Alterations in peptide levels in Parkinson's disease and incidental Lewy body disease


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Summary
The levels of the neuropeptides Met- and Leu-enkephalin (MET-ENK, LEU-ENK), substance P and neurotensin were measured by a combined high performance liquid chromatography/radioimmunoassay (HPLC/RIA) method in post-mortem samples of basal ganglia from Parkinson's disease patients, incidental Lewy body disease patients (presymptomatic Parkinson's disease) and matched controls. Dopamine (DA) levels were reduced in the caudate nucleus and putamen in Parkinson's disease, but unaltered in incidental Lewy body disease. The levels of MET-ENK were reduced in the caudate nucleus and putamen in Parkinson's disease. Met-enkephalin levels were decreased in the putamen and were undetectable in the substantia nigra in Parkinson's disease. Leu-enkephalin levels were unchanged in incidental Lewy body disease, although there was a tendency to a reduction in putamen. Substance P levels were reduced in the putamen in Parkinson's disease. No significant changes in substance P content were observed in incidental Lewy body disease. Neurotensin levels were increased in the substantia nigra in Parkinson's disease. Neurotensin levels in incidental Lewy body disease were not altered significantly, but tended to parallel the changes in Parkinson's disease. The changes in basal ganglia peptide levels in incidental Lewy body disease generally followed a trend similar to those seen in Parkinson's disease, but were less marked. This suggests that they are an integral part of the pathology of the illness and not secondary to DA neuronal loss or a consequence of prolonged drug therapy.

Keywords: Parkinson's disease; incidental Lewy body disease; basal ganglia; neuropeptides; high performance liquid chromatography

Abbreviations: DA = dopamine; HPLC = high performance liquid chromatography; l-dopa = l-3,4-dihydroxyphenylalanine; LEU-ENK = Leu-enkephalin; MET-ENK = Met-enkephalin; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA = 6-hydroxypamidine; RIA = radioimmunoanalysis

Introduction
The destruction of the nigrostriatal pathway with the presence of Lewy bodies in remaining nigral neurons underlies the motor deficit which characterizes Parkinson's disease (Gibb, 1987; Gibb and Lees, 1988). A marked reduction in striatal DA is the main biochemical deficit occurring in Parkinson's disease. Replacement therapy with l-3,4-dihydroxyphenylalanine (l-dopa) initially reverses the motor symptoms. However, with continued treatment the therapeutic response diminishes and motor complications (fluctuations and dyskinesias) usually appear. The involvement of neurotransmitter systems other than DA may contribute to the symptomatology of Parkinson's disease. In particular, a highly regional pattern of changes in neuropeptide levels in basal ganglia occurs in Parkinson's disease (Agid and Javoy-Agid, 1985).

In studies of post-mortem brain material in Parkinson's disease, MET-ENK and LEU-ENK levels have been reported to be reduced in the caudate nucleus and putamen, and there is also a reduction of MET-ENK content in substantia nigra (Taquet et al., 1983; Llorens-Cortés et al., 1984; Fernandez et al., 1992). Substance P levels have been reported to be decreased in the caudate nucleus, globus pallidus and substantia nigra (Mauborgne et al., 1983; Fernandez et al.,
1992). In contrast, neurotensin levels have been reported as either unaltered throughout basal ganglia (Bissette et al., 1985; Emson et al., 1985) or increased in substantia nigra (Fernandez et al., 1995). Immunocytochemical studies show a different pattern of change with substance P and MET-ENK immunostaining either unaltered (Grafe et al., 1985; Waters et al., 1988) or even increased in striatum and globus pallidus (Grafe et al., 1985; Goto et al., 1990) and unchanged in the substantia nigra (Waters et al., 1988).

