Lower nucleus accumbens α-synuclein load and D3 receptor levels in Parkinson's disease with impulsive compulsive behaviours

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Abstract
Impulsive compulsive behaviours in Parkinson’s disease have been linked to increased dopaminergic release in the ventral striatum and excessive stimulation of dopamine D3 receptors. Thirty-one patients with impulsive compulsive behaviours and Parkinson’s disease who donated their brains to the Queen Square Brain Bank for Neurological Disorders were assessed for alpha-synuclein neuropathological load and tyrosine hydroxylase levels in the nucleus accumbens, dorsal putamen and caudate using immunohistochemistry. Dopamine D2 and dopamine D3 receptors protein levels in the nucleus accumbens, frontal cortex and putamen were determined using western blotting. Results were compared to 29 Parkinson’s disease cases without impulsive compulsive behaviours matched by age, sex, disease duration, age at Parkinson’s disease onset and disease duration. The majority of patients with impulsive compulsive behaviours had dopamine dysregulation syndrome. Patients with Parkinson’s disease and impulsive compulsive behaviours had lower alpha-synuclein load and dopamine D3 receptor levels in the nucleus accumbens. No differences were seen between groups in the other brain areas and in the analysis of tyrosine hydroxylase and dopamine D2 receptor levels. Lower alpha-synuclein load in the nucleus accumbens of individuals with Parkinson’s disease and impulsive compulsive behaviours was confirmed on Western blotting. Downregulation of the dopamine D3 receptor levels may have occurred either as a consequence of the degenerative process or of a pre-morbid trait. The lower levels of alpha-synuclein may have contributed to an excessive stimulation of the ventral striatum resulting in impulsive compulsive behaviours.

Authors affiliations:
Introduction

Running title: Post mortem study of impulsive compulsive behaviours in Parkinson’s disease

Keywords: Parkinson’s disease; impulsive compulsive behaviours; alpha-synuclein; dopaminergic D2 receptor; dopaminergic D3 receptor.

Abbreviations: D3R – BSA – bovine serum albumin; CERAD - Consortium to Establish a Registry for Alzheimer’s Disease; D2R – dopaminergic D2 receptors; dopaminergic D3 receptors; DA – dopamine agonists; DDS – dopamine dysregulation syndrome; ICBs – impulsive compulsive behaviours; LEDD – levodopa equivalent daily dose; MAOi – monoamine oxidase inhibitors; NIA-AA - National Institute of Aging-Alzheimer’s Association; PD+ICB – patients with Parkinson’s disease and impulsive compulsive behaviours; PD-ICB – patients with Parkinson’s disease without impulsive compulsive behaviours; PET – positron emission tomography; QSBB – Queen Square Brain Bank for Neurological Disorders; ROI – region of interest; TH – tyrosine hydroxylase; TRI – Tris-buffered saline.
Pathophysiological similarities between drug addiction and impulsive compulsive behaviours (ICBs) in Parkinson’s disease suggest shared pathways between the two conditions involving the dopaminergic system of the meso-cortico-limbic circuit. Recreational drugs have the ability to increase dopamine in the basal ganglia to a supra-physiological level, and this is believed to be an important step in the promotion of addiction (Wise and Rompre, 1989). Evidence from imaging studies in drug addiction using $^{[11]}C$-raclopride positron emission tomography (PET) scanning shows that large and rapid increases in extracellular dopamine may be the encoding mechanism through which dopamine attributes salience to an event in normal conditions (Drevets et al., 2001; Probst and van Eimeren, 2013). Dopaminergic drugs such as amphetamines and cocaine with fast brain uptake and clearance more closely mimic this natural process and tend to be more implicated in the experience of euphoric “highs” and drug-induced reinforcement (Volkow et al., 2004).

A similar pattern of excessive release of dopamine in the ventral striatum to that described in drug abusers has been reported in Parkinson’s disease patients with ICBs. Using $^{[11]}C$-raclopride PET in patients with Parkinson’s disease, Evans and colleagues showed that individuals with dopamine dysregulation syndrome (DDS) have enhanced dopamine release in the ventral striatum induced by levodopa (Evans et al., 2006). O’Sullivan and colleagues further demonstrated that medicated Parkinson’s disease patients with ICBs (including compulsive sexual behaviour, compulsive eating, punding, compulsive shopping, DDS, pathological gambling and reckless generosity) have increased dopamine release in the ventral striatum after exposure to reward-related cues (O’Sullivan et al., 2011).

Dopamine D3 Receptors (D3R) are widely expressed in the limbic system of the human brain, particularly in cell populations within the nucleus accumbens (NAc) (Suzuki et al., 1998), and have a higher affinity for dopamine compared to dopaminergic D1 and D2 receptors, making them more sensitive to changes in dopamine levels (Payer et al., 2014). It has been postulated that ICBs are a consequence of overstimulation of D3R by dopamine agonists (DA). One study using data from observational clinical studies suggested the higher selectivity of a dopamine agonist for D3R might be associated with higher uncorrected average prevalence of ICBs (Seeman, 2015). However, it may be that in the situation of increased dopamine, high affinity receptors may be saturated and not sensitive to change, compared to low affinity receptors (Durstewitz and Seamans, 2008). Furthermore, despite previous findings that D3R expression
is upregulated in drug addiction (Payer et al., 2014), in Parkinson’s disease patients with ICBs, no changes in D2R expression and no evidence of upregulation of D3R in the limbic striatum was found in a PET study (Payer et al., 2015).

