-receptor availability in RLS patients contrasts with the results of previous imaging studies. We here discuss four methodological issues that can account for this discrepancy. (i) All previous studies but one have been performed using SPET, which compared with PET has a lower resolution. (ii) No head fixation procedures are described in any of the studies. Actions to control for head movement are of particular concern when studying RLS. Leg movement during examinations (as observed in the present study) could result in small but nevertheless significant head movement, leading to signal blurring with false low BP values as a consequence. The striatum may be particularly sensitive to this effect, since it is an anatomically small structure with a high signal in contrast to surrounding tissue. (iii) In some studies, control subjects were not age-matched. For instance, in one of the studies (Staedt *et al.*, 1995) the control subjects were significantly younger than patients (mean age 42 versus 58 years). Such a difference may substantially influence results since dopamine D2-receptor density is known to decrease by ~5–10% per decade (Volkow *et al.*, 1996; Kaasinen *et al.*, 2000). (iv) In several studies (Turjanski *et al.*, 1999; Eisensehr *et al.*, 2001; Tribl *et al.*, 2002) the sample included RLS patients who had been exposed to dopaminergic drugs. As discussed above, chronic receptor stimulation can lead to receptor downregulation, resulting in lower radioligand binding.

With regard to methodological concerns in the present study, it should be noted that there was a difference between groups with respect to injected mass in [^{11}C]FLB 457 evening examinations, albeit not statistically significant. Increased receptor occupancy of unlabelled radioligand could lead to lower BP values in control subjects, constituting a possible source of error. In simulation studies, an injected mass of 0.5 μg has been shown to result in a radioligand occupancy of 5% while 1 μg corresponded to 8% occupancy (Olsson *et al.*, 2004). On the basis of this analysis, the reported difference of 0.44–0.58 should account for 1 or 2% difference in BP. A further issue is that of partial volume effects (PVE), a phenomenon related to the limited resolution of PET where activity in a brain region might be contaminated by

that of neighbouring regions. However, the issue is of importance primarily when studying diseases with known tissue degeneration or other forms of structural change, which would then introduce systematic differences in PVE between groups. This has generally not been the case for RLS although a recent publication has suggested a grey matter increase in the pulvinar nuclei of thalamus in patients with RLS (Etgen *et al.*, 2005).

In conclusion, our results provide support for hypoactive dopaminergic neurotransmission as a key component in the pathophysiology of RLS. The study supports that extrastriatal as well as striatal brain regions are involved. Furthermore, it might be speculated that the specific anatomical location of the findings reflects a disturbance of the central processing of sensory input, suggesting a possible pathway for sensory symptoms of RLS. The results do not support variability in dopamine D2-receptor availability as a correlate to the diurnal rhythm of RLS symptoms.

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