IN SITU HYBRIDIZATION STUDIES OF HUMAN SUBSTANTIA NIGRA: PATHOPHYSIOLOGY OF PARKINSON'S DISEASE

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science of the University of London

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Abstract

This thesis describes the development of a protocol for semi-quantitative in situ hybridization, to examine and quantify brain gene expression in Parkinson’s disease (PD), particularly in dopaminergic neurons of the substantia nigra. mRNA was stable post-mortem and appeared unaffected by post-mortem delay and freezer storage time. Agonal status, assessed by measurement of brain pH, was the major determinant of mRNA preservation post-mortem, therefore brain pH was used to match case-control groups for semi-quantitative studies.

mRNA encoding the rate-limiting dopamine synthetic enzyme, tyrosine hydroxylase (TH) was not upregulated in surviving and nigral neurons in PD, and there was no evidence for intrinsically higher levels of dopamine synthesis in the ventral neuronal tier. These findings indicate that compensatory changes in PD do not involve regulation of TH gene expression and that the preferential vulnerability of ventral neurons is not due to toxic effects of endogenous dopamine. Similarly, there was no evidence of any effect of therapeutically-administered L-DOPA on mRNA expression.

mRNA encoding the ND1 subunit of mitochondrial complex I was reduced in surviving nigral neurons PD. mRNA encoding the glycolytic enzyme, aldolase C was less strongly expressed in nigral than other brain stem neurons. The combination of impaired energy metabolism and intrinsically low glycolytic capacity may predispose these neurons to degeneration in PD. Alpha synuclein mRNA expression was also reduced in nigral neurons in PD, indicating that alpha synuclein accumulation in nigral Lewy bodies was not caused by over-expression of the gene; down-regulation was also seen in frontal cortex, a region similarly
prone to Lewy body formation. DNA fragmentation, a marker of apoptotic cell
death was seen commonly in nigral neurons in hypoxic control cases, possible
evidence for the metabolic vulnerability of these cells. However, there was no
evidence that apoptosis was the primary mechanism of cell death in PD.
Acknowledgements

My thanks are owed to Dr Oliver Foster for the supervision of this PhD and for his support and encouragement throughout its (rather long) execution. My work has benefited greatly from his foresight and excellent scientific direction and from his sceptical and witty approach to the problems encountered in setting up the post-mortem in situ hybridization studies.

I am also deeply indebted to the late Professor David Marsden for giving me the opportunity to work in this fascinating field and for his support and enthusiasm for the development of the flash-frozen brain collection and for the for the human in situ hybridization studies.

Professor Stafford Lightman very kindly offered me the hospitality of his laboratory at the Westminster Hospital Medical School for a period of training in in situ hybridization techniques.

Professor Andrew Lees, the Clinical Director of the Queen Square Brain Bank, is warmly thanked for his past and continuing support of the project.

The following colleagues were closely involved in some aspects of the work presented here: Mrs Louise Hewson took part in the development of a protocol for combined labelling by immunohistochemistry and in situ hybridization, Dr David Eve in the studies of the relationship between pH and mRNA preservation (both described in Chapter 2), Ms Hardev Sangha and Ms Sarah Eisen each contributed to the study of alpha synuclein mRNA expression in the cortex and substantia nigra (described in Chapter 6). Dr Mark Cooper, Department of Neurological Sciences, Royal Free Hospital Medical School kindly provided the probe for mitochondrial ND1 mRNA. Dr Susan Daniel carried out the expert neuropathological evaluation
fundamental to the success of this work. I have been greatly helped, also, by
discussions with members of the Neurochemistry Department of the Institute of
Neurology, in particular Dr Christine Hall and Dr Clinton Montfries. Mrs Susan
Stoneham supplied expert advice on the production of photographic images.
In addition to scientific support, the kindness and friendship of my all my Brain
Bank and Institute colleagues, including Ms Linda Kilford, and of Professor Lees’
clinical research fellows, in particular Dr Alice Manson and Dr Andrew
Churchyard, is gratefully acknowledged and greatly enlivened the conduct of this
work.
I am indebted to the Reta Lila Weston Institute of Neurological Studies for
financial support.
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LIST OF ABBREVIATIONS

PD Parkinson's disease (note: the term PD is used throughout to denote the sporadic (idiopathic) form of Parkinson’s disease

