

Clinical correlates of selective pathology in the amygdala of patients with Parkinson's disease

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Summary

The amygdala exhibits significant pathological changes in Parkinson's disease, including atrophy and Lewy body (LB) formation. Amygdala pathology has been suggested to contribute to some clinical features of Parkinson's disease, including deficits of olfaction and facial expression. The degree of neuronal loss in amygdala subnuclei and the relationship with LB formation in non-demented Parkinson's disease cases have not been examined previously. Using stereological methods, the volume of neurones and the number of neurones in amygdala subdivisions were estimated in 18 prospectively studied, non-demented patients with Parkinson's disease and 16 age- and sex-matched controls. Careful exclusion (all cortical disease) and inclusion (non-demented, levodopa-responsive, idiopathic Parkinson's disease or controls) criteria were applied. Seven Parkinson's disease cases experienced well-formed visual hallucinations many years after disease onset, while nine Parkinson's disease cases and three controls were treated for depression. Anatomically, the amygdala was subdivided into the lateral nucleus, the basal (basolateral and basomedial) nuclei and the corticomедial (central, medial and cortical nuclei) complex. LB and Lewy neurites were identified by immunohistochemistry for α -synuclein and ubiquitin and were assessed semiquantitatively. LB were found throughout the amygdala in Parkinson's disease, being present in ~4% of neurones. Total amygdala volume was reduced by 20% in

Parkinson's disease ($P = 0.02$) and LB concentrated in the cortical and basolateral nuclei. Lewy neurites were present in most cases but did not correlate with any structural or functional variable. Amygdala volume loss was largely due to a 30% reduction in volume ($P = 0.01$) and the total estimated number of neurones ($P = 0.007$) in the corticomедial complex. The degree of neurone loss and the proportion of LB-containing neurones in the cortical nucleus within this complex were constant across Parkinson's disease cases and neither variable was related to disease duration ($R^2 < 0.03$; $P > 0.5$). The cortical nucleus has major olfactory connections and its degeneration is likely to contribute to the early selective anosmia common in Parkinson's disease. There was a small reduction in neuronal density in the basolateral nucleus in all Parkinson's disease cases, but no consistent volume or cell loss within this region. However, the proportion of LB-containing neurones in the basolateral nucleus was nearly doubled in cases that exhibited visual hallucinations, suggesting that neuronal dysfunction in this nucleus contributes to this late clinical feature. Detailed quantitation of the other amygdala subdivisions failed to reveal any other substantial anomalies or any associations with depression. Thus, the impact of Parkinson's disease on the amygdala is highly selective and correlates with both early and late clinical features.

Keywords: amygdala; Lewy body; neuronal loss; Parkinson's disease; α -synuclein

Abbreviations: LB = Lewy body; LN = Lewy neurite

Introduction

Lewy bodies (LB) are the major pathology associated with idiopathic Parkinson's disease and the amygdala is one of the most common brain regions likely to contain LB (Braak *et al.*, 1994, 1995; Schmidt *et al.*, 1996; Churchyard and Lees, 1997; Mattila *et al.*, 2000). Located in the medial temporal

lobe, the amygdala is a major component of the limbic system (Braak *et al.*, 1994; Rezaie *et al.*, 1996; Gloor, 1997; Swanson, 2000), yet the clinical manifestations of amygdala pathology are poorly understood. Our understanding of the functions of the amygdala has been based either on extrapo-

lation of the results from animal studies or on studies of stroke or surgical patients in whom the amygdala has been partially or wholly damaged and in whom the accompanying changes in behaviour have been determined. Examples of functional changes that have been shown to occur following amygdala damage include olfactory dysfunction (Aggleton, 1992), visual disturbance (Anderson and Phelps, 1998; Broks *et al.*, 1998; Gray, 1999), autonomic and endocrine dysfunction (de Olmos, 1990; Gloor, 1997), and emotional impairment, including increased fear, panic and anxiety (Aggleton, 1992; Broks *et al.*, 1998; Shekhar *et al.*, 1999).

The amygdala contains a diversity of nuclei with specific anatomical connections. However, the localization of functions to specific nuclei within the amygdala remains a difficult task, which is further complicated because most amygdala subnuclei have multiple afferent and efferent connections (Sims and Williams, 1990). The central, medial and cortical nuclei together make up the corticomедial complex of the amygdala (Gloor, 1997). The central nucleus is mainly concerned with autonomic function, with prominent projections to brainstem autonomic nuclei, while the cortical and medial nuclei have functions related to aspects of olfaction. The cortical nucleus has major connectivity with olfactory brain regions, including prominent input from the olfactory bulb, and therefore exerts a significant influence on olfactory function (Sims and Williams, 1990; Swanson and Petrovich, 1998). The basal nuclei can be further subdivided into the basolateral and basomedial nuclei and have significant connectivity with limbic regions and association cortices (Sims and Williams, 1990; Braak *et al.*, 1994; Gloor, 1997; Swanson and Petrovich, 1998; Iseki *et al.*, 2001; Petrovich *et al.*, 2001). The lateral nucleus is the largest nucleus of the amygdala in humans (Sims and Williams, 1990; Gloor, 1997). The lateral and basolateral nuclei both have significant connections with the association neocortex and modulate some of the many neocortical functions (Swanson, 2000; Petrovich *et al.*, 2001). Overall, the amygdala contains a diversity of nuclei with specific anatomical connections and a range of diverse functions.

Clinically, Parkinson's disease is characterized by the presence of at least two of bradykinesia, rigidity and tremor (Gelb *et al.*, 1999). These deficits appear after ~70% loss of dopaminergic neurones from the substantia nigra and are responsive to dopamine-replacement therapy (Fearnley and Lees, 1991; Halliday *et al.*, 1996). In addition to these classical clinical features, there are other features of Parkinson's disease that are only mildly responsive or even non-responsive to dopamine replacement therapy. These include deficits in olfaction and autonomic function, subtle cognitive changes, postural instability and also facial hypomimia, often referred to as a masked or expressionless face (Smith *et al.*, 1996; Gelb *et al.*, 1999). At least some of these additional features of Parkinson's disease have been attributed to pathology in the amygdala (Adolphs *et al.*, 1994; Cahill *et al.*, 1995; Jacobs *et al.*, 1995; Young *et al.*, 1995).

The majority of previous studies analysing amygdala pathology have included cases with clinical dementia (e.g. Double *et al.*, 1996; Schmidt *et al.*, 1996; Churchyard and Lees, 1997; Darvesh *et al.*, 1998; Cordato *et al.*, 2000; Yamazaki *et al.*, 2000; Iseki *et al.*, 2001; Harding *et al.*, 2002a). In a mixed population of Parkinson's disease cases with and without dementia, it is known that LB and Lewy neurites (LN) concentrate regionally in certain subnuclei, with only minor inter-individual variation (Braak *et al.*, 1994). The most prominent accumulation of LB and LN described occurs in the cortical and central subregions of the amygdala, with less prominent changes elsewhere (Braak *et al.*, 1994; McShane *et al.*, 2001). Neuronal density calculations have shown no significant neuronal loss associated with LB amygdala pathology in Parkinson's disease cases with or without dementia (Churchyard and Lees, 1997). Our recent work has shown a strong association between intracellular LB accumulation in the amygdala and visual hallucinations in cases with dementia (Harding *et al.*, 2002a). To fully understand the clinical correlates of amygdala pathology in Parkinson's disease, it is necessary to analyse cases without dementia, as atrophy of the amygdala is still significant in such Parkinson's disease cases (Cordato *et al.*, 2000), despite previous assertions of no neuronal loss (Churchyard and Lees, 1997). Furthermore, the relationships between LB, neuronal densities and volume-corrected neuronal numbers are unknown, as previous studies have not performed unbiased quantitation. The aim of the present study was to systematically quantify regional cell loss and LB formation in the amygdala and evaluate clinicopathological relationships.

