The Basal Ganglia Cholinergic Neurochemistry of Progressive Supranuclear Palsy and Other Neurodegenerative Diseases

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Abstract

Background: Progressive Supranuclear Palsy (PSP) is a progressive neurodegenerative disorder involving motor and cognitive dysfunction. Currently there is no effective treatment either for symptomatic relief or disease modification. This relates, in part, to a lack of knowledge of the underlying neurochemical abnormalities, including cholinergic receptor status in the basal ganglia.

Aim: To measure muscarinic M2 and M4 receptors in the basal ganglia in PSP.

Methods: We measured, autoradiographically, the muscarinic M2 (presynaptic) and M4 (postsynaptic) receptors in the striatum, pallidum and adjacent insular cortex of pathologically confirmed cases of PSP (n=18), and compared them to cases of Lewy body dementias (LBD) (n=45), Alzheimer's Disease (AD) (n=39) and controls (n=50).

Results: In PSP there was a reduction in M2 and M4 receptors in the posterior caudate and putamen compared to control, but no significant changes in the pallidum. AD cases had lower M2 receptors in the posterior striatum. LBD and AD groups had higher M2 binding in the insular cortex compared to controls.

Conclusions: The results suggest loss of posterior striatal cholinergic interneurons in PSP, and reduction of medium spiny projection neurons bearing M4 receptors. These results should be taken in context of more widespread pathology in PSP, but may have implications for future trials of cholinergic therapies.
Introduction
PSP was first described by Steele, Richardson and Olszewski in 1964, as a disease causing vertical gaze and pseudobulbar palsy, early falls, parkinsonism and dementia. Patients typically present in their 60’s and the average time from symptom onset to death ranges from 5-8.6 years. Neuropathological hallmarks of PSP include neurofibrillary tangles, neuropil threads and tufted astrocytes which are found predominantly within the basal ganglia, midbrain, pontine reticular formations and to a lesser extent the thalamus. The pathological inclusions comprise insoluble aggregates of four-repeat tau phosphoprotein. The basal ganglia, in particular the striatum (caudate and putamen) and its connections, are involved in motor and cognitive aspects of PSP. Neurochemical abnormalities are found within the dopaminergic, cholinergic and possibly GABAAergic systems. Cholinergic deficits are thought to be responsible for the early postural instability and cognitive impairment commonly found in PSP. Trials of a muscarinic M1/M2 cholinergic agonist and cholinesterase inhibitors have, however, failed to show improvement in motor function, quality of life or cognitive impairment.

In PSP there are abnormalities within the cholinergic system based on pathology in known cholinergic nuclei and reduced markers for acetylcholine (ACh) synthesis and release, but there is a dearth of knowledge about the status of cholinergic receptors. M1, M2 and M4 receptors are the predominant muscarinic receptors found in the striatum. We have recently demonstrated no significant difference in the M1 receptors in the striatum of PSP patients compared to controls. This suggests preservation of the medium spiny neurons bearing these receptors, which are predominantly involved in the indirect pathway (projecting to the external globus pallidus (GPe)). M2 receptors are found presynaptically on striatal cholinergic interneurons but also on corticostriatal and thalamostriatal afferents. Their activation inhibits neurotransmitter release, and therefore, via the cholinergic interneurons they provide a negative feedback mechanism for acetylcholine release. M4 receptors are predominantly located on the postsynaptic membranes of striatal medium spiny projection neurons expressing D1 receptors, which form part of the direct pathway, projecting to the internal globus pallidus (GPi). When activated M2 and M4 receptors inhibit adenylyl cyclase via a complex cascade of events through a G protein.

We aimed to define the status of the muscarinic M2 and M4 receptors in the striatum and pallidum to provide a more detailed understanding of the basal ganglia cholinergic neurochemistry of PSP. Cases with Lewy body dementia (LBD, a group comprising dementia with Lewy Bodies (DLB) and Parkinson’s Disease with dementia (PDD)) and Alzheimer’s Disease (AD) were chosen as disease controls as they share some clinico-neurochemical similarities with PSP. Notably, cholinesterase inhibitors improve cognitive and neuropsychiatric symptoms in DLB, PDD and AD patients but are not beneficial in PSP, showing no demonstrable improvement in cognition and a possibly detrimental effect on motor symptoms.