6OHDA 6-hydroxydopamine
AADC aromatic amino acid decarboxylase
AD Alzheimer's disease
AEC 3-amino-9-ethyl carbazole
Ca carcinoma
COMT catechol-O-methyltransferase
dATP deoxy adenosine triphosphate
DEPC diethyl pyrocarbonate
DLB dementia with Lewy bodies
DLBD diffuse Lewy body disease
DOPAC 3,4-dihydroxyphenylacetic acid
DRG dorsal root ganglion
DTT dithiothreitol
dUTP deoxy uridine triphosphate
GABA γ-aminobutyric acid
GAD glutamate decarboxylase
GFAP glial fibrillary acidic protein
GMP guanosine monophosphate
GSH reduced glutathione
GSSG oxidised glutathione
GTP guanosine triphosphate
HVA homovanillic acid
ILBD incidental Lewy body disease
ISEL in situ end labelling
ISHH in situ hybridization histochemistry
LB Lewy bodies
L-DOPA levadopa
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MAO-B</td>
<td>monoamine oxidase B</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-methyl-4-phenyl-pyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSA</td>
<td>multiple system atrophy</td>
</tr>
<tr>
<td>NAC</td>
<td>non-amyloid component</td>
</tr>
<tr>
<td>NACP</td>
<td>non-amyloid component precursor</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PAG</td>
<td>phosphate-activated glutaminase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PNMT</td>
<td>phenylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PSP</td>
<td>progressive supranuclear palsy</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SNAP25</td>
<td>synaptic vesicle associated protein 25 kd</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
</tr>
<tr>
<td>Tdt</td>
<td>terminal deoxynucleotide transferase</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>THIR</td>
<td>tyrosine hydroxylase immunoreactive</td>
</tr>
<tr>
<td>TIQs</td>
<td>tetrahydroisoquinolines</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotide-mediated 3'end labelling</td>
</tr>
<tr>
<td>UCH-L1</td>
<td>ubiquitin carboxyterminal hyrolase L1</td>
</tr>
<tr>
<td>VMAT2</td>
<td>vesicular monoamine transporter 2</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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Chapter 1: INTRODUCTION

1.1 Parkinson’s disease

Parkinson’s disease (PD) is a common, progressive, neurodegenerative condition affecting between 30 and 190 individuals per 100,000 population. The prevalence of Parkinson’s disease increases with age, with a mean age of onset of 61 years (Lowe et al 1997), although juvenile (onset under 21 years) and early-onset (onset under 40 years) forms are also seen. The first clinical description of the disease is credited to James Parkinson in his Essay on the Shaking Palsy, written in 1817 and the clinical features that he noted - bradykinesia, paucity of movement, tremor, festinant gait and a flexed posture, were elaborated upon in the years that followed. At present, clinical diagnosis of PD is based on a syndrome of two out of three of rigidity, resting tremor and bradykinesia. In addition to disorders of movement, individuals may also suffer autonomic dysfunction, dysphagia and dementia, which is thought to occur in between 20 and 30 percent of cases (Marsden 1990). The average duration of the disease is 13 years, during which time symptoms become more severe and sufferers progressively more disabled, although many die of causes unrelated to PD.

The clinical signs of the disease are common to a number of conditions, including multiple system atrophy, progressive supranuclear palsy, vascular parkinsonism and fronto-temporal neurodegenerative disorders. Parkinsonian features may be dominant in some of these disorders or part of more general and widespread neurological signs. The precise nature of parkinsonian disease is
defined neuropathologically, by the presence of characteristic inclusion bodies in neural cells. In the case of idiopathic Parkinson’s disease (PD), Lewy bodies, the hyaline, intraneuronal inclusions found in a number of brain regions and in the peripheral nervous system, are its diagnostic hallmark.

1.1.1. Neuropathology of Parkinson’s disease

The major pathological signs of Parkinson’s disease were first observed nearly a century after Parkinson’s essay. The loss of pigmented neurons from the midbrain and in several brain regions were described by Tretiakoff in 1919. The occurrence of intraneuronal inclusions in the substantia innominata and the dorsal vagal nucleus was first observed by Lewy in 1913. These Lewy bodies (LB) were subsequently shown to be present in the degenerating substantia nigra (Tretiakoff 1919). Cell loss in Parkinson’s disease occurs in both the midbrain and the locus ceruleus, resulting in visible depigmentation of these nuclei. Otherwise there are no macroscopic changes and other regions, in particular the striatum and globus pallidus appear normal (Jellinger 1998). On microscopy, free neuromelanin from degenerating neurons is found in the neuropil of and in macrophages in the substantia nigra. Astrocytosis is also usually present.

Surviving neurons of both the nigra and locus ceruleus contain LB. These inclusions are also seen in other brain stem nuclei, the serotonergic neurons of the raphe, the motor vagal nuclei, the pedunculopontine nucleus, the Edinger-Westphal nucleus, in the cortex in the frontal, insular and temporal regions and in the basal forebrain (nucleus basalis of Meynert). Cell loss of varying degrees
is also observed in some of these nuclei. LB are also found in the spinal cord in PD, in neurons of the peripheral nervous system and in enteric plexuses. A proportion of LB are apparent upon staining with haematoxylin and eosin but are more readily visualised and in larger numbers by immunohistochemistry for ubiquitin (Lennox et al, 1989; Sugiyama et al, 1994) and alpha synuclein (Spillantini et al, 1997; Mezey et al, 1998) (see below).