Subjects and methods

Cases

The present study included non-demented Parkinson's disease cases and age- and sex-matched controls. Prospective consent for brain donation was obtained from all cases and their next of kin through the Parkinson's New South Wales brain donor programme. This programme was approved by Human Ethics Committees of the Universities of New South Wales and Sydney and the Central and South Eastern Area Health Services and prospectively follows patients on a regular basis (usually annually) using standardized recording procedures. Controls had no history of neurological or psychiatric symptoms. Control cases underwent the same clinical follow-up as the Parkinson's disease cases with the same standardized recording procedures. Particulars of the extensive clinical and neuropathological data collection procedures have been published recently (Harding *et al.*, 2002a). For the present study, all cases that had not been seen by their physicians within 6 months preceding death were excluded. Shortly after the patient's death, standardized questionnaires were sent to the next of kin, as well as to the patient's general and specialist medical practitioners to

Table 1 Demographic and clinical data in controls and Parkinson's disease cases

	Controls	Parkinson's disease patients
Number of cases	16	18
Male : female	11 : 5	13 : 5
Age at disease onset (years)	–	60 ± 2
Age at death (years)	73 ± 3	73 ± 3
Post-mortem delay (h)	22 ± 3	16 ± 3
Movement disorder severity (Hoehn and Yahr)		
Stage 2–3	0	4
Stage 4	0	6
Stage 5	0	8
Other symptoms		
Late visual hallucinations	0	7
Depression	3	9
Medication used		
Levodopa (mg/day)	0	1279 ± 273
Neuroleptics	0	3
Anti-depressants	3	9
Anxiolytics	0	4
Sedative-hypnotics	3	6
Antihypertensives	5	5
Anticoagulants	1	2
Anti-inflammatories	2	5
Analgesics	3	5

Data are number of patients or mean ± standard error of the mean

update relevant details (covering the period from the time of last assessment). No control or Parkinson's disease case was included that had dementia according to both DSM-IV (American Psychiatric Association, 1994) and the Clinical Dementia Rating criteria (Morris *et al.*, 1989). Following careful neuropathological screening, cases with significant neuropathology other than Parkinson's disease were excluded (Gelb *et al.*, 1999).

Eighteen levodopa-responsive cases with Parkinson's disease (13 male; 5 female) and 16 age- and sex-matched controls (11 male; 5 female) were selected for the present study (Table 1). There were no significant differences between groups in the age at death or post-mortem delay (Table 1). The causes of death were mainly related to cardiorespiratory complications and cancer, which affected similar numbers of patients in the control and Parkinson's disease groups. Recorded Mini-Mental State Examination scores were >25. None of the selected Parkinson's disease cases had more than isolated cortical LB in the cingulate, hippocampal or association cortices. Those fulfilling diagnostic criteria for dementia with LB were excluded (McKeith *et al.*, 1996). Controls did not exhibit LB. Cases with substantial tau- or silver-positive neurofibrillary tangles in the amygdala were also excluded. Since there was no neuropathological evidence of dementing disorders or clinical history of dementia, and relatively long disease durations for Parkinson's disease cases, we are confident of these

cases being representative of non-demented Parkinson's disease. All tissue analyses were performed blinded to classification.

Evaluation of clinical features

Data from clinical records and standardized forms were used to assess the presence (including date of symptom onset) or absence of the following clinical features for correlations: bradykinesia and rigidity; rest tremor; hypomimia; well-formed visual hallucinations; and depression. Medications and doses taken were recorded and updated, where applicable, at each clinical assessment. The dominant clinical parkinsonian feature (tremor-dominant or akinetic-rigid) was identified (Table 1). The age of onset of Parkinson's disease was estimated from initial diagnosis and the severity of Parkinson's disease was assessed using the Hoehn and Yahr scale (Hoehn and Yahr, 1967). The types of visual hallucinations considered have been discussed in detail previously (Harding *et al.*, 2002a) and were mostly complex features relating to people, objects and landscapes reported in the presence of the examiner on at least one occasion and/or when there were consistent reports by the subject to a caregiver. Flashing lights and similar phenomena reported to occur with hallucinogenic drugs (Fénelon *et al.*, 2000) were not considered well-formed visual hallucinations. The presence of depression was ascertained from the clinical diagnosis combined with the nature of the treatment (antidepressant medications or other recognized treatments, such as electroconvulsive therapy), or if patients scored greater than 10 out of 30 on the geriatric depression scale (Yesavage *et al.*, 1983). Reactive depression to circumstances was included if medical practitioners deemed the depression of sufficient concern to record in clinical notes.

Tissue preparation

Brains were removed at either full or limited brain-only autopsy (mean post-mortem delay 19 ± 2 h, range 3–45 h), and the entire brain was fixed by suspension in 15% buffered formalin for 2 weeks. The brains were then weighed and the anteroposterior dimensions recorded prior to the brainstem and cerebellum being separated from the cerebrum at the level of the superior colliculus. The cerebrum was embedded in agar and cut into ~3 mm thick coronal slices using a rotary slicer. The brainstem was embedded in agar and similarly cut into 3 mm thick slices in the transverse plane, as described previously (Halliday *et al.*, 1996). Tissue samples were prepared for routine neuropathological examination. Briefly, samples were taken from the frontal (Brodmann area 9), temporal (area 20), parietal (area 39), occipital (areas 17 and 18) and anterior cingulate (area 24) cortices, as well as from the hippocampus at the level of the lateral geniculate nucleus, amygdala, anterior and posterior basal ganglia (including the basal forebrain), thalamus, hypothalamus and cerebellum. These were embedded in paraffin and sectioned at 10 µm. The