A number of in vivo imaging studies have identified reduced ventral striatum dopamine transporter binding in Parkinson’s disease patients with ICBs and these have been shown to predate onset of these disorders (Vriend et al., 2014a). Dopaminergic depletion in Parkinson’s disease affects the meso-cortico-limbic circuit (including the nucleus accumbens, anterior cingulate cortex, amygdala and hippocampus) to lesser extent than basal ganglia motor loops (Cilia and van Eimeren, 2011), leading to the concept that dopamine replacement therapy overstimulates these relatively intact circuits. In Parkinson’s disease patients with pathological gambling, resting state technetium SPECT showed overactivity in the meso limbic system compared with controls with Parkinson’s disease (Cilia et al., 2008). However, van Eimeren and colleagues (van Eimeren et al., 2010) showed that dopamine agonists lead to ventral prefrontal cortex deactivation only in patients with ICBs receiving dopaminergic drugs compared to controls, suggesting ICBs are characterised by impaired inhibitory control in vulnerable patients. Another recent study using functional and structural MRI identified factors associated with ICBs including decreased nucleus accumbens dopamine synthesis, decreased connectivity between the accumbens and rostral cingulate cortex, and increased cortical thickness of the subgenual rostral anterior cingulate cortex. Reduced meso-limbic dopaminergic projections in conjunction with a dysfunctional rostral anterior cingulate cortex may be important risk factors (Hammes et al., 2019) in ICBs. The role of mesolimbic dopaminergic projection neurons has also been highlighted by the finding of dysfunctional activation of dopamine autoreceptors in the midbrain and low dopaminergic tone in the anterior cingulate cortex in a PET study (Ray et al., 2012).

No post-mortem studies have been conducted with individuals who had Parkinson’s disease and developed ICBs in life. In this study, we have assessed alpha-synuclein neuropathological load and protein levels of D2R and D3R in the nucleus accumbens, and other brain areas, of individuals with Parkinson’s disease who developed ICBs and compared these with a matched group without ICBs.

Materials and Methods
**Selection of patients and brain regions**

Cases with pathologically proven Parkinson’s disease who developed ICBs in life were identified from the archives of the QSBB. Using the same database, cases with Parkinson’s disease without ICBs matched by sex, age at disease onset, disease duration and age at death were selected as controls in a consecutive fashion, starting with more recent cases.

The medical records comprising primary and specialist care notes were systematically reviewed. Demographic and clinical data were collated, including information on ICBs and detailed medication history. Symptoms that were not mentioned in the notes were considered absent. The QSBB is licensed by the Human Tissue Authority to store brain tissue, and its brain donation protocols were approved by a London Multi-Centre Research Ethics Committee. Written consent for donation was obtained in all cases.

Additional brain regions were chosen to confirm whether any differences found were restricted to the nucleus accumbens. For immunohistochemistry, the dorsal putamen and dorsal caudate were selected as areas not implicated in the development of ICBs. For western blotting, one area with high levels of D2R and D3R (the dorsal putamen) and another with low levels (the inferior frontal cortex), were chosen (Luquin-Piudo and Sanz, 2011).

**Sample preparation**

Brain donations were processed according to QSBB standard operating procedures. After post-mortem, the brain was hemi-dissected and one half of the brain (usually the right) was sliced, frozen and stored at -80°C whilst the other half was fixed in 10% formalin for 3 weeks.

Paraffin-embedded blocks of the anterior striatum, including the NAc, are routinely obtained during neuropathological examination of all Parkinson’s disease cases. Initially, a case by case macroscopic review of the paraffin-fixed blocks was conducted to confirm the presence of the NAc, putamen and caudate. Subsequently, three 8 µm sections and one 12 µm section were cut and mounted on glass slides and left to dry in a 60°C oven overnight. One 8 µm section was stained with haematoxylin and eosin (H&E) and one 12 µm section with Luxol fast blue (LFB)
using standard procedures. After confirmation of adequate sampling of the NAc using microscopy, the remaining 8 µm sections were stained with alpha-synuclein and tyrosine hydroxylase. The antibodies and other materials used for immunohistochemistry are detailed in supplementary material.

**Image analysis of alpha-synuclein and TH immunoreactivity**

Digital images of TH and alpha-synuclein stained slides were obtained using a high-resolution digital scanner (Leica SCN400). The NAc, putamen and caudate were extracted from the original picture using the software Aperio ImageScope (Leica Biosystems) and the images processed with ImageJ. To optimise the sampling of the regions of interest, a Bland-Altman plot (Bland and Altman, 1999) was used to identify the ideal number of random squares needed to be sampled from each brain region: 5 random squares of 1000 pixels each for the NAc and caudate, and 10 for the putamen. The threshold was adjusted to correctly identify the two-dimensional area of alpha-synuclein (Lewy bodies and neurites) and TH immunoreactivity. Areal fraction, defined as the ratio of immune-stained pixels to the total number of pixels in the whole field, was calculated and expressed as percentage. Mean values for the random squares were obtained and exported to SPSS 22 for statistical analysis. All the analysis was conducted blinded to the presence of ICBs. To account for learning effect, all cases were analysed twice and only the second round of analysis was used for comparison.