1.1.2 Lewy bodies

Examination of post-encephalitic parkinsonism cases revealed that brains from these individuals contained few or no LB, in spite of the extensive loss of nigral neurons (Hallervorden 1933, 1935), suggesting that the Lewy body had special pathological significance for idiopathic Parkinsons' disease. Even so, LB are not specific to PD and have been found in other conditions, such as Hallervorden-Spatz disease, in which they are also immunoreactive for alpha synuclein (Wakabayashi et al, 1999).

In brain stem structures, such as the nigra and locus ceruleus, LB are spherical, with a dense granular core and a pale halo when visualised with eosin staining. Ultrastructural studies show that the halo is composed of radially-arranged intermediate filaments. Cortical LB, in contrast, are less regular in shape, have no core and halo structure and intermediate filaments are arranged in a dense, felt-like mass (Kosaka 1978).

The immunohistochemical profile of these inclusions has been the subject of
intense study. LB are generally immunoreactive for structural proteins such as phosphorylated and non-phosphorylated neurofilament protein (Forno et al, 1986; Hill et l, 1991; Schmidt et al, 1991), for tubulin (Galloway et al, 1988; Schmidt et al, 1991), microtubule-associative proteins, MAP1 and MAP2 (Galloway et al, 1988; Wakabayashi et al, 1992) and for proteins associated with synaptic and vesicular structures, including synaptic vesicle-specific 38 kDa protein (SVP38) (Wakabayashi et al, 1992), synaptophysin and chromogranin A (Nishimura et al, 1994). Tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis has also been shown to be present in the outer layers of substantia nigra LB but not in those in non-catecholaminergic nuclei, such as dorsal raphe (Nakashima and Ikuta, 1984). LB are immunoreactive for alpha synuclein (Spillantini et al, 1997; Mezey et al, 1998), a transmembrane protein thought to be involved in vesicular trafficking at the synapse. The discovery that LB are also immunoreactive for a number of proteins associated with ubiquitin-mediated proteolysis, such as ubiquitin (Lowe et al, 1993; Sugiyama et al, 1994), ubiquitin carboxy-terminal hydrolase (PGP9.5) (Lowe et al, 1990) and multicatalytic protease (Kwak et al, 1991) has lead to the suggestion that the formation of inclusion bodies is associated with disturbed function of protein degrading mechanisms. This view is supported by the finding that mutations of ubiquitin carboxy-terminal hydrolase and parkin, both of which are proteins of the ubiquitin-proteasome pathway, are associated with familial PD (see below).

1.1.3 Additional pathology in Parkinson’s disease

In spite of the normal appearance of the cortical ribbon immunohistochemical
studies have demonstrated extensive and severe pathological change in the cortex. Ubiquitin immunoreactive abnormal neurites are seen, predominantly in hippocampal neurons (CA2/3) but also occur in the nucleus basalis of Meynert and in the dorsal vagal nucleus. Their formation has been considered to be an example of intra-neuritic Lewy body formation (Lowe et al, 1997). Alpha synuclein immunoreactive LB have been shown in the cingulate gyrus, temporal cortex and amygdala; Lewy neurites have also been demonstrated in the amygdala, the transentorhinal cortex, and in the basal forebrain. In general, it seems that these pathological changes are associated with long-projection neurons of a number of phenotypes, including GABAergic, glutamatergic, cholinergic and noradrenergic neurons (Braak and Braak, 2000).

1.1.4 Neuropharmacology of Parkinson’s disease

The use of phenothiazines to treat psychosis and of reserpine for the treatment of hypertension showed that both classes of drugs produced parkinsonian symptoms in humans. These could be reproduced in experimental animals and were shown to involve interaction with striatal dopamine systems. Reserpine blocks the uptake and storage of catecholamines and leads to depletion dopamine from striatal terminals. In rats, the parkinsonian symptoms induced by reserpine could be reversed by administration of L-3,4-dihydroxyphenylalanine (L-DOPA), the immediate precursor of dopamine, thus reinstating dopamine levels (Carlsson et al, 1957). Evidence for the physiological role of dopamine in the basal ganglia came from studies of its distribution, where it was found in the caudate and putamen, the globus pallidus.
and the nigra (Bertler and Rosengren 1959; Sano et al, 1959; Ehringer and Hornykiewicz 1960). The striatal dopaminergic deficit present in human parkinsonian subjects, which was implied by the findings in experimental animals, was first demonstrated by Ehringer and Hornykiewicz in 1960 and the clinical response of PD patients to treatment with L-DOPA was demonstrated soon after (e.g. Birkmayer and Hornykiewicz 1961; Barbeau, Soukses and Murphy 1962; Yahr et al, 1968). Subsequent anatomical studies of the distribution of catecholaminergic neurons and their projections, using fluorescence as a marker, demonstrated the correlation between the anatomical sites of cell destruction in PD and the loss of catecholaminergic input in the terminal fields of these neurons and established that the missing nigral neurons used dopamine as a transmitter and projected to the striatum (Carlsson et al, 1959, 1962; Dahlstrom and Fuxe 1964).