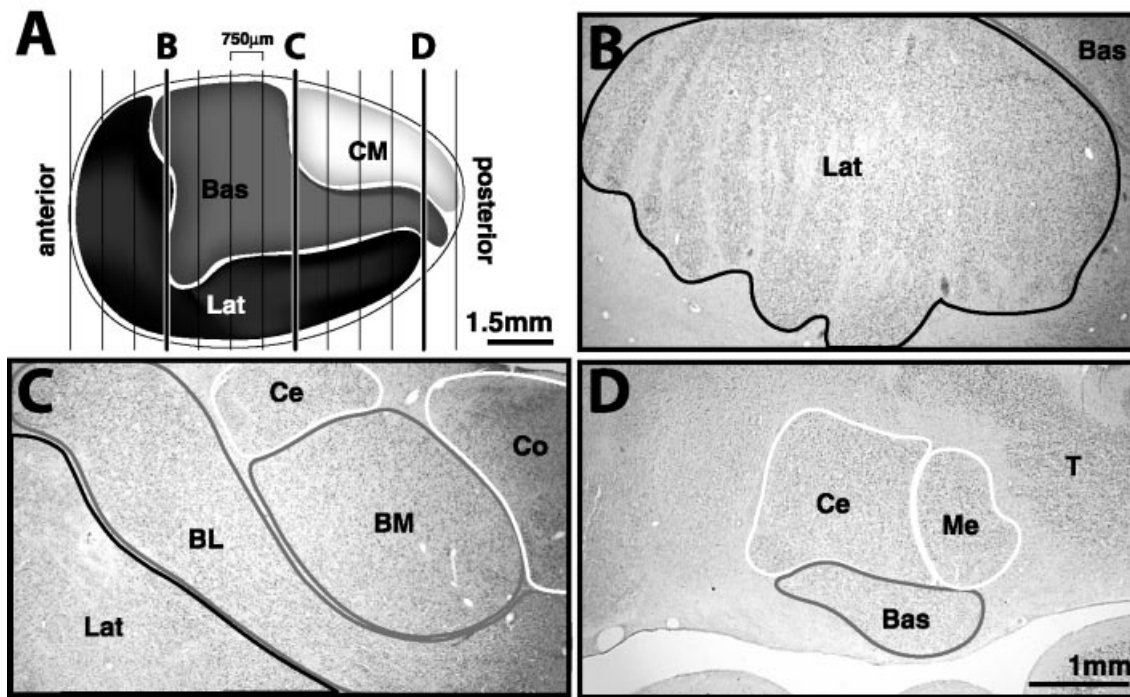


Fig. 1 (A). Diagram representing the systematic sampling scheme of the amygdala for the present study. Sections, represented by the vertical lines, are spaced 750 μm apart. Lines marked B–D in the diagram show the approximate anteroposterior locations of the photomicrographs (B–D), which are representative coronal sections from a control case, stained with cresyl violet to show the normal appearance and location of the amygdaloid subnuclei. Scale in D also applies to B and C. Bas = basal nuclei, which can be further subdivided into BM = basomedial nucleus and BL = basolateral nucleus; CM = corticomедial complex, which can be further subdivided into Ce = central nucleus, Co = cortical nucleus and Me = medial nucleus; Lat = lateral nucleus; T = corticoamygdaloid transition area.

midbrain, pons and medulla oblongata were cryoprotected in 30% sucrose in Tris buffer, then 20 and 30 μm thick sections were cut on a cryostat and the sections washed in buffer and mounted on gelatinized glass slides. Sections from all regions were stained, as necessary, for routine screening using currently recommended diagnostic protocols for Parkinson's disease (Gelb *et al.*, 1999), dementia with LB (McKeith *et al.*, 1996; Harding and Halliday, 1998) and Alzheimer's disease (Braak and Braak, 1991; Mirra *et al.*, 1991; National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997). Standard stains used included haematoxylin and eosin, congo red and the modified Bielschowsky silver stains, while immunohistochemistry was performed using antibodies to ubiquitin (Z0458, diluted 1:200; Dako, Glostrup, Denmark), tau II (T5530, diluted 1:10 000; Sigma, St Louis, MO, USA) and α -synuclein (18-0215, diluted 1:200; Zymed Laboratories, San Francisco, CA, USA).

Blocks of tissue containing the right amygdala were removed from the cerebral slices and cryoprotected in 30% sucrose in Tris buffer. Serial 50 μm thick sections were cut on a cryostat, the sections washed in buffer and mounted onto gelatinized glass slides, and every 15th section (i.e. 750 μm apart; e.g. Fig. 1A) was stained with cresyl violet for stereological analysis (see below). The block of tissue containing the left amygdala at its greatest cross-sectional

area was removed from the cerebral slice, embedded in paraffin and 10 μm coronal sections were cut. Adjacent sections were stained using anti- α -synuclein peroxidase immunohistochemistry (18-0215, diluted 1:200) and anti-ubiquitin peroxidase immunohistochemistry (Z0458, diluted 1:200). The specificity of the immunohistochemistry technique was tested by omitting the primary antibody as described previously (Henderson *et al.*, 2001). No peroxidase reaction was observed in these sections. All immunohistochemical stains were lightly counterstained with cresyl violet.

Quantitation of the amygdala and its subdivisions

Semiquantitation

The major amygdala subdivisions (Fig. 1) were examined for the presence of pathology. From the paraffin-embedded sections, the region with the greatest LB density was identified (a technique similar to that used for semiquantitation of plaques and tangles in Alzheimer's disease and for LB in dementia with LB (Mirra *et al.*, 1991; McKeith *et al.*, 1996; The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997; Harding and Halliday, 1998). The number of (α -

synuclein-positive) LB and neurones (identified by the presence of nucleoli) per 200 \times microscopic field (field diameter 1 mm) was determined for each amygdaloid nucleus and the proportion of neurones with LB calculated. There was <5% variation in counts made by two investigators and no difference between α -synuclein-positive LB counts and ubiquitin-positive LB counts performed in adjacent sections (paired Student's *t* test, $P > 0.26$). The density of LN within the same field was graded using previously described semiquantitative methods (Kim *et al.*, 1995). Briefly, LN were identified as thickened α -synuclein-positive deposits within neuronal processes and were semiquantitatively rated as absent, sparse, moderate or many in number (Kim *et al.*, 1995).

Stereological quantitation

The total volume of the amygdala was calculated by point-counting photographic images from the 3 mm coronal brain slices, as detailed previously (Cordato *et al.*, 2000). To determine the volume and number of neurones within the reliably identified amygdaloid subnuclei, stereological analyses of the tissue sections was performed as described previously (Harding *et al.*, 1994; Henderson *et al.*, 2000). The identification of exact boundaries for a number of smaller amygdaloid nuclei was unreliable for stereological analysis; consequently, estimates of total neurone number for these smaller amygdaloid nuclei could not be calculated. However, the three main amygdaloid subnuclei (the corticomедial complex, the basal nuclei and the lateral nucleus) could be identified reliably in 50 μ m thick sections spaced 750 μ m apart (e.g. Fig. 1A). The areas of each of the main subnuclei were determined by tracing the boundary (Fig. 1B–D) using a computer mouse and an integrated computerized microscopic system (NeuroLucida; MicroBrightField, Colchester, VT, USA). The volumes of the subnuclei were determined by multiplying the sum of their cross-sectional areas by the distance between the sections (750 μ m), using Cavalieri's principle. The optical disector technique was used to estimate neuronal number by optically placing on the tissue section disector frames with sides 160 \times 160 μ m, spaced 2.5 mm apart, and counting the number of neurones within the disector frame. Between cases, the number of sections used varied from 11 to 16 and the number of disector frames varied from 12 to 34. Only those neurones whose nucleolus fell entirely within the sampling frame or on adjacent inclusion borders were counted. The number of neurones counted varied between 156 and 249 for the corticomедial complex, 154 and 194 for the basal nuclei and 156 and 234 for the lateral nucleus. Repeated measures of the neurone number in multiple sections from multiple cases always gave similar results, even between different investigators (<5% variation between investigators on repeated measures, with Pearson's correlation 0.95).