Methods
Subjects
Eighteen pathologically confirmed cases of PSP, from which anterior striatal sections were available in 16 and posterior in 12, were acquired from the Newcastle Brain Tissue Resource and the Sara Koe PSP Research Centre at the Institute of Neurology, UCL. They were compared with age-matched controls (n=50, 27 anterior striatum, 33 posterior striatum) and patients with LBD (n=45 (27 DLB and 18 PDD), 37 anterior...
striatum, 34 posterior striatum) and AD (n=39, 27 anterior striatum, 27 posterior striatum). Clinical information for PSP patients was obtained where possible, via a retrospective notes review. DLB, PDD and AD patients were prospectively assessed annually. Patient demographics are shown in table 1. One patient with LBD was taking a cholinesterase inhibitor, and three were taking anticholinergics. None of the PSP or AD group had taken cholinergic medication. There were no significant inter-group differences in post mortem delay. A summary of the pathological findings in the PSP cases in relevant areas is shown in table 2. Consent for brain donation was obtained either from the patient, or/and the next of kin, in accordance with the Local Research Ethics Committee (Newcastle and North Tyneside) and the London Multi-Centre Research Ethics Committee.

**Tissue preparation**

The right cerebral hemisphere was fixed and used for pathological diagnosis and the left cerebral hemisphere was sliced into 1cm thick coronal blocks and rapidly frozen in liquid Arcton cooled over liquid nitrogen and stored at -70°C. Cases acquired in the last three years have been quickly frozen on copper plates maintained at -80°C. There is no difference in tissue quality between the two freezing methods. Frozen tissue sections containing basal ganglia at a level including the striatum, globus pallidus, and insular cortex were sub-dissected. Anterior striatum was up to, and including, coronal level 13, and posterior from coronal level 14, that is rostral and caudal of the anterior commissure. For autoradiography, 20 micron cryostat sections were cut and dried onto Vectabond (Vecta, UK) coated slides before storage at -70°C. Prior to assay, slides were taken to room temperature and air dried for 1-2 hours.

**Table 1: Demographic Details of Study Groups**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=50)</th>
<th>PSP (n=18)</th>
<th>LBD (n=45)</th>
<th>AD (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at death in years (mean ± SD)</strong></td>
<td>79.6 (8.8)</td>
<td>76.0 (8.2)</td>
<td>79.3 (7.0)</td>
<td>83.9 (6.1)*</td>
</tr>
<tr>
<td><strong>Male/Female</strong></td>
<td>17/33</td>
<td>11/7</td>
<td>25/20</td>
<td>19/20</td>
</tr>
<tr>
<td><strong>Disease duration years (mean ± SD)</strong></td>
<td>-</td>
<td>6.1 (3.5)</td>
<td>7.8 (5.6)</td>
<td>5.5 (2.9)</td>
</tr>
<tr>
<td><strong>PM delay hours (mean ± SD)</strong></td>
<td>38.4 (22.5)</td>
<td>45.7 (23.4)</td>
<td>37.4 (21.2)</td>
<td>44.9 (23.8)</td>
</tr>
</tbody>
</table>
*AD age higher vs. controls and LBD p<0.05, and vs. PSP p<0.01
PM = post mortem  SD = standard deviation

Table 2: Range of PSP pathology found in 18 cases

<table>
<thead>
<tr>
<th></th>
<th>Caudate (anterior &amp; posterior levels)</th>
<th>Putamen (anterior &amp; posterior levels)</th>
<th>GPe</th>
<th>GPi</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL NFT</td>
<td>0/+ to  +</td>
<td>+ to ++</td>
<td>+++</td>
<td>++ to</td>
</tr>
<tr>
<td></td>
<td>+++ to +</td>
<td>+ to ++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

+ = mild, ++ = moderate, +++ = severely affected. NL = neuronal loss, NFT = neurofibrillary tangles.

Autoradiographic ligand binding assays
The total and selective binding was determined in triplicate contiguous sections and non-specific binding established in one section by the addition of 2µM atropine. At room temperature sections were prewashed in a buffer (10mM KH₂PO₄, 10mM Na₂HPO₄ pH 7.4) for 15 minutes to remove any endogenous ligand, for example acetylcholine or residual drugs. M2 and M4 combined receptor density was measured using ³H AFDX 384, which labels both receptors (total binding) and, in adjacent sections, selectively blocking M4 binding with dicyclomine. After pre-washing, 4.8 nM ³H AFDX 384 alone, with dicyclomine (10nM) or with atropine was added and incubated for one hour at room temperature. Sections were washed in the buffer for two times two minutes before being dipped in ice-cold water, dried and apposed to tritium-sensitive Hyperfilm (Amersham) for seven weeks.