1.1.5 A model for movement disorder in Parkinson's disease

The currently accepted, though simplified, model of the origin of movement dysfunction in PD postulates that movement is modulated by a balance between excitatory and inhibitory signals, integrated through a series of interconnecting loops in specific pathways of the basal ganglia. The basal ganglia include the substantia nigra pars compacta and pars reticulata, the caudate nucleus and putamen (striatum), the internal and external globus pallidus, the amygdala, the subthalamic nucleus and nuclei of the thalamus. The three main transmitter systems of the basal ganglia are glutamate, GABA and dopamine. The striatum receives excitatory glutamatergic inputs from the cortex and has inhibitory
GABAergic outputs through two main pathways which connect with other basal
ganglia structures: i) the direct pathway from the striatum to the medial globus
pallidus and the substantia nigra pars reticulata, which, in turn, send GABAergic
projections to thalamic nuclei which have an excitatory input to the motor
cortex (the pallido-thalamo-cortical projection) ii) the indirect pathway, from
the striatum to the lateral globus pallidus and a second projection from the
lateral globus pallidus to the subthalamic nucleus which projects in turn to the
substantia nigra pars reticulata and the medial globus pallidus, thus modulating
the pallido-thalamo-cortical projection. The dopamine projection to the
striatum from the substantia nigra has both excitatory and inhibitory effects on
striatal neurons, through activation of D1 and D2 receptors respectively. D1
receptors activate the direct output pathway and D2 receptors the indirect
pathway (Figure 1.1). In the normally-functioning basal ganglia it is thought
that the output of the indirect pathway results in a tonic inhibition of the
thalamo-cortical projections, through the subthalamus, thus inhibiting
movement. The direct pathway is thought to suppress this inhibition in normal
individuals, maintaining a balance between the two output pathways.

As a result of the loss of dopaminergic neurons of the substantia nigra, striatal
dopamine is severely depleted in PD, together with dopaminergic pre- and post-
synaptic markers. The loss of dopamine innervation on projection neurons of
the caudate and putamen results in overactivity of the inhibitory neurons
projecting to the indirect output pathway via the lateral globus pallidus and,
consequently of the subthalamic nucleus, and thus, inhibition of the
Diagram of basal ganglia circuits
Simplified representation of basal ganglia pathways. Black lines, excitatory pathways (believed to be glutamatergic) except nigral da projection, which is both inhibitory and excitatory; dashed lines, inhibitory projections, believed to be GABAergic. The direct pathway is activated by D1 receptors; the indirect pathway, though the external globus pallidus and subthalamic nucleus is activated by D2 receptors.
da, dopamine; enk, enkephalin; GPe, external (lateral) globus pallidus; GPi, internal (medial) globus pallidus; thal, thalamus; sp, substance P; SNpc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus.
thalamocortical pathways. The loss of excitatory output via the direct pathway results in a failure of the direct pathway to suppress tonic inhibition of the thalamus. The net effect of these changes is an increase in inhibition of the thalamo-cortical projection, which is thought to result in the characteristic akinesia and rigidity of PD (Albin et al, 1989; DeLong et al, 1990; Gerfen 2000 and refs therein).

1.2 Nigral cell loss in Parkinson’s disease

Nigral degeneration in PD has been studied extensively but, as yet, no single factor has been identified which could be considered as being the primary cause of cell death. Nevertheless studies of human brain and animal models have indicated a number of the processes which might be involved.

1.2.1 Anatomy of the midbrain dopaminergic systems

The substantia nigra pars compacta extends through the ventral part of the midbrain from the rostral lip of the pons ventrally and the caudal border of the inferior colliculus dorsally, to the subthlhamic region of the forebrain. It forms two symmetrical concave groups of cells on either side of the midline and overlies the fibre tracts of the corticopontine and corticospinal connections. The nucleus comprises many large, generally polygonal and a smaller number of fusiform neurons which are characteristically melanised, giving the nucleus its black appearance and its name. At the midline, around the interpeduncular fossa, the substantia nigra is continuous with the neurons of the ventral tegmental area (VTA), which extend vertically into the region ventromedial to the red nucleus.
Caudally, melanised neurons extend into the retro-rubral area close to the medial geniculate nucleus, forming the pars lateralis. Neuromelanin-containing neurons are also found singly throughout the midbrain, in the periaqueductal grey matter and posteriorly are continuous with the pigmented neurons of the locus ceruleus (van Domberg and ten Donkaaler, 1991).