It is not known whether these peptide changes are part of the primary pathology of Parkinson's disease, whether they occur secondary to the loss of DA neurons, or whether they are a result of prolonged drug therapy with L-dopa. We have examined these questions by utilizing animal models of Parkinson's disease such as the unilateral 6-hydroxydopamine (6-OHDA) lesioned rat (Taylor et al., 1992), in combination with L-dopa treatment for 6 months, or the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated parkinsonian primate (Jenner et al., 1986; Taquet et al., 1988; Taylor et al., 1991). Overall, the effects of nigrostriatal degeneration on basal ganglia peptide levels did not parallel the alterations in peptide content observed in Parkinson's disease. This suggested that they may be part of the primary pathological process occurring in Parkinson's disease.

To confirm this conclusion using post-mortem tissue from patients with Parkinson's disease is difficult. Brain material from early or untreated parkinsonians is not available. Consequently, we have investigated peptide content in basal ganglia in Parkinson's disease and in brain material from the 8–10% of normal individuals who at, post-mortem, are found to have evidence for nigral cell death and Lewy bodies (incidental Lewy body disease). Incidental Lewy body disease is considered to be the presymptomatic phase of Parkinson's disease (Forno and Alvord, 1971; Fearnley and Lees, 1991), since the distribution of pigmented cell loss observed in incidental Lewy body disease, although less severe, mirrors that of Parkinson's disease (Gibb, 1987; Fearnley and Lees, 1991).

**Patients and methods**

**Patients**

Brain tissue was obtained from the Parkinson's Disease Society Brain Bank (London, UK) and from the Department of Pathology (University of Innsbruck, Austria). The dissection of all brain material was undertaken by Dr F. R. Wells at the Parkinson's Disease Society Brain Bank. The brains were divided midsagittally with one-half immersion fixed in 10% buffered formalin and the other frozen at −70°C. The brain tissue was dissected from frozen brain and stored at −70°C until biochemical analysis (Dexter et al., 1989).

Controls consisted of five males and one female (mean age±SE, 68.3±5.7 years), who died without any neurological or psychiatric disease. Pathological examination of the brains did not show cell loss or Lewy bodies in haematoxylin–eosin stained sections of substantia nigra. Fifty control brains were screened to detect cases of incidental Lewy body disease. Incidental Lewy body disease was diagnosed in six apparently normal individuals (three males and three females; mean age±SE, 68.2±5.8 years) whose brains exhibited cell loss in the substantia nigra and the presence of Lewy bodies in remaining neurons at post-mortem examination. Other basal ganglia regions showed no overt pathological changes. However, other nuclei such as the locus coeruleus and nucleus basalis of Meynert were also involved, indicating that the regional distribution of the lesions was identical to that in Parkinson's disease. These brains were pathologically defined and validated as incidental Lewy body disease cases (Gibb and Lees, 1988; Fearnley and Lees, 1991). Patients found to have incidental Lewy body disease were not known to have neurological symptoms or signs in life and had not received drug treatment. Parkinson's disease patients consisted of four males and two females (mean age±SE, 78.7±2.1 years), who were clinically diagnosed and histopathologically defined by the presence of Lewy bodies and a characteristic pattern of neuronal loss in the substantia nigra. The age of onset of Parkinson's disease ranged between 59 and 73 years (mean±SE, 69.2±3.1 years) and the duration of the disease ranged from 5 to 14 years (mean±SE, 10.4±1.6 years). All the Parkinson's disease patients were receiving L-dopa (with a peripheral dopa decarboxylase inhibitor) at the time of death. The doses ranged between 200 and 1000 mg kg day−1 (mean±SE, 550±154 mg kg day−1). The average time between death and refrigeration of the body did not differ between the three groups of subjects (mean±SE, controls 2.9±0.2 h; incidental Lewy body disease 2.7±0.3 h and Parkinson's disease 2.8±0.4 h), and the average times between death and brain removal were similar (mean±SE, controls 14.3±1.5 h; incidental Lewy body disease 21.5±2.2 h and Parkinson's disease 16.6±1.7 h).