Developing and Analysis
Films were developed after warming to room temperature (2hrs) before development using 500ml D19 for 5 minutes, stopped using 500ml 1% aqueous acetic acid for 1 minute, fixed using 500ml 25% Unifix for 6 minutes and washed for 20 minutes in running water. Films were dried and binding assessed by comparison to ³H autoradiographic microscale standards (Amersham) using MCID M5+ image analysis (Imaging Research Inc.) to give binding in fmols/mg. The specific binding for combined M2 and M4 receptors was calculated by subtracting the non-specific binding (nsb) from the total. Similarly, M2 was determined by subtracting nsb from total plus dicyclomine. M4 was then determined by taking the M2 binding value away from the total.

Statistical Analysis
Statistical analysis was performed using MINITAB (version 13). For the neurochemical data, results were compared between PSP, controls, LBD and AD. Where possible, data were correlated with clinical measures. To ensure a normal distribution results were logged prior to analysis, and the means antilogged. Results are presented as geometric means and standard deviations. Statistical tests included one way ANOVA between disease groups, with post-hoc analysis using Tukey's pair wise comparisons where indicated. Regression analysis was used to explore potential correlations between clinical variables, demographics and neurochemical data.
### Results

**Table 3** M2 receptor binding in anterior (bold) and posterior striatum

<table>
<thead>
<tr>
<th>M2 Anterior M2 Posterior</th>
<th>Control</th>
<th>PSP</th>
<th>LBD</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caudate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32.5 (0.1)</td>
<td>29.7 (0.1)</td>
<td>36.4 (0.1)</td>
<td>36.9 (0.2)</td>
</tr>
<tr>
<td></td>
<td>28.7 (0.1)</td>
<td>18.0 (0.2)</td>
<td>25.2 (0.1)</td>
<td>22.1 (0.2)</td>
</tr>
<tr>
<td><strong>Putamen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32.4 (0.1)</td>
<td>33.0 (0.1)</td>
<td>38.6 (0.1)</td>
<td>38.6 (0.2)</td>
</tr>
<tr>
<td></td>
<td>33.1 (0.1)</td>
<td>23.0 (0.1)</td>
<td>30.0 (0.1)</td>
<td>25.4 (0.2)</td>
</tr>
<tr>
<td><strong>GPe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.9 (0.3)</td>
<td>5.0 (0.3)</td>
<td>4.4 (0.3)</td>
<td>5.2 (0.3)</td>
</tr>
<tr>
<td></td>
<td>2.9 (0.3)</td>
<td>2.8 (0.1)</td>
<td>3.6 (0.2)</td>
<td>3.0 (0.5)</td>
</tr>
<tr>
<td><strong>GPi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3 (0.4)</td>
<td>3.9 (0.2)</td>
<td>3.7 (0.2)</td>
<td>2.6 (0.5)</td>
</tr>
<tr>
<td><strong>Insular Cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.0 (0.1)</td>
<td>17.1 (0.2)</td>
<td>19.7 (0.2)</td>
<td>18.8 (0.2)</td>
</tr>
<tr>
<td></td>
<td>15.6 (0.1)</td>
<td>11.4 (0.2)</td>
<td>15.7 (0.1)</td>
<td>13.5 (0.2)</td>
</tr>
</tbody>
</table>

Values expressed as geometric means (standard deviation) in fmol/mg.

1 Disease v Control p<0.05  2 Disease v Control p<0.01  3 Disease v Control p<0.001  4 PSP v LBD p<0.05

Abbreviations: PSP = Progressive Supranuclear Palsy, LBD = Lewy body dementias, AD = Alzheimer’s Disease, GPe/i = Globus pallidus external/internal segment.

### Progressive Supranuclear Palsy

**M2 Receptor Binding**

The results for M2 receptor binding are shown in table 3 and figures 1 and 2. M2 binding in controls was highest in the caudate and putamen, and lowest in the pallidum. There was a striatal rostrocaudal reduction in M2 binding. This gradient was more marked in the PSP group. The density of M2 receptor binding was significantly reduced in the posterior caudate (-37%, p<0.001) and putamen (-31%, p<0.01) in PSP compared to controls. In the anterior striatum there was no significant change in M2 binding. There was no statistically significant difference in M2 binding in either the internal or external globus pallidus. There was reduced M2 binding in the posterior insular cortex compared to controls (-27%, p<0.05).
Table 4  M4 receptor binding in the anterior (bold) and posterior striatum