Neuronal density (coefficient of error range 0.03–0.06) was determined from the total number of neurones within the sample volume. Regional neurone number was estimated by multiplying the density and volume.

Statistical analysis

Statistical analysis was performed with the Statview 5.0 program (Abacus Concepts, Berkeley, CA, USA). Differences in clinical and pathological parameters between Parkinson's disease and control groups and between subgroups of Parkinson's disease patients were analysed using unpaired *t* tests. Relationships within and between clinical variables (i.e. age at onset of Parkinson's disease, disease severity at death, disease duration) and pathological variables (volume, neurone number and LB density in the amygdala and its subregions) were analysed using multiple regression analyses. Mean and standard error of the mean are given for all variables and a *P* value less than 0.05 was accepted as being statistically significant.

Results

Clinical indices

All patients presented with asymmetrical motor deficits of Parkinson's disease (nine had tremor, two bradykinesia, seven tremor and bradykinesia) without evidence of cognitive dysfunction. All patients were levodopa-responsive and displayed hypomimia early in the disease course (Table 1). Medication use was recorded for all cases and controls (Table 1). All Parkinson's disease cases regularly took antiparkinsonian medication, including levodopa (Table 1). Seven controls took no medications, while six Parkinson's disease patients took no medications other than antiparkinsonian medication. Neuroleptic medications were taken by three Parkinson's disease patients and antidepressants were taken by three controls and nine Parkinson's disease patients (Table 1). At the time of death, all patients had mild to advanced disease (Table 1) according to the Hoehn and Yahr scale (Hoehn and Yahr, 1967), with disease duration varying from 8 to 28 years (Table 1). No patient exhibited symptoms considered atypical of Parkinson's disease according to current diagnostic criteria (Gelb *et al.*, 1999), and in particular none had clinical dementia.

Seven Parkinson's disease cases reported visual hallucinations, which were episodic, and occurred late in the disease course (Harding *et al.*, 2002a) (Table 1). The medical records for the Parkinson's disease patients with late visual hallucinations did not show any change in medication use associated with the phenomenon. There was a non-significant trend for the Parkinson's disease cases who experienced visual hallucinations to be older (78 ± 2 versus 70 ± 4 years; $P = 0.18$), to use less levodopa (836 ± 143 versus 1510 ± 354 mg/day; $P = 0.15$) and to have a longer duration of Parkinson's

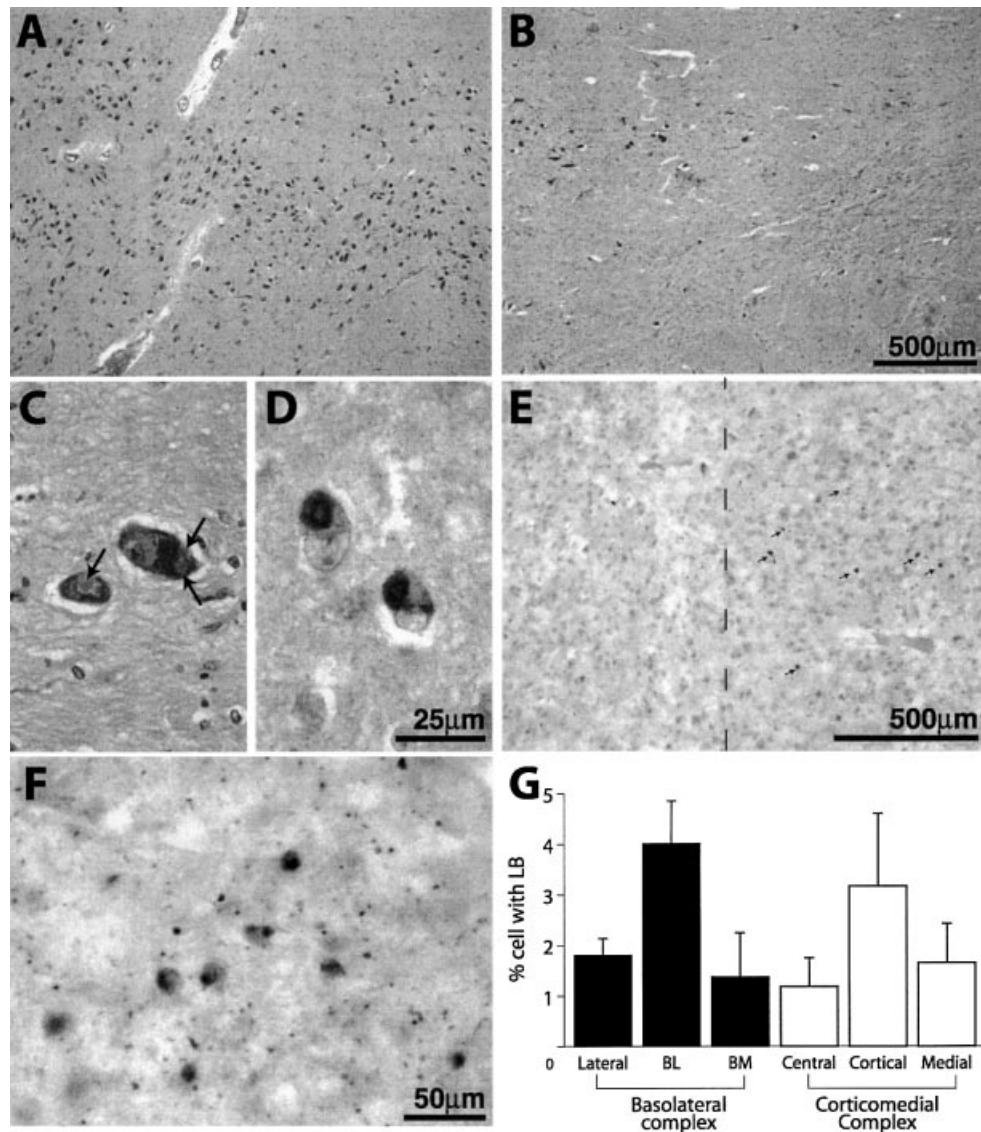


Fig. 2 (A and B) Haematoxylin–eosin-stained sections showing the pigmented neurones of the substantia nigra in (A) a normal control case and (B) a case with Parkinson's disease. Whilst the dorsomedial nigra is relatively spared in Parkinson's disease (top left quadrant of B), note the severe loss of pigmented neurones from the ventrolateral nigra (lower left and right quadrants in B). Scale in B also applies to A. (C) Haematoxylin–eosin-stained section from the substantia nigra. Typical Lewy bodies (arrowed) are observed in pigmented neurones. Scale in F also applies to C. (D) Section from amygdala stained immunohistochemically for α -synuclein and lightly counterstained with cresyl violet. Lewy bodies typical of those found within neurones in the cortical nucleus of the amygdala are observed in a representative Parkinson's disease case. (E) Thick α -synuclein-stained section with a cresyl violet counterstain from the amygdala of a representative Parkinson's disease case showing a relative absence of Lewy bodies in the lateral nucleus (left of dashed line) compared with an abundance of Lewy bodies (arrows) in the adjacent basolateral nucleus (right of dashed line). (F) Thick ubiquitin-stained section counterstained with cresyl violet from the basolateral nucleus of the amygdala of a representative Parkinson's disease case, showing intracellular staining of Lewy bodies and many Lewy neurites within the neuropil. (G) Histogram of the proportion of neurones containing LB (mean \pm standard error of the mean) in the subnuclei comprising the basolateral and corticomedial complexes of the amygdala. Note the similarly high proportions of neurones containing LB in the basolateral (BL) and cortical amygdaloid nuclei in Parkinson's disease. BM = basomedial nucleus.

disease symptoms (17 ± 2 versus 12 ± 2 years; $P = 0.16$). Six cases not experiencing visual hallucinations were taking multiple medications. Two Parkinson's disease

cases given neuroleptic medications had experienced visual hallucination but had no significant improvement following neuroleptic treatment.