**Western immunoblotting**

As none of the commercially available D3 receptor antibodies were suitable for immunohistochemistry we opted for analysis of protein levels by western blot. However, as Western blot analysis requires flash frozen brain tissue, only 31 cases were available for this part of the study. 16 PD+ICB and 15 Parkinson’s disease controls. Samples from the NAc, dorsal putamen and the inferior frontal inferior gyrus were dissected and prepared for western blotting according to the QSBB standard operating procedure.

Protein concentrations were determined using the BioRad DC Protein Assay following the manufacturer’s instructions. Proteins were separated according to molecular weight using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immobilisation by transfer
to a nitrocellulose membrane and detection of specific proteins by antibodies as previously described (Laemmli, 1970). Thirty µL samples were loaded into the wells of the gel alongside 5 µL of SeeBlue (Invitrogen) pre-stained protein size markers.

Excess protein binding sites of the membranes were blocked by incubation for 1 hour with 5% bovine serum albumin (Sigma-Aldrich) in tris buffered saline (TBS). Primary antibodies (Supplementary table 1) against the proteins of interest were diluted at defined ratios in TBS containing 0.05% (v/v) Tween-20 (TBSt) and incubated with the membranes for a defined period. After washing, the membrane was then incubated for 1 hour at room temperature with secondary fluorescence-labelled detection antibody (Li-Cor) against the primary antibody diluted 1:20,000 in TBSt. Membranes were scanned on a Li-Cor Odyssey 3000 for fluorescence detection of immunolabelled protein bands. Materials used in Western blotting are detailed in supplementary material.

Densitometry of immunoblots

Densitometry was performed on two gels. To allow statistical comparison between gels, clinical data was used to choose one Parkinson’s disease case without ICBs that was included in every gel (internal control). Immunoblot scans were analysed by densitometry using Image Studio Lite software (Li-Cor). A region of interest (ROI) was drawn around the largest/brightest band, and ROIs of the same dimensions drawn around protein bands for all other samples to be quantified. Mean intensities of each ROI were then analysed in Microsoft Excel. In order to calculate net band intensity, background readings were subtracted from band intensity readings. Signal intensity values of each band were normalised for beta-actin and, subsequently, for the optimal internal control. All comparisons were run three times and the mean values used for statistical analysis. Immunoblot results were expressed as proportional/fold change in intensity between cases and controls.

Lewy pathology and Alzheimer’s pathology analysis

Parkinson’s disease pathology was assessed using Braak staging (Braak et al., 2003) and cortical Lewy pathology using the McKeith criteria (McKeith et al., 1996) according to
published criteria (Alafuzoff et al., 2009). Alzheimer’s disease pathology was quantified based on the Braak and Braak staging system (Braak and Braak, 1991) for tau neurofibrillary deposits, the Thal staging of amyloid beta deposition (Thal et al., 2002), the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) score of neuritic plaques (Mirra et al., 1991), and the National Institute of Aging-Alzheimer’s Association (NIA-AA) scores (Hyman et al., 2012).

**Statistical analysis**

All variables were tested for normality. Parametric data were analysed using independent samples t-test and non-parametric data Mann-Whitney U and Wilcoxon matched pairs accordingly. Mann-Whitney U test was used for all variables obtained from western blotting. Proportions were compared with the Pearson chi-square test, except if the minimum expected cell count was less than five, when the Fisher’s exact test was used. A p-value of less than 0.05 was considered significant. Data was analysed using SPSS 22.

**Results**

**Clinical and demographic data**

Thirty patients with Parkinson’s disease and ICBs (PD+ICB) were identified from the QSBB archives and matched by sex, age at Parkinson’s disease onset, age at death and disease duration with thirty Parkinson’s disease patients without ICBs (PD-ICB). The study population was composed mostly of males with early onset Parkinson’s disease. All patients had been evaluated and followed by hospital specialists throughout the disease course. One patient in the control group had DDS, therefore 31 patients were included in the PD+ICB group and 29 in the control group (PD-ICB). In the PD+ICB group, ten individuals (32.2%) had multiple behavioural addictions. The most predominant ICB was DDS, seen isolated in 16 patients and in combination with other ICBs in 8. Details on the types of ICBs can be seen in Table 1.
Table 1 – Types of ICBs

<table>
<thead>
<tr>
<th>N</th>
<th>ICBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Isolated DDS</td>
</tr>
<tr>
<td>4</td>
<td>Isolated CSB</td>
</tr>
<tr>
<td>1</td>
<td>Isolated punding</td>
</tr>
<tr>
<td>2</td>
<td>DDS and CSB</td>
</tr>
<tr>
<td>1</td>
<td>CSB and compulsive shopping</td>
</tr>
<tr>
<td>1</td>
<td>DDS and punding</td>
</tr>
<tr>
<td>1</td>
<td>DDS and pathological gambling</td>
</tr>
<tr>
<td>1</td>
<td>CSB and punding</td>
</tr>
<tr>
<td>2</td>
<td>DDS, CSB and compulsive shopping</td>
</tr>
<tr>
<td>1</td>
<td>DDS, CSB and punding</td>
</tr>
<tr>
<td>1</td>
<td>DDS, CSB, compulsive eating and punding</td>
</tr>
</tbody>
</table>