<table>
<thead>
<tr>
<th>M4 Anterior</th>
<th>Control</th>
<th>PSP</th>
<th>LBD</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4 Posterior</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate</td>
<td>59.7 (0.1)</td>
<td>47.3 (0.3)</td>
<td>62.6 (0.2)</td>
<td>54.7 (0.2)</td>
</tr>
<tr>
<td></td>
<td>62.8 (0.1)</td>
<td>30.7 (0.4)</td>
<td>62.7 (0.2)</td>
<td>54.4 (0.2)</td>
</tr>
<tr>
<td>Putamen</td>
<td>56.8 (0.1)</td>
<td>43.4 (0.4)</td>
<td>56.3 (0.2)</td>
<td>48.5 (0.2)</td>
</tr>
<tr>
<td></td>
<td>52.5 (0.2)</td>
<td>33.8 (0.3)</td>
<td>54.8 (0.2)</td>
<td>45.5 (0.2)</td>
</tr>
<tr>
<td>GPe</td>
<td>4.1 (0.3)</td>
<td>5.7 (0.4)</td>
<td>6.3 (0.3)</td>
<td>7.4 (0.2)</td>
</tr>
<tr>
<td></td>
<td>3.7 (0.3)</td>
<td>4.4 (0.2)</td>
<td>5.7 (0.2)</td>
<td>5.0 (0.3)</td>
</tr>
<tr>
<td>GPi</td>
<td>6.3 (0.2)</td>
<td>10.4 (0.1)</td>
<td>3.6 (0.6)</td>
<td>3.6 (0.7)</td>
</tr>
<tr>
<td>Insular Cortex</td>
<td>28.1 (0.1)</td>
<td>23.6 (0.4)</td>
<td>31.0 (0.4)</td>
<td>24.9 (0.2)</td>
</tr>
<tr>
<td></td>
<td>26.8 (0.2)</td>
<td>17.9 (0.3)</td>
<td>32.0 (0.1)</td>
<td>23.9 (0.2)</td>
</tr>
</tbody>
</table>

Values expressed as geometric means (standard deviation) in fmol/mg.

1 Disease v Control p<0.05  2 Disease v Control p<0.001  3 PSP v LBD p<0.05  4 PSP v LBD p<0.001  5 PSP v AD p<0.01

Abbreviations: PSP = Progressive Supranuclear Palsy, LBD = Lewy body dementias, AD = Alzheimer's Disease, GPe/i = Globus pallidus external/internal segment.

M4 Receptor Binding
The results for M4 receptor binding are shown in table 4 and figures 1 and 2. Highest binding in controls was again in the striatum with low binding in the pallidum. There was a rostrocaudal reduction in M4 receptors in PSP, which was not evident in the other groups. The posterior striatum showed significantly lower M4 binding than controls (caudate reduced by 51%, p<0.001, and putamen by 36%, p<0.05). M4 binding in the caudate was also significantly lower than LBD and AD groups and in the putamen lower than LBD. GPi M4 binding was increased in PSP relative to all groups, but this did not reach statistical significance. M4 receptor binding in the insular cortex was lower in PSP than controls (-33%, p<0.05) and LBD (-44%, p<0.001).

Lewy Body Dementias
M2 receptor binding in the striatum and pallidum was normal. In the anterior insular cortex M2 binding was increased compared to controls (+40%, p<0.01), and in the posterior insular cortex compared to PSP (+38%, p<0.05). There was a trend towards higher M4 binding in the GPe in LBD compared to controls although this did not reach statistical significance.

Alzheimer’s Disease
There was a decrease in M2 binding in the posterior striatum of 23%, caudate (p<0.05) and putamen (p<0.001). There was a significant increase in M2 binding in the anterior insular cortex (+34%, p<0.05). There was a trend towards reduced M4 binding in the striatum but this was not significant.
**Effect of Demographic Variables**

In the control group there was no correlation between age or PM delay and receptor binding in any brain area. In PSP there was no correlation between M2 or M4 receptor binding and age, disease duration or PM delay. There was no difference in binding of M2 or M4 receptors between those patients noted to have frontal executive dysfunction or dementia and those without, although numbers where data was available in each group were small. In LBD and AD there were no correlations between M2 or M4 receptors and age, PM delay, or disease duration in the striatum or pallidum.

**Discussion**

We have shown a reduction in M2 and M4 receptors in the posterior striatum in PSP. Although subtypes of muscarinic receptor have not been previously measured in PSP, two studies using $[^3]H$N-methyl-scopolamine which binds to all muscarinic receptors, found a 18-30% reduction in the striatum 7, 18. In contrast, the total number of striatal muscarinic receptors measured by $[^3]H$ quinuclidynyl benzilate ($[^3]H$QNB) was similar between controls and PSP patients 19.