Table 2 Quantitative data in controls and Parkinson's disease cases

	Controls	Parkinson's disease patients	<i>P</i>
Brain volume (ml)	1307 ± 40	1254 ± 32	0.31
Amygdala volume (ml)	1.82 ± 0.12	1.45 ± 0.08	0.02*
Corticomedial complex			
Volume (ml)	0.52 ± 0.05	0.36 ± 0.02	0.01*
Neurone number (× 10 ⁶)	1.92 ± 0.13	1.35 ± 0.13	0.007*
Neurone density			
Cortical	174 ± 5	122 ± 4	<i>P</i> < 0.0001*
Medial	119 ± 8	112 ± 5	0.44
Central	129 ± 6	128 ± 8	0.92
Basal nuclei			
Volume (ml)	0.49 ± 0.04	0.43 ± 0.03	0.24
Neurone number (× 10 ⁶)	1.75 ± 0.12	1.62 ± 0.11	0.46
Neurone density			
Basolateral nucleus	134 ± 4	103 ± 6	0.002*
Basomedial nucleus	124 ± 7	114 ± 5	0.22
Lateral nucleus			
Volume (ml)	0.85 ± 0.04	0.76 ± 0.05	0.18
Neurone number (× 10 ⁶)	2.95 ± 0.18	2.72 ± 0.23	0.43
Neurone density	138 ± 8	142 ± 9	0.80

Data are mean ± standard error of the mean from one hemisphere; neurone density is expressed as number per microscopic field (200× magnification, field diameter 1 mm). *Significant.

Nine Parkinson's disease patients and three controls had a history of clinical depression, all of whom were prescribed antidepressant medication (Table 1). Depression was also a late feature in the Parkinson's disease cases, and led to suicide in one instance. Five depressed Parkinson's disease cases also experienced late visual hallucinations. There were no significant differences in age (76 ± 4 versus 70 ± 4 years, $P = 0.31$), levodopa use (1367 ± 419 versus 1081 ± 134 mg/day, $P = 0.55$) or duration of Parkinson's disease symptoms (15 ± 2 versus 15 ± 2 years) for cases with and without depression. There was also no significant difference in the age of depressed controls compared with controls without depression (75 ± 3 versus 72 ± 3 years, $P = 0.72$).

Pathology and cell loss

As required for diagnosis, there was substantial pigmented cell loss (compare Fig. 2A with Fig. 2B) and LB formation (Fig. 2C) in the midbrain in all Parkinson's disease cases. No LB were observed in the substantia nigra pars compacta or amygdala of control brains. LB were found within the amygdala in all Parkinson's disease cases (Fig. 2D), the distribution of LB creating a distinctive pattern (Fig. 2G). The presence of amygdala LB correlated with a 20% reduction in the volume in Parkinson's disease ($P = 0.02$) (Table 2), with no left-right asymmetry ($P > 0.05$). LN were present throughout the amygdala in all but one of the cases examined. There was no correlation between the presence of LN in any region and any other variable (all $P > 0.05$).

Corticomedial complex

Both the volume and the estimated number of neurones in the corticomedial complex were reduced by 30% in Parkinson's disease ($P = 0.01$ and $P = 0.007$, respectively) (Table 2). There was little overlap in the estimates of corticomedial complex neurone number between individuals in the control and Parkinson's disease groups, indicating that cell loss in the cortical nucleus is relatively consistent across Parkinson's disease cases. Only one Parkinson's disease case had a neuronal estimate for this region within the control range (2.14 million neurones), despite also demonstrating high densities of LN and LB (maximum of 3 LB per 200× field). The cortical nucleus is the largest structure within the corticomedial complex and is the only region of the corticomedial complex that exhibited a significant reduction in neurone density (30% decrease in Parkinson's disease, $P < 0.0001$) (Table 2, and compare Fig. 3A with Fig. 3B), consistent with selective degeneration of this region. There was no significant relationship between neurone loss in the cortical nucleus and the duration or severity of Parkinson's disease, indicating that the consistent cell loss in the cortical nucleus is likely to occur early in the course of the disease.

The highest densities of LB in the corticomedial complex were in the cortical nucleus (3.5 ± 0.7 LB per 200× field) (Fig. 2D and G), being present in all but two Parkinson's disease cases. Relatively few neurones ($3.0 \pm 0.7\%$) contained LB even in the area of maximum LB density (Fig. 2G). The remaining nuclei of the corticomedial complex (central and medial nuclei) contained inconsistent and variable LB