ICBs – impulsive compulsive behaviours; DDS – dopamine dysregulation syndrome; CSB – compulsive sexual behaviour

The most common cause of death was pneumonia (13 PD+ICB and 12 PD-ICB), followed by end stage Parkinson’s disease (5 PD+ICB and 7 PD-ICB) and sepsis (1 PD+ICB and 5 PD-ICB). Causes of death according to group are displayed in supplementary material. From careful review of records, 16/24 cases of DDS (+/- other ICB), and 5/7 cases of ICB alone appeared to have evidence of ICBs in their last year of life, amounting to 68% of total PD+ICB cohort.

The proportion of males, age at disease onset, Parkinson’s disease duration, age at death, and prevalence of dyskinesias and dementia did not differ between groups (Table 2). A similar proportion of patients in the two groups used DA throughout the course of the illness but there was a non-significantly higher proportion of PD+ICB patients using DA at the time of death. There was a trend for higher DA peak dose, measured in levodopa equivalent daily dose (LEDD) (Tomlinson et al., 2010), but this also failed to reach statistical significance. Duration of DA use, DA end-dose and proportion of patients using monoamine oxidase inhibitors (MAOi) did not differ between groups. Lifetime cumulative dose of levodopa was calculated as described elsewhere (Parkkinen et al., 2011) and did not differ between groups. The dose of
all Parkinson’s disease medications at death was calculated in LEDD revealing that the PD+ICB group used more medication than controls.

Table 2 – Demographic and clinical data

<table>
<thead>
<tr>
<th></th>
<th>PD+ICB</th>
<th>PD-ICB</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>83.8%</td>
<td>75.8%</td>
<td>0.438^A</td>
</tr>
<tr>
<td>Age at PD onset (years)</td>
<td>51.29 (± 11)</td>
<td>51.82 (± 5)</td>
<td>0.906^†</td>
</tr>
<tr>
<td>PD duration (years)</td>
<td>20.46 (± 5.8)</td>
<td>21.48 (± 3.6)</td>
<td>0.421^p</td>
</tr>
<tr>
<td>Age at death (years)</td>
<td>71.74 (± 8.9)</td>
<td>73.24 (± 4.7)</td>
<td>0.789^†</td>
</tr>
<tr>
<td>Dyskinesias (%)</td>
<td>87%</td>
<td>75.8%</td>
<td>0.327^§</td>
</tr>
<tr>
<td>Dementia (%)</td>
<td>52%</td>
<td>62%</td>
<td>0.414^A</td>
</tr>
<tr>
<td>LEDD at death</td>
<td>1104.66 (± 631.2)</td>
<td>777.39 (± 448.5)</td>
<td>0.025^p</td>
</tr>
<tr>
<td>Levodopa lifetime cumulative dose (g)</td>
<td>4914.91 (± 3982)</td>
<td>3475.1 (± 2651)</td>
<td>0.149^†</td>
</tr>
<tr>
<td>DA use anytime (%)</td>
<td>90.3%</td>
<td>86.2%</td>
<td>0.702^p</td>
</tr>
<tr>
<td>DA at death (%)</td>
<td>51.6%</td>
<td>34.4%</td>
<td>0.181^A</td>
</tr>
<tr>
<td>DA peak dose in LEDD</td>
<td>601.6 (± 631) N = 28</td>
<td>317.6 (± 255) N = 25</td>
<td>0.051^†</td>
</tr>
<tr>
<td>DA end dose in LEDD</td>
<td>285.8 (± 202) N = 11</td>
<td>173.8 (± 105) N = 18</td>
<td>0.188^‡</td>
</tr>
<tr>
<td>DA duration (years)</td>
<td>8.47 (± 5.7)</td>
<td>6.91 (± 5.5)</td>
<td>0.428^†</td>
</tr>
<tr>
<td>MAOi anytime (%)</td>
<td>77.4%</td>
<td>89.6%</td>
<td>0.302^§</td>
</tr>
</tbody>
</table>

PD – Parkinson’s disease; ICBs – impulsive compulsive behaviours; PD+ICB – patients with PD and ICBs; PD-ICB – PD patients without ICBs; LEDD – levodopa equivalent daily dose; DA – dopamine agonists; MAOi – monoamine oxidase inhibitors. ^Chi-square; † Mann-Whitney test; p Independent samples t-test; § Fisher’s exact test. Significant results in bold.