**Determination of levels of dopamine and its metabolites**

The levels of DA and its metabolites in samples from the caudate nucleus and putamen were measured by a standard HPLC technique with electrochemical detection (Wagner et al., 1982).

**Peptide measurements**

For peptide measurements, frozen samples were weighed and boiled for 15 min in 100 volumes of a mixture of 1 N acetic acid and 0.02 N HCl containing 2-mercaptoethanol. Samples were homogenized with an Ultraturrax homogenizer (20 000 r.p.m., 6 s) and centrifuged at 12 000 g for 10 min at 4°C. After centrifugation, aliquots of the supernatant were freeze-dried and stored at −20°C until assayed.

Met-enkephalin, LEU-ENK and neurotensin were measured by radioimmunoanalysis (RIA), following HPLC...
Table 1 Levels of dopamine, dihydroxyphenylacetic acid, homovanillic acid and dopamine turnover ratio in the caudate nucleus and putamen from normal subjects, incidental Lewy body disease patients and Parkinson’s disease patients

<table>
<thead>
<tr>
<th></th>
<th>DA</th>
<th>DOPAC</th>
<th>HVA</th>
<th>HVA+DOPAC/DA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caudate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.09±0.56</td>
<td>0.26±0.07</td>
<td>3.48±0.57</td>
<td>1.37±0.24</td>
</tr>
<tr>
<td>+Lb</td>
<td>3.08±0.82</td>
<td>0.25±0.15</td>
<td>3.51±0.32</td>
<td>1.89±0.66</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>0.42±0.23**</td>
<td>0.11±0.04</td>
<td>2.50±0.53</td>
<td>16.04±5.99***</td>
</tr>
<tr>
<td><strong>Putamen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.36±0.42</td>
<td>0.38±0.13</td>
<td>8.27±0.87</td>
<td>2.82±0.48</td>
</tr>
<tr>
<td>+Lb</td>
<td>3.33±0.47</td>
<td>0.23±0.11</td>
<td>6.86±1.10</td>
<td>2.29±0.35</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>0.16±0.06***</td>
<td>0.06±0.01*</td>
<td>1.85±0.38**</td>
<td>23.77±3.53***</td>
</tr>
</tbody>
</table>

Results are given as mean±SE expressed as micrograms per gram wet weight of tissue (n = 6). DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid; +Lb = Lewy body disease patients. *P < 0.05; **P < 0.01; ***P < 0.001: compared with control subjects (ANOVA, Student’s t test).

**Fig. 1** Met-enkephalin levels in control subjects (C–Lb), incidental Lewy body (C+Lb) and Parkinson’s disease (PD) patients in basal ganglia regions. CN = caudate nucleus; Put = putamen; GPL = globus pallidus lateral segment; GPM = globus pallidus medial segment; SN = substantia nigra. The results are means±SE (n = 6). *P < 0.05 (ANOVA, Student’s t test).

**Fig. 2** Leu-enkephalin levels in control subjects (C–Lb), incidental Lewy body (C+Lb) and Parkinson’s disease (PD) patients in basal ganglia regions. CN = caudate nucleus; Put = putamen; GPL = globus pallidus lateral segment; GPM globus pallidus medial segment; SN = substantia nigra. The results are means±SE (n = 6). *P < 0.05 (ANOVA, Student’s t test).

Statistical analysis
Analysis of differences between groups was performed using one-factor ANOVA followed by unpaired Student’s t test. Correlations between individual biochemical levels with different ante- and post-mortem parameters were sought using Pearson’s coefficient.
Dopamine levels in Parkinson’s disease and incidental Lewy body disease

Dopamine levels were significantly reduced in the caudate nucleus (by 87%) and putamen (by 95%) in patients with Parkinson’s disease compared with controls (Table 1). Levels of homovanillic acid and dihydroxyphenylacetic acid were unaltered in the caudate nucleus, but were reduced in the putamen in Parkinson’s disease. Dopamine turnover measured by the ratio of (homovanillic acid + dihydroxyphenylacetic acid)/DA was enhanced in both caudate and putamen in Parkinson’s disease. In individuals with incidental Lewy body disease, there was no change in the caudate or putamen DA content or in DA metabolite levels or DA turnover (Table 1).