There have been a number of studies of the sub-regional anatomy of the substantia nigra pars compacta. Hassler described 50 subregions, based on the anatomical position of the cell groups and the fibre fields which surrounded them (Hassler 1937). Subsequently, Olszewski and Baxter divided the nigra into four zones, which included the substantia nigra pars reticulata, a non-melanised nucleus at the more rostral regions of the midbrain, underlying the pars compacta and forming a caudal-wards extension of the medial globus pallidus (Olszewski and Baxter 1954). Olszewski and Baxter’s classification introduced the important concept of nigral tiers. The remaining three nigral groups comprised a dorsal tier, which the authors interpreted as being composed of neurons which did not form irregularly-shaped groups but were more evenly distributed and continuous medially with the VTA and laterally with the retrorubral area, designated β, a ventral tier, composed of irregularly shaped congregations of neurons, was designated α and a third group comprising neurons located in the ventral regions of the red nucleus capsule, designated γ (Olszewski and Baxter 1954). Although possibly an oversimplification of the nigral sub-regional structure, most recent subregional studies of the nigra are based on this classification.
The substantia nigra, the ventral tegmental area and the pars lateralis were allocated to areas, A9, A10 and A8 respectively by Dahlstrom and Fuxe in their study of catecholaminergic cell groups in the brain (Dahlstrom and Fuxe 1964). The dopaminergic projections of the substantia nigra (A9) and ventral tegmental area (A10) groups are different; broadly speaking, A9 neurons project to the caudate nucleus and putamen, while neurons of the ventral tegmental area (part of A10) innervate the limbic cortex, the amygdala and ventral striatum. Primate and human studies show that the dorsomedial parts of the substantia nigra (A9) and a component of the VTA (A10) innervate the ventral regions of the striatum (Gaspar et al, 1985) and the basal forebrain, which has extensive tyrosine hydroxylase immunoreactivity. Cat and primate studies also demonstrated that the projections to the caudate nucleus arose in rostral regions of the substantia nigra (A9) whereas posterior regions innervated the putamen. The nigro-striatal projection was shown to be organised so that the dorsal part of the caudate nucleus received projections from the medial substantia nigra and, within this subdivision, ventral neurons projected to the dorsal caudate and dorsal neurons to medial and ventral parts of the dorsal striatum. The lateral and posterior nigra innervated the dorsal and lateral putamen, while projections to the ventral putamen arose in the medial and central nigra. (Szabo 1980 a, b). In addition to the mesolimbic projections of the VTA, midbrain dopaminergic innervation of the human cortex is extensive (Gaspar et al, 1989).

1.2.2 Patterns of nigral cell loss in Parkinson’s disease

Neuronal death in the nigra produces a gradient of loss of striatal innervation,
reflecting the nigral pattern of cell death, the putaminal input from the
ventrolateral regions of the nigra being more severely affected than the input to
the caudate from the more medial regions of the nigra. The loss of striatal
dopamine innervation in PD was confirmed by Bernheimer and colleagues
(Bernheimer et al, 1973). Biochemical analysis of striatal dopamine and
homonanillic acid (HVA) showed that mean values for extracted dopamine were
significantly reduced in the caudate nucleus (16% of control) and the putamen
(1% of control) in PD. Mean values for dopamine were higher in control
putamen than in the caudate nucleus but demonstrated greater proportional loss.
HVA values were reduced to 30% of control in the caudate nucleus and 20% in
the putamen, thus the ratio of HVA to its precursor, dopamine, was increased in
both the putamen and caudate, compared with controls, a finding that was
thought to indicate increased turnover of dopamine. The reduction of striatal
dopamine and HVA values correlated strongly with the degree of cell loss in the
substantia nigra. This correlation was much stronger for the putamen than the
caudate nucleus, indicating that the former had a more pronounced loss of
dopaminergic terminals. The dopamine and homovanillic acid content of the
nigra was also found to be reduced, evidence which the authors concluded,
strongly supported the hypothesis that dopamine containing neurons in the
midbrain projected to the striatum.

In addition, it was noted that patterns of nigral cell loss distinguished between
idiopathic Parkinson’s disease and post-encephalitic and vascular parkinsonism.
Both the latter had extensive and diffuse nigral cell loss, in comparison with
idiopathic PD, where cell loss in the nigra was less severe but more focal. In the striatum in PD, the dopamine deficiency was greater in the putamen than in the caudate nucleus, even in cases with less severe degeneration. This was held to reflect the somatotopic organisation of the nigro-striatal pathways and the possibility that the nigro-putaminal projections were affected earlier in the disease and ultimately more severely than the nigral projections to the caudate. In idiopathic PD the authors noted that the conspicuous differences in the decrease of dopamine concentrations caudate nucleus and putamen were reflected in the more severe cell loss they observed in the caudal regions of the nigra which projected to the putamen (Bernheimer et al 1973).