**Alpha-synuclein quantification**

Alpha-synuclein pathology was identified by immunohistochemistry. Dorsal caudate from 27 brains and dorsal putamen from 29 in each group were available for analysis. One NAc sample from a patient with ICB was poorly represented and had to be removed from the data analysis. Representative image of Lewy bodies and neurites were seen in all three regions as displayed in Supplementary Figure 1.
Aereal fraction analysis revealed a significantly lower alpha-synuclein load in the nucleus accumbens of patients with ICBs. No differences between groups were seen in the putamen and caudate (Figure 2). Total alpha-synuclein load was calculated as the sum of alpha-synuclein stained area from all three structures and was significantly lower in the PD+ICB group (supplementary material). Western blot analysis of alpha-synuclein levels confirmed the immunohistochemical findings. Samples from 16 PD+ICB and 15 PD-ICB cases were available. Alpha-synuclein was identified as a band of 19 kDa molecular weight (see supplementary material, Figure S2). Comparison between groups revealed significantly lower signal intensity in the NAc of patients with ICBs, whereas no differences were seen in the putamen and frontal cortex (Figure 1 and Supplementary Material).

The caudate from 27 cases and putamen from 29 cases in each group were available for TH immunohistochemical analysis. Similar levels of immunoreactivity were found in both groups and in all three regions as displayed in figure 1. Total TH level, calculated as the sum of TH levels from all three regions, was also similar between PD+ICB and PD-ICB (Supplementary Material).
**Figure 1. Quantification of alpha-synuclein and tyrosine hydroxylase.** Lower alpha-synuclein load was seen only in the nucleus accumbens (NAc) of patients with ICBs, with no differences in the caudate and putamen. In top and bottom images the y axis represents the ratio of immuno-stained pixels to the total number of pixels in the whole field expressed as percentage. In the middle image, the y axis represents signal intensity normalised for beta-actin and for the optimal internal control. No differences were detected in tyrosine hydroxylase levels. PD+ICB – patients with PD and ICBs; PD-ICB – patients with PD without ICBs. *Mann-Whitney U test. Bars represent median values, boxes interquartile range and whiskers minimum and maximum values. Significant results in bold.
Comparison of alpha-synuclein load and tyrosine hydroxylase levels between the three regions was conducted. Whilst no differences were found for alpha-synuclein when both groups were analysed together (Friedman test; \( p = 0.744 \)), post hoc analysis revealed higher alpha-synuclein levels in the NAc of PD-ICB (Figure 3). TH levels were significantly different (Friedman test; \( p < 0.001 \)). Post hoc analysis revealed that both groups had higher TH levels in the NAc compared to the caudate and in the caudate compared to the putamen as displayed in Figures 2 and 3.

**Figure 2. Comparison of alpha-synuclein load and tyrosine hydroxylase expression (TH) in different structures according to the presence of ICBs.** Levels of alpha-synuclein pathology were higher in the NAc of PD-ICB whereas no differences were seen in PD+ICB. Higher levels of TH were detected in the NAc followed by the caudate and the putamen in both groups. The y-axis represents the ratio of immune-stained pixels to the total number of pixels in the whole field and expressed as percentage. PD+ICB – patients with Parkinson’s disease and impulsive compulsive behaviours; PD-ICB – patients with Parkinson’s disease without impulsive compulsive behaviours; NAc – nucleus accumbens. Bars represent median values, boxes interquartile range and whiskers minimum and maximum values. *Wilcoxon matched pairs.
Figure 3. Tyrosine hydroxylase staining in different brain regions from a patient with ICB. A - the nucleus accumbens (NAc); B – caudate; C – putamen. Scale bar represents 50µm. TH immunoreactivity was higher in the NAc, followed by the caudate and the putamen with no difference between patients with and without impulsive compulsive behaviours.

**Dopaminergic D2 and D3 receptors**

Frozen tissue samples from 16 PD+ICB and 15 PD-ICB were available. Immunoblot analysis of D2R revealed a single band of 49 kDa of molecular weight (see supplementary material). The analysis of dopaminergic D2R values revealed no differences between groups, although lower values of D2R in the NAc of PD+ICB were found, it did not reach significance (Figure 4 and supplementary material Figure S3). Immunoblot analysis of D3R revealed a single band of 44 kDa molecular weight as detailed in supplementary material. Figure 4 shows D3R levels in the different brain regions separated by groups. Lower D3 signal in the NAc of PD+ICB was detected, whereas no differences were seen in the putamen and frontal cortex.
Figure 4. Comparison of D2 (D2R) and D3 receptor (D3R) signal intensity between PD patients with ICBs (PD+ICB) and PD controls (PD-ICB). No significant differences were found in D2R protein levels. There was a statistically significant difference of D3R in the NAc, with lower signal seen among patients with ICBs. The y-axis represents signal intensity normalised for beta-actin and for the optimal internal control. *Mann-Whitney U test. Bars represent median values, boxes interquartile range and whiskers minimum and maximum values. Significant results in bold.