Basal ganglia neuropeptide alterations in Parkinson’s disease and incidental Lewy body disease

There was a parallel reduction (~50%) in MET-ENK levels in the caudate nucleus in Parkinson’s disease and in incidental Lewy body disease compared with controls (Fig. 1). There was a reduction in MET-ENK levels in putamen (90% decrease versus controls) and in substantia nigra (60% decrease versus controls) in Parkinson’s disease. Met-enkephalin levels were also reduced by over 50% in the putamen (but not in substantia nigra) in incidental Lewy body cases. Met-enkephalin concentrations were increased in both segments of the globus pallidus in Parkinson’s disease but the changes were not statistically significant. A similar increase was found in incidental Lewy body disease patients, but again the differences did not reach statistical significance.

Leu-enkephalin levels in the caudate nucleus and both segments of globus pallidus were unaltered in Parkinson’s disease and in incidental Lewy body disease compared with controls (Fig. 2). In contrast, LEU-ENK concentrations were markedly reduced in the putamen and were undetectable in the substantia nigra in Parkinson’s disease. There was a reduction of LEU-ENK content in the putamen in incidental Lewy body disease, but this was not statistical significant.

There was a decrease in substance P concentrations in the putamen in Parkinson’s disease (Fig. 3). Substance P content was non-significantly increased in the lateral and medial globus pallidus in both Parkinson’s disease and incidental Lewy body disease (Fig. 3). Substance P levels in the caudate nucleus and substantia nigra were unaltered in either Parkinson’s disease or incidental Lewy body disease compared with controls.

The small amount of tissue available and the low levels of neurotensin in striatal areas precluded the analysis of this peptide in the caudate nucleus and putamen. Neurotensin levels were increased (two-fold) in the substantia nigra of Parkinson’s disease patients (Fig. 4). Neurotensin concentrations in the incidental Lewy body disease group followed the same trends to those seen in Parkinson’s disease but the changes did not reach statistical significance.

Correlations of peptide levels and with ante- or post-mortem parameters

There was no correlation of individual peptide levels with either sex, age, time to refrigeration or to autopsy (data not shown). There were significant positive correlations between MET-ENK and LEU-ENK levels in the caudate nucleus, putamen and globus pallidus. For example, in the putamen both peptides were correlated in control subjects (0.903), in incidental Lewy body disease cases (0.856) and in Parkinson’s disease patients (0.904). Individual MET-ENK and substance P levels in medial globus pallidus were correlated in incidental Lewy disease patients and Parkinson’s disease patients. In agreement with previous studies, DA content in caudate...
nucleus in Parkinson’s disease was positively correlated with the age of onset of the illness (0.8644), but was negatively correlated with the duration of the disease (-0.8357). There was no correlation between individual peptide levels in any region and L-dopa dose.

### Discussion

The relative distribution of neuropeptide levels in control subjects was in general agreement with those previously reported (Buck et al., 1981; Cooper et al., 1981; Manberg et al., 1982; Mauborgne et al., 1983; Taquet et al., 1983; Llorens-Cortés et al., 1984). However, in this work the ratio of enkephalin content in globus pallidus lateral segment to either caudate nucleus or putamen was not in accordance with previous studies (e.g. Taquet et al., 1983; Llorens-Cortés et al., 1984). This a consequence of the HPLC/RIA technique used for the peptide measurements. As we previously reported (de Ceballos et al., 1991), there is a disparity between apparent peptide levels in crude extracts with those of authentic peptides as determined after HPLC purification. Indeed, in recent studies using another series of controls and Parkinson’s disease brains, we have shown that MET-ENK levels in globus pallidus lateral segment are double those in caudate nucleus or putamen when measured in crude extracts, but are very similar in HPLC purified controls (de Ceballos et al., unpublished observations).