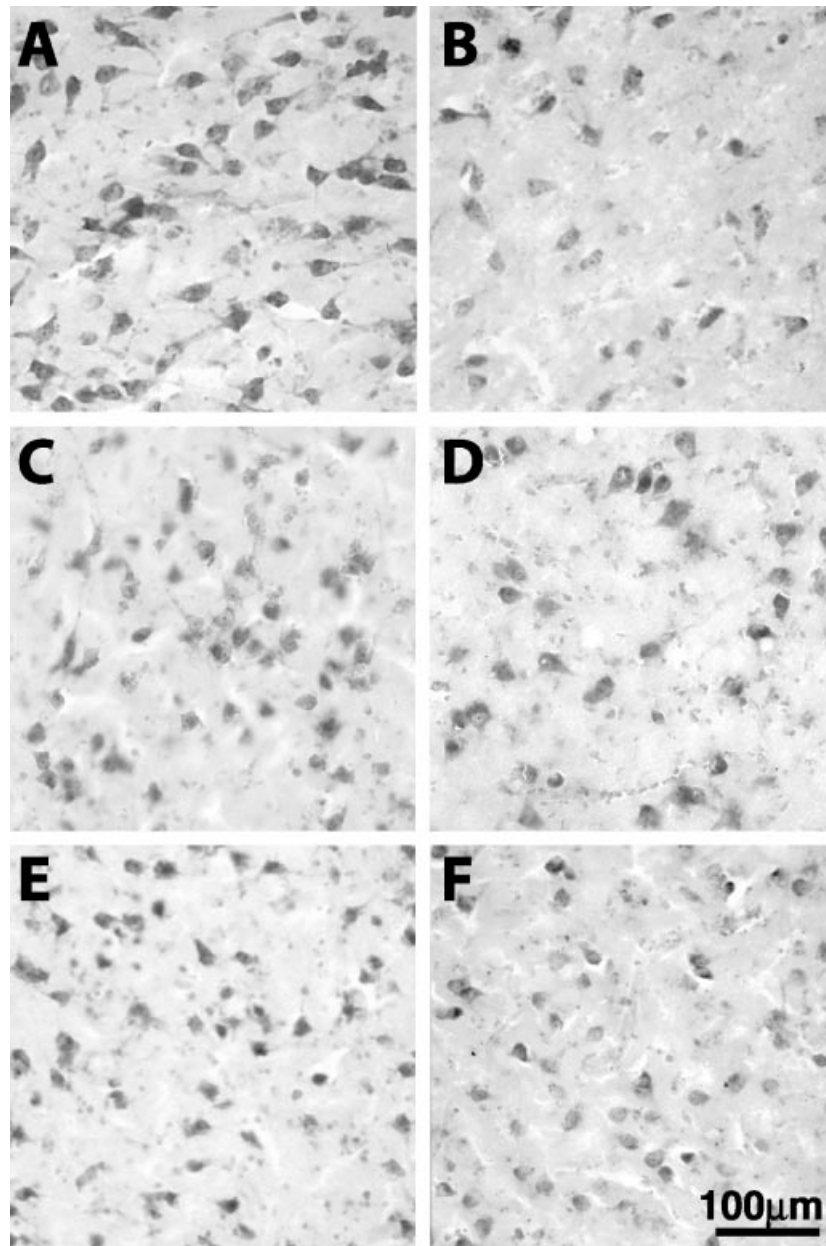


Fig. 3 High-power view of 50 μm thick sections stained with cresyl violet showing the morphology and density of neurones in the cortical (**A, B**), basolateral (**C, D**) and lateral (**E, F**) nuclei in a Parkinson's disease case (**B, D, F**) and a control (**A, C, E**). Note the obvious reduction in neurone density in the cortical nucleus of this representative Parkinson's disease case (**B**) compared with the control (**A**). In the basolateral nucleus, an example is given showing a severe reduction in neurone density in a Parkinson's disease case (**D**) when compared with the same region of a control (**C**). Scale in **F** also applies to **A–E**.

pathology. LN, however, were consistently found within the corticomедial complex and were present in high densities in nearly half of the Parkinson's disease cases. We could not find a significant relationship between the estimated number of neurones and the level of LB pathology in this region (all $P > 0.05$). There was also no significant relationship between LB density and the duration or severity of Parkinson's disease ($P > 0.05$).

Basal nuclei

There was no atrophy of the basal nuclei, nor was there an overall loss of neurones using volume-corrected measures (Table 2). However, there was a significant reduction in neuronal density in the basolateral nucleus, but not in the basomedial nucleus (compare Fig. 3C with Fig. 3D; Table 2), suggesting a small, selective loss of neurones in this subnucleus.

LB were present in relatively high concentrations in the basal nuclei of the amygdala in all Parkinson's disease cases, especially in the basolateral nucleus (Fig. 2E–G) and were accompanied by a few LN. The distribution of LB varied within the basolateral nucleus such that the most basal regions had the greatest density of LB (4.5 ± 0.7 LB per $200\times$ field). Relatively few neurones within this area contained LB ($4.2 \pm 0.5\%$) (Fig. 2G); however, there was a relationship between the densities of neurones and LB in the basolateral nucleus ($R^2 = 0.24$, $P = 0.046$). There was no such relationship for the basomedial nucleus ($R^2 = 0.01$, $P = 0.68$). There was no relationship between the number or proportion of basolateral or basomedial LB with either disease duration or severity ($P > 0.05$) (Fig. 3C–F), nor was there any relationship with variables quantified from other amygdala nuclei (all comparisons $P > 0.05$).

Lateral nucleus

There was no significant atrophy or neurone loss in the lateral nucleus in Parkinson's disease (Table 2). In every Parkinson's disease case, at least one LB was found in the lateral nucleus. Although the maximum density of LB in the lateral nucleus was 2.4 ± 0.3 LB per $200\times$ field (Fig. 2G), the average density of LB and LN within the lateral nucleus was quite low, as many cases had only a few LB within the vast expanse of the lateral nucleus. Consequently, the majority of microscopic fields of view in the lateral nucleus showed mostly no LB within the neurones (Fig. 2E) and careful scanning of sections containing the lateral nucleus was required to locate LB in this region. There was no relationship between the number or proportion of LB or LN in the lateral nucleus and any clinical or pathological variable ($P > 0.05$).

Clinicopathological correlations

Using multiple regression analyses, there were no correlations between the pathological variables (total neuronal number, number of LB, presence or severity of LN, the estimated volume of the amygdala or its subregions) and the clinical variables (severity or duration of Parkinson's disease). In particular, there was no correlation with duration of Parkinson's disease and the loss of neurones in the cortical nucleus. Nine Parkinson's disease patients had tremor-dominant disease, two were mainly akinetic–rigid, and in seven cases both types of motor deficits were prominent. Using unpaired *t* tests, there were no significant differences between tremor-dominant and akinetic–rigid patients in the estimated amygdala volume, neurone number or number of LB or LN in any subregion (all $P > 0.05$). This lack of significance was also the case when Parkinson's disease cases were grouped according to the presence or absence of depression (all $P > 0.05$).

Using unpaired *t* tests, there was a significant increase in the density of LB in the basolateral nucleus (nearly double) in Parkinson's disease cases that suffered from hallucinations

compared with those Parkinson's disease cases that did not report them (6.3 ± 1.3 versus 3.2 ± 0.4 ; $P = 0.02$). Likewise, the proportion of neurones containing LB was also nearly double in those with hallucinations (5.7 ± 0.9 versus 3.1 ± 0.4 ; $P = 0.01$); however, there was no significant correlation with neurone loss in the basolateral nucleus and the presence of visual hallucinations. In no other region was there any difference between these groups (all $P > 0.05$).

Discussion

It is now well established that the amygdala is a preferential site in the brain for LB formation in a variety of disorders, including Parkinson's disease (Lippa *et al.*, 1998, 1999; Gelb *et al.*, 1999; Hamilton, 2000; Yamazaki *et al.*, 2000). Although the amygdala contains the characteristic pathology of Parkinson's disease, our understanding of the importance of amygdala pathology in the pathogenesis of the disease is limited. In this study, well-characterized patients with Parkinson's disease and no dementia were analysed to determine any clinical correlations for the pathological changes previously noted in the amygdala (see Introduction). We identified a highly selective pattern of neuronal loss in the Parkinson's disease amygdala, cell loss which was accompanied by high concentrations of LB formation. The two amygdala regions affected appear to impact on specific clinical features prevalent in Parkinson's disease. One of these, the cortical nucleus, has a consistent significant loss of both volume and neurone number, as well as substantial LB formation. The cortical nucleus has a well-known involvement in olfactory function (Gloor, 1997; Swanson and Petrovich, 1998). The second region affected was the basolateral nucleus, which had a reduction in neurone density in all Parkinson's disease cases, and also an increased proportion of neurones containing LB in cases who had experienced well-formed visual hallucinations during life. Our results show that selective nuclei of the amygdala have considerable degenerative changes in Parkinson's disease and that the topography of the pathology correlates with specific clinical features.