Assessment of Lewy pathology and Alzheimer’s disease neuropathological changes

Lewy pathology and Alzheimer’s disease pathological changes were assessed in 29 PD+ICB and 29 PD-ICB. The majority of patients were classified as Braak stage 6 and the remaining as stage 5 with no significant differences between groups. Cortical Lewy pathology assessment with the McKeith criteria revealed that all cases in both groups had either limbic or neocortical pathology. With respect to the presence of Alzheimer’s disease pathological changes, no differences were seen in the Braak and Braak and Thal staging systems. Similarly, the CERAD and NIA-AA scores did not differ between groups, showing absent or low levels of Alzheimer’s disease neuropathological changes in the majority (table 3).
<table>
<thead>
<tr>
<th></th>
<th>PD+ICB N = 29</th>
<th>PD-ICB N = 29</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>Braak Lewy pathology staging</td>
<td>5: 17.2% 6: 82.8%</td>
<td>5: 3.4% 6: 96.5%</td>
<td>0.194*</td>
</tr>
<tr>
<td>McKeith criteria</td>
<td>Limbic: 17.2% Neocortical: 82.8%</td>
<td>Limbic: 6.9% Neocortical: 93.1%</td>
<td>0.423*</td>
</tr>
<tr>
<td>Thal staging</td>
<td>0: 31% 1: 20.7% 2: 13.8% 3: 17.2% 4: 13.8% 5: 3.4%</td>
<td>0: 37.9% 1: 17.2% 2: 17.2% 3: 10.4% 4: 13.8% 5: 3.5%</td>
<td>0.970‡</td>
</tr>
<tr>
<td>Braak and Braak tau staging</td>
<td>0: 17.2% 1: 34.5% 2: 31% 3: 6.9% 4: 10.4%</td>
<td>0: 13.8% 1: 51.7% 2: 27.6% 3: 6.9% 4: 0%</td>
<td>0.383‡</td>
</tr>
<tr>
<td>CERAD score</td>
<td>0: 55.1% 1: 27.6% 2: 17.3% 3: 0%</td>
<td>0: 58.6% 1: 24.2% 2: 13.8% 3: 3.4%</td>
<td>0.751‡</td>
</tr>
<tr>
<td>NIA-AA score</td>
<td>Low: 58.7% Intermediate: 3.4% Not: 37.9%</td>
<td>Low: 58.6% Intermediate: 10.3% Not: 31.1%</td>
<td>0.549‡</td>
</tr>
</tbody>
</table>

PD – Parkinson’s disease; ICBs – impulsive compulsive behaviours; PD+ICB – patients with PD and ICBs; PD-ICB – PD patients without ICBs; *Fisher’s exact test; ‡Chi-square test.

**Analysis of DDS subgroups**

To address the question of whether DDS represents a different subgroup of ICBs with separate pathogenesis, we performed two additional *posthoc* data analyses: comparison of all subjects with PD+DDS (n = 24) regardless of whether there was additional ICB present versus PD-ICB, and comparison of a subgroup of PD+pure DDS (n = 16) versus PD-ICB .
For PD+DDS vs PD-ICB the alpha synuclein load in NAc remained significantly lower than in the PD-ICB group by aerial fraction analysis ($p = 0.06$) and Western blot ($p = 0.031$). Western blot analysis of D3 receptor protein no longer reached statistical significance between the groups ($p = 0.091$). No other significant results were found (data in supplementary material). For PD+pure DDS vs PD-ICB again the aerial fraction analysis of alpha synuclein load in NAc was significantly lower ($p = 0.01$) whilst the protein quantification by western blotting was no longer significant ($p = 0.115$). D3 receptor protein level by western blotting was not significant between groups ($p = 0.333$). No other significant results were found (data in supplementary material).

**Discussion**

This is the first *post mortem* study of alpha-synuclein, D2R and D3R levels in Parkinson’s disease patients with ICBs. The principal findings were of lower alpha synuclein load and D3R levels in the NAc in patients with PD+ICB compared with PD-ICB.

The disease duration of slightly more than 20 years was higher than that described in a general population of Parkinson’s disease patients (Kempster *et al.*, 2010), corroborating previous findings that younger age of onset patients have a slower disease progression (Wickremaratchi *et al.*, 2009). The first formal description of DDS was published by our centre in 2000 (Giovannoni *et al.*, 2000) and the higher proportion of DDS patients may reflect a local research interest in this relatively uncommon behavioural disturbance in Parkinson’s disease. Previous research has identified oral dopamine receptor agonists as the strongest risk factor for ICBs, especially pathological gambling (Averbeck *et al.*, 2014), whereas the main risk factor for DDS is the use of levodopa (O’Sullivan *et al.*, 2009). Therefore, the lack of differences in DA use found in this study might reflect the higher number of patients with DDS. This could also explain the higher total dopaminergic treatment dose seen in the PD+ICB group, as patients with DDS are characterised by inappropriate overuse of dopaminergic medication (O'Sullivan *et al.*, 2009).

It has been suggested that the differing primary risk factors for DDS (levodopa) and other ICBs (DA), as well as the co-occurrence of dyskinesias with DDS and different age of onset, may mean DDS has a different pathophysiology. We therefore undertook further analyses using the
broad PD+DDS group (which included cases with additional ICBs) and PD+pure DDS versus PD-ICB. The results for aerel fraction alpha synuclein load in NAc was lower in both DDS groups, but the results using western blotting lost significance. The same was also true for D3R levels in NAc. This suggests that those patients with only ICB, but not DDS, were contributing to the significant results for both alpha synuclein load and DR3 levels seen in the total PD+ICB group.