Some neuropeptide alterations occurring in the brains of those with established Parkinson’s disease on L-dopa treatment are also present in incidental Lewy body disease. Since these individuals were normal in life and did not receive L-dopa therapy, and since the extent of pathological change in the substantia nigra was small and was not associated with any change in caudate or putamen DA content, such alterations in neuropeptide levels may reflect part of the primary pathology of Parkinson’s disease.

In agreement with previous studies MET-ENK content was decreased in the caudate nucleus (Fernandez et al., 1992; Sivam, 1991), putamen and substantia nigra in Parkinson’s disease (Taquet et al., 1983; Llorens-Cortés et al., 1984). In incidental Lewy body disease a significant reduction in MET-ENK levels was also observed in the caudate nucleus and the putamen. In 6-OHDA lesioned rats or in MPTP treated monkeys, there were increased striatal MET-ENK levels (Thal et al., 1983; Sivam et al., 1987; Dacko and Schneider, 1991; Taylor et al., 1991, 1992). Therefore, the decrease observed in Parkinson’s disease and in incidental Lewy body disease does not appear to be a consequence of nigrostriatal pathway degeneration. In contrast, in both segments of the globus pallidus, MET-ENK content tended to be increased in both Parkinson’s disease (Grafe et al., 1985; Goto et al., 1990; de Ceballos et al., 1993) and incidental Lewy body disease. This change is similar to that seen in 6-OHDA and MPTP lesioned animals (Thal et al., 1983; Sivam et al., 1987; Taylor et al., 1991), so may be a reflection of nigrostriatal loss. The changes observed in incidental Lewy body disease cannot be ascribed to L-dopa therapy, but may reflect the underlying disease process (Table 2).

In 6-OHDA lesioned rodents and in MPTP treated primates, the lesion induced increase in MET-ENK levels is a consequence of increased preproenkephalin gene expression (Angulo et al., 1986; Young et al., 1986; Sivam et al., 1987; Augood et al., 1989; Gerfen et al., 1990). It has been recently reported that preproenkephalin gene expression is enhanced in parkinsonian caudate nucleus and putamen (Nisbet et al., 1995). Although this might explain the tendency to an increase in MET-ENK levels in globus pallidus, the main projection area of the striopallidal enkephalin pathway, is difficult to reconcile with the observation of a decrease in striatal levels of MET-ENK in Parkinson’s disease. However,
increased biosynthesis accompanied by decreased levels may be a result of increased release of MET-ENK.

Changes in LEU-ENK levels partly paralleled those of MET-ENK. Thus, LEU-ENK levels were reduced in putamen (Taquet et al., 1983) and substantia nigra of patients with Parkinson’s disease. The marked correlation between the levels of MET-ENK and LEU-ENK strongly suggests that both peptides derive from the same precursor, preproenkephalin (Gubler et al., 1982). There was no statistically significant changes in LEU-ENK levels in incidental Lewy body disease, although those in putamen were considerably reduced. Changes in LEU-ENK levels appear to be characteristic of Parkinson’s disease since a nigrostriatal lesion does not alter striatal LEU-ENK levels (Jenner et al., 1986; Taylor et al., 1991, 1992). Furthermore, although there is a lesion-induced decrease in LEU-ENK levels in substantia nigra, this was reversed by L-dopa, such that it increased seven-fold compared with values for control rats (Taylor et al., 1992).