The number of cases studied is small but the Parkinson's disease cases examined were selected from a larger, longitudinally studied population (132 cases), as stringent selection criteria were necessary to evaluate the role of the amygdala in the clinical features found in Parkinson's disease. While for some variables correlations may be more evident in larger samples, the careful case selection procedure used in this study greatly strengthens any positive clinicopathological correlations for the symptoms of Parkinson's disease. The amygdala is a region in which age-related pathology is common (Iseki *et al.*, 1996; Anderton, 1997; Davis *et al.*, 1999), and the exclusion of cases with age- or dementia-related neurofibrillary tangles in this region significantly restricted the number of Parkinson's disease cases that could be examined. Our study sample is similar in number to

that described by Churchyard and Lees (1997) and significantly larger than the samples used in other published studies of the amygdala in Parkinson's disease. As the majority of Parkinson's disease cases examined by Churchyard and Lees (1997) were demented, our cohort is the largest non-demented sample of Parkinson's disease patients yet studied, and thus contributes significantly to the literature on the degree of pathology typical in such Parkinson's disease cases with restricted cortical disease.

In the non-demented Parkinson's disease patients examined, much of the amygdala contained little or no pathology. Like other authors, we found substantial LB in the cortical nucleus in Parkinson's disease (Braak *et al.*, 1994; Churchyard and Lees, 1997). However, in previous studies ubiquitin immunohistochemistry was used to identify LB, and other structures may have been stained, including corpora amylacea and neurofibrillary tangles. The use of α -synuclein immunohistochemistry (which does not identify these structures) provides a means of ensuring that the inclusions identified in the present study were indeed LB or LN (Dickson, 1998; Dickson, 1999; Hurtig *et al.*, 2000). In our stringently selected Parkinson's disease cases, comparable densities of LB or LN were identified using α -synuclein and ubiquitin immunohistochemistry, confirming that these pathologies are consistent features in the amygdala in Parkinson's disease. LN in the amygdala did not correlate with any other pathological or clinical variable, a finding similar to our observations in the hippocampus (Harding and Halliday, 2001). Our study confirms that both the lateral and the basomedial nuclei are less consistently affected by LB (Braak *et al.*, 1994), and that there is no change in neurone density for the central or lateral nuclei (Churchyard and Lees, 1997; Iseki *et al.*, 2001). The LB pathology in the central amygdaloid nucleus in Parkinson's disease may underlie autonomic dysfunction (Braak *et al.*, 1994), although autonomic dysfunction correlates with degeneration in peripheral and other central autonomic nuclei (Hague *et al.*, 1997; Wakabayashi and Takahashi, 1997).

The majority of previous studies analysing amygdala pathology have included cases with clinical dementia (e.g. Double *et al.*, 1996; Schmidt *et al.*, 1996; Churchyard and Lees, 1997; Darvesh *et al.*, 1998; Cordato *et al.*, 2000; Yamazaki *et al.*, 2000; Iseki *et al.*, 2001; Harding *et al.*, 2002a); some studies report that non-demented Parkinson's disease cases have little amygdala pathology (Mattila *et al.*, 2000) while others suggest substantial pathology in Parkinson's disease cases with or without dementia (Braak *et al.*, 1994; Churchyard and Lees, 1997). Our results in non-demented Parkinson's disease cases support these latter studies and data generated from patients with epilepsy which suggest that substantial loss of neurones in the lateral and basolateral amygdaloid nuclei do not necessarily result in dementia (Yilmazer-Hanke *et al.*, 2000). The high LB densities shown in the Parkinson's disease amygdala in the present study cannot be a causative factor for dementia (which was absent in the present study). It is likely, however,

that dementia is related to significant pathology in other associated nearby cortical regions, as we have shown recently (Harding *et al.*, 2000, 2002a, b). Our results show that the conclusions of studies considering amygdala LB formation in relation to dementia should be interpreted with caution if non-demented Parkinson's disease cases are not included as disease controls (Schmidt *et al.*, 1996; Yamazaki *et al.*, 2000; Iseki *et al.*, 2001).

One of the main functions of the amygdala is considered to be emotional integration (Aggleton, 1992; Broks *et al.*, 1998; Shekhar *et al.*, 1999), and depression is particularly common in patients with Parkinson's disease (Poewe and Luginger, 1999). In the present study, several of the Parkinson's disease cases and controls had depression, but there was no relationship between depression and the concentration of histopathology, the size of the structure(s), or the density or number of neurones in either the Parkinson's disease cases or controls. A number of studies have reported hypertrophy of the amygdala in patients with major depression (Tebartz van Elst *et al.*, 2001; Davidson *et al.*, 2002) and with psychosis of epilepsy (Tebartz van Elst *et al.*, 2002). However, such hypertrophy was not related to depression in the present study and is not generally seen in geriatric depression (Davidson *et al.*, 2002). It is interesting to note that increases in glucose metabolism and blood flow in the amygdala have been reported with depression (Drevets, 2001), suggesting that increased activity in the amygdala rather than hypertrophy may be the dominant clinical correlate.

Atrophy of the amygdala has been noted previously in Parkinson's disease (de la Monte *et al.*, 1989; Cordato *et al.*, 2000); however, this atrophy is subtle and not easily detected either visually (Braak *et al.*, 1994) or using quantitative single-section area analysis (Churchyard and Lees, 1997). This is consistent with the 20% atrophy noted using serial section analysis in the present study. The atrophy was relatively small because it concentrates in the cortical nucleus, where there is a corresponding decrease in neurone number and density. Although this discrete change was not detected in a previous study (Churchyard and Lees, 1997), we have analysed greater numbers of sections and used unbiased stereological techniques, which probably accounts for the different result obtained. The cortical nucleus receives input from the olfactory bulb and has projections to the olfactory cortices (Gloor, 1997; Swanson and Petrovich, 1998; Swanson, 2000), and olfactory dysfunction is a common problem in Parkinson's disease (Doty *et al.*, 1988; Hawkes and Shephard, 1993;). Although the patients in the present study were not formally tested to determine if they had olfactory deficits, it has been well documented that Parkinson's disease patients exhibit marked deficits in odour identification, recognition and detection threshold early in the disease course (Meshulam *et al.*, 1998; Potogas *et al.*, 1998; Tissingh *et al.*, 2001) and that odour discrimination declines with increasing disease severity (Tissingh *et al.*, 2001). This latter deficit is likely to correlate with the progressive neuronal loss noted in the anterior olfactory

nucleus in Parkinson's disease (Pearce *et al.*, 1995). The finding in the present study of consistent neuronal loss in the cortical amygdaloid nucleus early in the disease course may account for the earlier olfactory deficits in Parkinson's disease. Only one study has performed formal olfactory testing on controls and patients with neurodegenerative disorders with subsequent neuropathological examination (McShane *et al.*, 2001). Dementia with LB patients with anosmia had higher cortical LB counts than patients without olfactory impairment (McShane *et al.*, 2001) and it was suggested that LB may be pathological markers of anosmia (Mann, 2001). However, an alternative possibility is that, like Parkinson's disease, the selective anosmia in dementia with LB reflects similar involvement of the cortical amygdaloid nucleus.