The dorsal parts of the striatum are more severely damaged in Parkinson’s disease (Kish et al., 1988). As a consequence of the nigral degeneration there is reduction of TH staining in the striatum which correlates with disease duration and begins in the putamen, extends into the caudate and eventually extends to the ventral striatum, including the NAc (Jellinger, 2011). This pattern of progression was confirmed in the present study, with TH levels highest in the NAc, followed by the caudate and the putamen. The relative preservation of the ventral striatum has led to the hypothesis that both ICBs and downstream frontal cognitive deficits may be a consequence of iatrogenic overstimulation of a relatively preserved reward pathway (Gotham et al., 1988; Cools et al., 2003).

TH staining in the nucleus accumbens, however, showed no difference between the PD+ICB and PD-ICB groups, which does not support imaging studies of reduced dopamine transporter binding in the ventral striatum (Vriend et al., 2014a) and decreased NAc dopamine synthesis capacity (Hammes et al., 2019). Post mortem TH staining reflects the numbers of dopaminergic nerve terminals within the region studied, and our data suggests that the in vivo imaging studies demonstrate a functional change in DAT availability and DA synthesis, rather than cell loss, possibly as an aberrant response in those with ICB.

There was no gradient of alpha-synuclein staining between the NAc, dorsal putamen and caudate when both groups were analysed together, but PD-ICB showed higher alpha-synuclein load in the nucleus accumbens compared to the dorsal striatum. Comparison between groups revealed that PD+ICB had a lower alpha-synuclein load in the accumbens compared to controls. The fact that the NAc was the only region with different levels of alpha-synuclein in this study points to a possible role of alpha-synuclein pathology and the nucleus accumbens in the genesis of ICBs. Individual susceptibility may underlie the lower levels of aggregated alpha synuclein, and hence disease burden, found in patients with ICBs.
Lower levels of alpha-synuclein pathology in PD+ICB groups may indicate a better preserved ventral striatum. Alpha-synuclein is a negative regulator of dopamine release as suggested by studies with cultured neurons and animal models that have reported inhibition of vesicle exocytosis by overexpression of this molecule (Abeliovich et al., 2000; Nemani et al., 2010). Although the role of pathogenic aggregated alpha-synuclein on dopamine release is not known, it is nevertheless conceivable that alpha synuclein could potentially play a role in a hyper-dopaminergic state.

Upregulation of D3R has been described in animal models after exposure to drugs of abuse and levodopa (Payer et al., 2014) and in the NAc of individuals following death from cocaine overdose (Mash and Staley, 1999). However, functional neuroimaging studies in humans with behavioural and drug addiction have consistently shown reduced D2R/D3R availability, which has been attributed to excessive synaptic dopamine (Payer et al., 2014). Two PET studies have assessed patients after stimuli presentation, one examined patients with DDS after levodopa intake (Evans et al., 2006), and the other patients with diverse ICBs after presentation of visual cues (O’Sullivan et al., 2011). The reduction in tracer binding seen in both studies is probably a consequence of excessive dopamine release to salient stimuli. Other PET studies have assessed patients with pathological gambling (Steeves et al., 2009) and Parkinson’s disease with different impulse control disorders (Payer et al., 2015) at baseline, without stimulus presentation. In this case, reduced tracer binding does not necessarily reflect increased dopaminergic tone and could be a consequence of reduced expression of dopaminergic receptors (Stark and Claassen, 2017). Only one of these studies used a highly selective D3R tracer and the authors reported lower binding of the tracer in the limbic striatum, attributed to excessive synaptic dopamine (Payer et al., 2015). Although no patients with DDS were included in the study, it may be that different behavioural addictions are associated with similar pathophysiological findings (O’Sullivan et al., 2011). Behavioural traits of ICBs, such as reduced loss sensitivity and slower loss learning rates (van Eimeren et al., 2009) may be due to a lack of tonic dopamine level dip. Thus, it may be dysfunction at a synaptic level rather than a simple “too much” or “too little” dopamine in the ventral striatum that is responsible for ICBs (Probst and van Eimeren, 2013).

Indirect evidence to support an interaction between dopamine overdose of the ventral striatum and reduced D3R levels in the NAc leading to ICBs can also be gleaned from post mortem studies of D3R levels in Parkinson’s disease patients without behavioural addictions. One study
reported an association between reduced D3R levels and loss of response to dopaminergic drugs (Joyce et al., 2001). The observed decrease in D3R levels in the NAc, caudal striatum and globus pallidus was also associated with cognitive impairment (Joyce et al., 2002). We were unable to investigate loss of response to treatment in the present study, but if lower D3R levels in the PD+ICB group is associated with loss of response to dopaminergic treatment, these patients would require more medication to achieve symptomatic control, which, in turn puts them at greater risk of dopamine overdose of the ventral striatum and development of ICBs. The higher doses of dopaminergic treatment at death in the PD+ICB group would be in keeping with this notion. It is also possible that low D3R levels might be a biological pre-morbid trait increasing susceptibility to ICBs in individuals with nigral degeneration.