In the present study, substance P levels were decreased in the putamen (Mauborgne et al., 1983), tended to increase in globus pallidus (Grafé et al., 1985; Goto et al., 1990; de Ceballos et al., 1993) and were unchanged in substantia nigra (Grafé et al., 1985; Waters et al., 1988) in Parkinson’s disease in agreement with some previous findings. In 6-OHDA lesioned rats, decreased striatal (Lindefors et al., 1989) and nigral substance P content and decreased preprotachykinin mRNA has been reported (Young et al., 1986; Sivam et al., 1987; Gerfen et al., 1990; Engber et al., 1991; Taylor et al., 1992). L-Dopa treatment completely reversed the reduction in substance P content in substantia nigra in such animals (Engber et al., 1991; Taylor et al., 1992) (Table 2). Thus, the absence of change in nigral substance P levels in Parkinson’s disease (Grafé et al., 1985; Waters et al., 1988) may be viewed as a combined effect of nigrostriatal pathway degeneration and L-dopa treatment. Indeed, pre-protachykinin mRNA levels are also unaltered in Parkinson’s disease patients treated with L-dopa (Nisbet et al., 1995).

Neurotensin content in substantia nigra was increased in Parkinson’s disease (Fernandez et al., 1995), and no statistically significant changes were observed in globus pallidus. In another series of Parkinson’s disease patients, neurotensin levels were shown to be increased in both zona compacta and zona reticulata of the substantia nigra (Fernandez et al., 1995). In incidental Lewy body disease, neurotensin levels only tended to similar changes in these structures. Following a nigrostriatal lesion there are increased striatal and pallidal neurotensin levels, which are not modified by L-dopa treatment. However, nigral neurotensin levels, which were unchanged following a 6-OHDA lesion, were increased by L-dopa treatment (Taylor et al., 1992). Thus, changes in neurotensin levels appear to be secondary to DA neuron loss in combination with prolonged drug therapy. The fact that in incidental Lewy body disease patients only tendencies were observed, further support this conclusion.

Hypokinetic disorders, such as Parkinson’s disease, have been postulated to result from the selective changes in the different striofugal pathways (Albin et al., 1989), which contain various neuropeptides as cotransmitters. Activity in the pathway from striatum to lateral globus pallidus (colocalizing enkephalins) is thought to be increased, while activity in the pathway from striatum to medial globus pallidus and substantia nigra (colocalizing tachykinins) is thought to be decreased in Parkinson’s disease. In this study some of the observed alterations in peptide levels are compatible with this scenario. However, the stationary levels of a given peptide in a particular structure may not indicate activity in a neuronal pathway. Thus, as argued above, the reduced levels of MET-ENK and LEU-ENK in the striatum in Parkinson’s disease, especially in the putamen, and the similar but less marked trends in incidental Lewy body disease, may reflect increased release of the peptides due to overactivity of such neurons containing enkephalins. This is supported by the finding of increased preproenkephalin mRNA message in the striatum in Parkinson’s disease (Nisbet et al., 1995). With regard to substance P, the matter is more complex, for L-dopa treatment reverses the decrease in preprotachykinin mRNA message in experimental animals with lesions of the dopaminergic pathway (see above), and such message is unchanged in Parkinson’s disease striatum (Nisbet et al., 1995). This might explain why substance P levels were not significantly altered in medial globus pallidus and especially in substantia nigra in Parkinson’s disease.

The most important finding of the present study is that some neuropeptide alterations occurring in Parkinson’s disease are also observed in incidental Lewy body disease. Most convincing are the reductions in MET-ENK levels in striatum in both disorders, which contrast with the increase found in 6-OHDA lesioned rats or MPTP treated monkeys. From these results several conclusions can be drawn. First, such changes in peptide levels may be characteristic of the disease and not just secondary to DA neuron loss. Secondly, since similar changes are observed in incidental Lewy body disease, they may be an early component of the pathological process rather than being secondary biochemical alterations resulting from loss of the nigrostriatal pathway or a drug induced event. Additional insight into the involvement of these basal ganglia peptide systems in the pathophysiology of Parkinson’s disease could provide new therapeutic strategies for the illness.

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