Hypomimia is one of the signs of Parkinson's disease that develops during the disease process, but usually not as a presenting symptom of Parkinson's disease. The Hoehn and Yahr scale (Hoehn and Yahr, 1967) uses hypomimia as a midline sign, indicating the progression from stage 1 to stage 1.5. Numerous studies have been performed investigating the role of the amygdala in a person's ability to identify facial expression in others (e.g. Gorno-Tempini *et al.*, 2001; Morris *et al.*, 2001), including a recent report that recognition of facial expression in Parkinson's disease is intact (Adolphs *et al.*, 1994). However, studies of the causes of hypomimia are significantly fewer (Smith *et al.*, 1996). It has been found that only spontaneous facial expression is affected in Parkinson's disease (Smith *et al.*, 1996), as forced smiles etc. can still be consciously elicited. At the present time, it is speculated that hypomimia is most likely due to combined loss of neurones in the substantia nigra, striatum and amygdala (Aggleton, 1992; Cordato *et al.*, 2000), which are significantly interconnected (Gloor, 1997). Location of a precise site in the amygdala in humans responsible for hypomimia is difficult, as all Parkinson's disease cases will have this clinical feature at the end stage and differential involvement over time cannot be determined. If the amygdala does play a role in the development of hypomimia, the consistent small loss of basolateral neurones in all the Parkinson's disease patients examined may contribute to this clinical feature.

Our recent clinicopathological analysis of demented patients with LB disease revealed high amygdala LB densities in patients who had well-formed visual hallucinations during life (Harding *et al.*, 2002a). In the present study of non-demented Parkinson's disease patients, a similar correlation was observed, with the subregion involved localized to the basolateral nucleus. Selective basolateral neurone loss has been reported previously in temporal lobe epilepsy (Yilmazer-Hanke *et al.*, 2000), where memory-like hallucinations and feelings of déjà vu were evoked from amygdala stimulation in 73% of cases, and every case with spontaneous symptoms had amygdala activation (Bancaud *et al.*, 1994). Detailed factor analysis of these types of well-formed visual hallucinations has revealed an emotional

content (either pleasant or unpleasant feelings) associated with the phenomenon (Santhouse *et al.*, 2000). However, visual dysfunction has also been associated with the amygdala (Anderson and Phelps, 1998; Broks *et al.*, 1998; Gray, 1999), with recent experiments showing an important role for the amygdala in the integration of parallel visual systems. In a patient with cortical blindness in his right hemifield, the amygdala, posterior thalamus and superior colliculus were activated following the visual presentation of emotional facial expressions to his blindside and accurate identification of these visual stimuli by the patient (Morris *et al.*, 2001). This indicates remarkable residual visual abilities mediated through subcortical extrageniculostriate pathways. The amygdala therefore appears to be the final processing centre for the ventral stream of the cortical visual system subserving conscious visual identification and discrimination (Morris *et al.*, 2001) and the extrageniculostriate (colliculo-thalamo-amygdala) visual system, which appears to subservise automatic, non-conscious emotional visual processing (blindsight) (Morris *et al.*, 2001). Dopamine receptor activation in the basolateral nucleus removes prefrontal cortex-induced suppression of neuronal output and enhances activity driven by sensory inputs (Rosenkranz and Grace, 2002). Thus, in the absence of other cortical changes, dopamine plays a significant role in the neural integration within the amygdala. In our Parkinson's disease patients, the dopamine medication usage did not appear to precipitate hallucinations, with no significant difference in dosage between hallucinators and non-hallucinators. This implicates the LB pathology in the basolateral amygdala as more critical for the phenomenon. The increased density of LB in the basolateral amygdaloid nucleus is likely to disrupt the ability of the amygdala to integrate coordinated behavioural responses between the two visual systems and, in association with dopamine replacement therapies, precipitate the visual hallucinations experienced by the patients with Parkinson's disease.

In our previous clinicopathological analysis of demented patients with LB, we showed that hallucinations presenting as an early clinical feature (i.e. either as the initial presenting symptom or presenting within the first 2 years of disease onset) were associated with higher densities of LB in the ventral temporal lobe stream of the cortical visual system (Harding *et al.*, 2002a). Such early cortical LB disruption appears to cause clinical features similar to those observed in people with well-formed visual hallucinations due to ocular pathology (Santhouse *et al.*, 2000). The well-formed visual hallucinations in these people are associated with increased temporal lobe activity due to reduced visual cortical inhibition (Santhouse *et al.*, 2000), and similar patterns of cortical activation have been identified in dementia patients with LB and well-formed visual hallucinations (Imamura *et al.*, 1999). These data show that the phenomenon of well-formed visual hallucinations in these cohorts is associated with altered cortical activity and that LB density appears to be associated with this increased cellular activity. Of course, all the

demented LB patients we studied previously also had substantial numbers of LB in the amygdala (Harding *et al.*, 2002a). In this regard, they are similar to the cases in this study in whom visual hallucinations were all of late onset. The main differences between these cohorts are the absence of significant neocortical LB and clinical dementia (see also Harding and Halliday, 2001). It is not surprising that changes in cortical activity patterns may be associated with a dementia syndrome, but all LB dementia cases also have significant numbers of LB concentrating in limbic neocortices (Harding and Halliday, 2001) and LB cases with early dementia have additional selective neuronal loss in the hippocampus (Harding *et al.*, 2002b). This suggests that the neural substrates for dementia and well-formed visual hallucinations are likely to differ in patients with LB disease. The data also suggest that changes driving increased cortical visual activity within the amygdala appear to produce the same phenomenon—well-formed visual hallucinations.

Overall, our data confirm that pathology in the amygdala is as consistent a feature in idiopathic Parkinson's disease as LB in the substantia nigra. We have also established a consistent relationship between mild neuronal cell loss and increasing densities of LB within the amygdala. The relationship between abnormal intracellular protein accumulations and cell death in Parkinson's disease is still somewhat contentious (Terry, 2000), although our data are consistent with the concept in Alzheimer's disease that the slow, abnormal build-up of such proteins renders neurones more vulnerable to degeneration (Kanazawa, 2001; Maccioni *et al.*, 2001; Lovestone and McLoughlin, 2002), possibly due to increased activity. Finally, the data presented provide evidence of a contribution of LB pathology in the amygdala to visual hallucinations in patients suffering from Parkinson's disease. The consistent, substantial cell loss identified in the cortical amygdaloid nucleus is compatible with an early deficit underlying the early olfactory dysfunction noted in a large proportion of patients with Parkinson's disease (Doty *et al.*, 1988; Hawkes and Shephard, 1993; Meshulam *et al.*, 1998). The late onset of well-formed visual hallucinations was directly related to increased numbers of LB in the basolateral amygdaloid nucleus in the non-demented Parkinson's disease patients analysed. This finding is consistent with literature on hallucinations in other disorders that identifies the amygdala as commonly dysfunctional in patients-experiencing these phenomena. Our data suggest that there is a substantial and restricted early impact of Parkinson's disease on the amygdala that is largely confined to the cortical nucleus, followed by an increase in the concentration of pathology in the basolateral nucleus, associated with the late presentation of visual hallucinations in Parkinson's disease. Thus, the largest regions of the amygdala have relatively newly formed pathology in non-demented Parkinson's disease patients, identifying these regions as important for further studies on the mechanisms of cell death in this disease.

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