A reduction of D3R correlated with dementia in the studies by Joyce and colleagues (Joyce et al., 2002), raising the possibility that the lower D3R levels might be linked with higher burden of cortical Lewy pathology. Our data, however, does not support this explanation as both groups had a similar prevalence of dementia and similar Braak, McKeith and Alzheimer’s disease staging. It is also unlikely that the lower levels of D3R are a consequence of the higher doses of dopaminergic treatment at death seen in the PD+ICB group. Rather than down-regulation, dopamine can induce ectopic expression of D3R in the denervated caudate and putamen in animal models (Bordet et al., 1997) and exposure to levodopa and drugs of abuse upregulate D3R (Payer et al., 2014).

D2R are widely expressed throughout the striatum (Hurd et al., 2001) and have been shown to be downregulated in the initial stages of Parkinson’s disease (Antonini et al., 1997) and to normalise following chronic exposure to levodopa (Luquin-Piudo and Sanz, 2011). In line with a previous PET study, no significant differences in D2R levels were seen between the PD+ICB and PD-ICB groups (Payer et al., 2015).

An alternative explanation for lower D3R levels in PD+ICB is that reduced D2/3 receptors in the midbrain caused by Parkinson’s disease degeneration are contributing to excessive ventral striatal dopamine levels. This may have a number of effects on D3R including potential downregulation of D3R in susceptible individuals and/or supersensitivity of the remaining D3R (reviewed in (Vriend et al., 2014b)). Our data also provides an alternative interpretation for the in vivo PET scan data which show reduced D2/3 receptor binding which may reflect a reduction in actual number of receptors rather than indicating a hyperdopaminergic state reduced receptor
availability. In addition, our D2 and D3 data includes receptors on the plasma membrane and also those internalised, usually via endosomes and the endoplasmic reticulum. Internalisation can be stimulated by chronic dopaminergic stimulation (Bloch et al., 2003) which may also play a susceptibility role in ICBs.

Clinical data was analysed retrospectively in our study, which is a problem inherent in most brain bank studies. However, all the patients were seen by specialists throughout disease course and the clinical data was detailed. ICBs are underreported (Perez-Lloret et al., 2012) increasing the risk of false negatives in the control group but we were able to determine that at least two thirds of the patients still had ICB/DDS close to the time of death. To minimise this problem our controls were selected consecutively starting with the most recent donations, when clinicians were more aware of ICBs. The lack of a control group without neurodegenerative disease also limits the generalisation of our findings. Finally, neither PD+ICB and PD-ICB cohorts were genotyped for the more common Parkinson’s disease genes which potentially may have influenced results, although the age matching of both groups would mitigate against this.

Our results need to be confirmed by future pathological and focused in vivo studies. Increased susceptibility to alpha synuclein pathology in the accumbens could be protective against ICBs in Parkinson’s disease and D3R levels could be used to identify individuals with Parkinson’s disease at higher risk of behavioural addictions. Future research should analyse the influence of genetic polymorphisms in the expression of D3R, measure activation products of D3R and study additional brain areas of the dopaminergic reward pathway.

Acknowledgements
The authors would like to thank Robert Courtney, Linda Parsons, Geshanti Hondhamuni, Bridget Benson and Christina Murray for their technical expertise and the Reta Lila Weston Institute of Neurological Studies for the support received during this research project. J LH is supported by the Multiple System Atrophy Trust; the Multiple System Atrophy Coalition; Fund Sophia, managed by the King Baudouin Foundation and Karin & Sten Mortstedt CBD Solutions. Queen Square Brain Bank is supported by the Reta Lila Weston Institute for Neurological Studies and the Medical Research Council UK. This research was supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre.
Data availability statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding sources
Pedro Barbosa is supported by a grant from Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Competing interests
The authors report no competing interests.

Legend for the figures
Figure 1. Quantification of alpha-synuclein and tyrosine hydroxylase. Lower alpha-synuclein load was seen only in the nucleus accumbens (NAc) of patients with ICBs, with no differences in the caudate and putamen. No differences were detected in tyrosine hydroxylase levels. PD+ICB – patients with PD and ICBs; PD-ICB – patients with PD without ICBs. *Mann-Whitney U test. Bars represent median values, boxes interquartile range and whiskers minimum and maximum values. Significant results in bold.

Figure 2. Comparison of alpha-synuclein load and tyrosine hydroxylase levels in different structures. NAc – nucleus accumbens. No differences in alpha-synuclein load were seen. Higher levels of TH were detected in the NAc followed by the caudate and the putamen. Bars represent median values, boxes interquartile range and whiskers minimum and maximum values. No significant differences were found. *Friedman test.

Figure 3. Tyrosine hydroxylase levels in different regions. Left - the nucleus accumbens (NAc); Middle – caudate; Right – putamen (4x objective). TH immunoreactivity was higher in the NAc, followed by the caudate and the putamen with no difference between patients with and without impulsive compulsive behaviours.

Figure 4. Comparison of D2 and D3 receptor signal intensity between PD patients with ICBs (PD+ICB) and PD controls (PD-ICB). No differences were found in the D2 analysis. There was a statistically significant difference in the NAc, with lower signal seen among patients...
with ICBs. *Mann-Whitney U test. Bars represent median values, boxes interquartile range and whiskers minimum and maximum values.

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