Subsequent morphological studies have confirmed the non-uniform rates of cell loss in nigral sub-regions in PD and show that over 90% of neurons degenerate in the ventro-lateral subdivision, while more dorsal sub-regions lose a smaller proportion of neurons, between 40 and 60%, particularly in cases with relatively minor cell loss in the earlier stages of the disease (Fearnley and Lees 1991). Neurons of the VTA (A10 group) are relatively less vulnerable to the disease and cell loss between 64% in the anterior part and 45% in posterior regions has been reported in this group (Jellinger 1998). German and colleagues have also demonstrated that loss of melanised dopaminergic neurons was greatest in the ventral and lateral portions of the substantia nigra. The authors noted a 68% loss of cells overall in PD, with a >95% loss in ventral regions. Surviving neurons in PD were generally found in the dorsal extent of the nucleus. The A8 and A10 regions were less severely affected, and numbers of surviving neurons in
these regions were more variable in number between individuals. The results obtained from cell counting were consistent with patterns of dopamine depletion observed in the striatum in PD, where the dorsal and lateral striatum are most severely affected, and which had been demonstrated by retrograde labelling experiments to represent regions innervated by the ventral and lateral regions of the nigra (German et al, 1989).

Fearnley and Lees also studied nigral neuronal numbers in a series of 36 control individuals between 21 and 91 years of age and 20 PD cases with ages ranging from 62 to 87 years, with similar findings. Dividing the nigra into six separate subregions, based on Hassler’s classification but taking into account the tiered arrangement of neurons described by Olszewski and Baxter, cell counts were made in a single section from each case, taken at the level of the third nerve exit from the midbrain, where dorsal and ventral tiers could be most easily delineated. In comparison with age adjusted values for control nigra, cell loss in the ventrolateral nigra was 91%, in the ventromedial nigra, 71%, while in the dorsal tier cell loss was between 47 and 51%. In contrast to the pattern of cell loss in PD, dorsal nigral regions were more affected by ageing than ventral regions. Nigral cell numbers of neurologically normal individuals declined with age, with a loss of about 5% per decade, in the dorso-medial, dorso-lateral and pars lateralis subregions. There was also a strong correlation between loss of nigral neurons and the recorded duration of the disease in the PD subjects and the authors identified an exponential decline of neuronal numbers in both total and regional counts with increasing symptom duration (Fearnley and Lees 1991).
Anatomy of midbrain subregions

Diagrammatic representation of nigral sub-regions in a hemi-section of midbrain at the level of the third nerve exit (III\textsubscript{n}). Cp, cerebral peduncle; rn, red nucleus, PL, pars lateralis. DL, DM, VL, VM, dorso-lateral, dorso-medial, ventro-lateral and ventromedial subdivisions of the substantia nigra pars compacta.

Cell loss in DL and DM subregions: 57 and 47\% respectively; cell loss in VL, 91\% and VM 71\%; PL 61\% (Fearnley and Lees 1991).

Nigral neurons examined in the present studies were selected from the VL and DL subregions, with some DM and VM cells, but care was taken in all studies to exclude neurons of the VTA and pars lateralis.
Gibb and Lees studied nigral cell loss throughout the nigra with respect to the degree of melanisation of nigral neurons. Neurons from dorsal nigral regions were found to be heavily and evenly pigmented, with neuromelanin covering between 0.48 and 0.57 of the cell area in control subjects. Neurons of the ventro-lateral regions were less heavily pigmented, with neuromelanin occupying between 0.18 and 0.28 of the cell area. The differential distribution of neuromelanin corresponded to the differential patterns of cell loss observed in the nigra, in that the more lightly-melanised ventral tier showed greater cell loss than the dorsal tier. The distribution of LB was also examined in incidental Lewy body disease (ILBD) cases, i.e. in individuals with LB present in the midbrain and some midbrain cell loss but no motor symptoms, thought to represent pre-symptomatic PD. LB distribution in these cases also co-incided with the lower concentrations of neuromelanin in the ventral regions (Gibb and Lees 1991). In contrast, a comparison of the degree of cell loss between different midbrain populations of tyrosine hydroxylase-positive (catecholaminergic) neurons indicated that neurons were preferentially lost from the more heavily-melanised groups such as the substantia nigra (around 77% of the total) in PD while degeneration of non-melanised neurons of the central grey substance was almost undetectable. This was taken as evidence that the presence of neuromelanin conferred selective vulnerability on catecholaminergic neurons (Hirsch et al, 1988).

1.2.3 Rates of nigral cell loss

Measurements of the rate of nigral cell loss suggest that this is an active
degenerative process, rather than a slow process of attrition. McGeer and colleagues examined representative sections from the substantia nigra of 9 control and 9 PD cases and counted cells undergoing phagocytosis. They found more numerous microglia in the nigra of the PD cases and that the number of cells undergoing phagocytosis was six times greater in the PD cases than the controls, indicating an active degenerative process. Based on a previous study of cell numbers in the nigra, they estimated that the PD cases had less than 50% of the total cell numbers expected in the nigra of age-matched controls and, based on the numbers of degenerating neurons seen in sections from the PD nigrae, they estimated that cell losses might be in the range of 1000 to 3600 neurons a month. The presence of microglia and phagocytosis made it unlikely that age-related attrition of cells added to losses resulting from an early but extinct disease process was the cause of cell death in PD since it would not be expected that active phagocytosis would still be taking place in these circumstances. Similarly, a process of accelerated ageing, a possibility which had been proposed to explain the late onset of cell loss in PD, was thought unlikely to produce the much greater degree of active phagocytosis observed in the PD nigra. The authors concluded that the results were only consistent with an active process of cell loss of a few years duration since the rate of loss calculated from their findings was not sustainable over many years without total depletion of nigral neurons (McGeer et al, 1988). The findings of Fearnley and Lees also made it unlikely that cell loss in PD was a form of accelerated aging and pointed to a similar conclusion. The exponential rate of neuronal loss which they identified in the ventromedial and ventrolateral neuronal sub-populations was
too great to be consistent with accelerated ageing and indicated that PD was a relatively acute monophasic illness.