The reduced striatal M2 receptor density is likely to reflect loss of posterior cholinergic interneurons, previously shown in vitro. Choline acetyltransferase (ChAT) is a marker of cholinergic neuron integrity and this was reduced by approximately 50% in the caudate and 40% in the putamen in nine PSP patients compared to controls 19. Another study demonstrated a 50% reduction in striatal ACh vesicular transporter expression and ChAT activity in 11 PSP patients 20. This reduction was more marked in the posterior striatum, as we found in the present study. Interestingly, nerve growth factor receptors are localised on intrinsic striatal cholinergic neurons 21 and were reduced by 30% in three PSP patients compared to controls and patients with PD 21. The percentage reduction in cholinergic interneurons we found is less than some previous reports, probably reflecting the presence of M2 receptors on cortico-striatal and thalamic afferents, which are not known to be affected in PSP.

The posterior striatum, particularly the putamen, is involved predominantly in motor function and the anterior striatum and most of the caudate in cognitive and associative functions 22. Posterior striatal neuronal loss suggests cholinergic dysfunction may be involved in motor as well as cognitive impairment, although cholinergic loss in the basal forebrain system may be more relevant to the cognitive deficits in PSP.

M4 receptors are predominantly located post-synaptically, on medium spiny neurons of the direct pathway and co-localise with D1 receptors. They may also be present presynaptically on striatal cholinergic interneurons 23, and to a lesser extent on spiny neurons of the indirect pathway 24. The marked reduction of striatal M4 receptors in our study could indicate loss of medium spiny projection neurons of the direct pathway, although D1 receptors were reported to be within the normal range in one case of PSP 25. M4 receptor loss may therefore be selective or, more likely, represent the loss of cholinergic interneurons. It is possible that the M4 receptors are down regulated, although this is unlikely as the reduction in ACh should, if anything, cause up regulation in functioning neurons.

GABA is the predominant neurotransmitter in the GP and cholinergic inputs to this nucleus are modest, as reflected by low overall binding in controls. The normal, or even increased, M4 receptor binding is supported by a previous study which measured total muscarinic receptors in the globus pallidus and found a significant increase in the GPi in PSP 7. Pathologically the GPe and GPi both show evidence of atrophy and
typical PSP pathology. M4 receptors may, therefore, be upregulated in the GP in response to neuronal loss as a compensatory mechanism. Reduced M2 receptor binding in the insular cortex suggests decreased cholinergic input. The insular cortex has been implicated in many functions including somatosensory, gustatory, vestibular-like functions and cardiovascular disturbances, but none of these symptoms are prominent in PSP. Failure to detect any difference in receptor binding between those PSP patients with and without cognitive impairment may be due to the fact that this information was available only for a small number of cases. The normal M2 and M4 receptor binding in the striatum in LBD suggests cholinergic interneurons and medium spiny projection neurones of the direct pathway are intact. This is supported by previous work demonstrating normal striatal M2/M4 and D1 receptors in DLB. Medium spiny neurones of the indirect pathway may be reduced, however, reflected by decreased numbers of striatal M1 and D2 receptors. The increase in M2 binding in the insular cortex in LBD and AD was not expected as cholinergic cortical projections are reduced in these dementing illnesses. However, an increase in presynaptic receptors has been shown previously in AD and may be due to axonal sprouting of the remaining cholinergic neurons. The reduction in posterior striatal M2 in AD may represent a reduction in cortical afferents rather than a reduction in intrinsic cholinergic neurones. Normal striatal M4 receptors in AD suggest medium spiny projection neurons are preserved. This is supported by a previous study, although muscarinic subtypes were not measured. In conclusion, this study highlights differences in cholinergic dysfunction between the three disease groups, and is compatible with marked striatal cholinergic pathology in PSP. Potential therapeutic approaches suggested by our findings include specific targeting of the remaining M2 receptors with an M2 antagonist, or use of an M1 agonist to stimulate intact postsynaptic receptors. The striatum, however, is only part of the basal ganglia-thalamo-cortico loop and dysfunction of other sites and neurotransmitter systems are likely to be clinically relevant.

Acknowledgements

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Legend to the Figures

**Figure 1** Autoradiography photographs demonstrating total M2/M4 binding using $^3$H AFDX; and M2 receptors only, using dicyclomine to block M4 receptors

**Figure 2** M2 and M4 receptors
References


30. Sakai N, Imada S. Bilateral lesions of the insular cortex or of the prefrontal cortex block the association between taste and odor in the rat. Neurobiol Learn Mem. 2003;80:24-31
Figure 1  Autoradiography photographs demonstrating total M2/M4 binding using $^3$H AFDX; and M2 receptors only, using dicyclomine to block M4 receptors
Figure 2  M2 and M4 receptors