1.2.4 Factors implicated in nigral cell loss in Parkinson’s disease

1.2.4.1 Oxidative stress

The interest in melanisation as a factor in the vulnerability of nigral neurons in PD stemmed from the possibility that neuromelanin might itself be cytotoxic. This could either be a consequence of free radicals generated during its formation by polymerisation of oxidation products of dopamine or by auto-oxidation (Graham 1978, 1984) or because the pigment itself might sequester free radicals or other toxic molecules, acting as a reservoir for compounds which could generate oxidative stress in dopamine neurons. There has been considerable research into the possible role of oxidative stress in the selective destruction of nigral neurons and a number of mechanisms which involve oxidative stress from free radical formation have been proposed to explain nigral cell death.

Free radicals include hydrogen and molecular oxygen, although in vivo, these are not very toxic. The more damaging oxygen radicals, superoxides, peroxyl radicals, nitric oxide and the hydroxyl radical, are generated from interactions involving molecular oxygen. Free radicals are highly reactive and participate in a majority of synthetic and metabolic processes in the cell, notably in the mitochondrial electron transport chain. They may also play a part in cellular defence mechanisms, e.g. the superoxide radical and nitric oxide are both
synthesised by brain microglia, but overproduction of free radicals or failure by
the cell to balance levels of oxidative reactions with anti-oxidative processes,
may permit free radicals to damage important molecules and cellular structures.

The evidence for oxidative stress in the nigra in PD comes from identification of
biochemical markers in post-mortem tissue nigral tissue. For instance, metal
ions, such as Fe, Cu, Co and Se which are co-factors in many enzymic reactions,
are crucial to the utilisation of molecular oxygen in oxidative phosphorylation
but can further generate reactive oxygen species. Hydrogen peroxide, although
not itself a free radical and probably not strongly toxic, gives rise to the highly
reactive hydroxyl radical by reaction with ferric ions (the Fenton reaction)
(Halliwell and Gutteridge 1990). The hydroxyl radical can theoretically damage
almost all cellular molecules, including DNA, in which it causes strand breaks,
and polyunsaturated fatty acids (PFA) which are major components of cellular
membranes. However, all charged molecules can cause lipid peroxidation by
abstraction of electrons from PFA side chains in membrane lipids, leaving
charged lipids in the membrane which go on to interact with molecular oxygen
and produce a number of free radicals including peroxyl radicals and superoxide
ions and can set up a chain reaction of lipid peroxidation. A number of other
toxic products can be formed by interaction of iron and copper with lipid
hydroperoxides, including, malondialdehyde (MDA), 4-hydroxy-2,3-trans-
nonenal, and aldehydes. All these toxic molecules could potentially compromise
membrane function, ion conductance and ligand-receptor interactions at the
Increased iron concentrations and decreased levels of the iron-binding protein, ferritin observed in post-mortem nigra from PD cases, suggests that nigral neurons could potentially be vulnerable to iron-dependent lipid peroxidation (Dexter et al, 1989, 1990). In contrast, increased iron with increased ferritin levels have also been demonstrated in post-mortem nigral samples in PD (Reiderer et al, 1989, Sofic et al, 1991). The cellular source of the increased iron in the nigra is not known but it has been suggested that increased transport into dopamine neurons may be a possibility, since expression of the iron-binding protein, lactoferrin and membrane lactoferrin receptors is up-regulated in surviving neurons in PD (Double et al, 2000). Alternatively, the source for increased iron may be intracellular release from neuromelanin, which binds a variety of heavy metals. In parallel with increased iron concentrations, increased malondialdehyde levels have been observed in PD nigra (Dexter et al,1989) and a ten-fold increase in the levels of lipid hydroperoxides (Dexter et al,1994a). Activity of magnesium-dependent superoxide dismutase, which removes the superoxide radical by catalysing the formation of hydrogen peroxide and molecular oxygen, has also been reported to be increased in the particulate fraction of PD nigra (Saggu et al, 1989), suggesting an elevated response to oxidative stress caused by the superoxide radical (Fahn and Cohen, 1992) and supporting the view that iron-dependent lipid peroxidation might be a factor in cell death.

Other studies have reported reduced anti-oxidant capacity in the nigra in PD. Hydrogen peroxide, generated by superoxide dismutase, is removed by the
action of glutathione peroxidase, which catalyses the transfer of one electron from hydrogen peroxide to reduced glutathione (GSH) to generate oxidised glutathione (GSSG) and water. Reduced GSH levels have been demonstrated in PD nigra (Sofic et al, 1992; Sian et al, 1994) while other brain regions which are neuropathologically affected in PD do not show a reduction (Sian et al, 1994; Gu et al, 1998) and the reduction in GSH has been shown to correlate with the severity of parkinsonism (Reiderer et al, 1989). There is some evidence that the activity of glutathione peroxidase might also be reduced (Kish et al, 1993) and γ-glutamyl transpeptidase activity, which is concerned with the degradation of GSSG and its efflux from cells, has been reported to be increased (Sian et al, 1994b).

The origin of these changes is obscure since biochemical data do not indicate which cellular components of the nigral population are involved and it is unclear whether oxidative stress is a primary cause of degeneration or whether it arises secondarily to other neurodegenerative changes. For instance, the nitration of nigral proteins, a marker for damage by nitric oxide, which is present in PD nigra (Shergill et al, 1996) could be the result of a primary toxic mechanism or could result from increased synthesis of nitric oxide by astroglia in response to degeneration of nigral neurons. Some insight into the relationship between oxidative stress and nigral degeneration is provided by studies of ILBD cases. Dexter and colleagues found that levels of GSH and the ratio of reduced to oxidised glutathione in the nigra in ILBD to be significantly lower than in control subjects, but the changes in reduced glutathione levels were not as great.
as in advanced PD. Levels of iron and ferritin were unaltered. These findings suggested that i) that the alteration of reduced glutathione could be an early change due to oxidative stress and ii) that increased iron and reduced ferritin levels seen in established PD might represent a secondary process (Dexter et al, 1994b). Recent studies suggest that the increase in iron concentration in the substantia nigra in PD is seen only in advanced disease, supporting the view that this may be a secondary event (Double 2000).

1.2.4.2 Nigral neurotoxins

The discovery that accidental poisoning by 1-methyl-4-phenyl 1,2,5,6 tetrahydropyridine (MPTP), a contaminant of an illegally-produced heroine substitute, led to chronic parkinsonism in drug abusers, has opened up fruitful research into the causes of cell death in the substantia nigra. In the late 1970s and early 1980s there were instances of intravenous drug users injecting illegally-synthesised meperidine and developing parkinsonism with a clinical picture very similar to that of PD, including immobility, dysphasia, facial seborrhoea, dribbling, cogwheel rigidity and flexed posture. One individual had a pill-rolling tremor, one shuffling gait and bradykinesia (Langston and Ballard 1983). All responded to combined L-DOPA/carbidopa therapy and one, additionally, to treatment with a dopamine agonist. The close similarity to Parkinson's disease was confirmed when one individual was examined post-mortem and was found to have extensive cell loss in the substantia nigra combined with focal glial scarring, extracellular neuromelanin and a single Lewy body. In common with PD, the most extensive cell loss was seen in the

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caudal nigra (Davis et al, 1979). No other gross abnormalities of the brain were found, indicating that the clinical signs of PD could be induced solely by degeneration of the substantia nigra (Ballard et al, 1985).

MPTP was found to be a major contaminant of the meperidine (Langston 1983) and its selective toxicity to the dopaminergic nigro-striatal projection was established experimentally in primate studies (Langston et al, 1984a). Squirrel monkeys treated with MPTP quickly developed parkinsonian symptoms closely similar to the human disease. All were found to have abnormalities of the substantia nigra, including cell loss and gliosis. No effect was observed in the locus ceruleus and other brain regions appeared normal. It was subsequently established that metabolism of MPTP to 1-methyl-4 phenyl pyridinium (MPP') was essential for its toxicity (Langston et al, 1984b; Markey et al, 1984). Autoradiographic studies of uptake and metabolism of $^3$H and $^{14}$C-labelled MPTP in squirrel monkey brain (Markey et al, 1984) established that 80% of accumulated radioactivity was an MPTP metabolite, assumed to be the major pyridinium oxidation product MPP', and was trapped in neurons. The major sites of accumulation were the striatum, hypothalamus, nucleus accumbens and parabralchial nucleus. Pretreatment of mice (Markey et al, 1984) or the squirrel monkey (Langston et al, 1984a) with the general monoamine oxidase inhibitor, pargyline, showed that oxidation of MPTP to MPP' could be blocked in vivo, with a concomitant reduction in toxicity demonstrated by preserved striatal dopamine levels and $^3$H dopamine uptake in striatal synaptosomes. The authors concluded that, since intoxicated human subjects
also responded to deprenyl (a specific monoamine oxidase B inhibitor), an analogous oxidation of MPTP to MPP⁺ was likely to be responsible for the ongoing toxicity seen in these cases. MPP⁺ either stereotaxically-administered or derived from the metabolism of MPTP was shown to be selectively accumulated by the nigro-striatal system and to have the same toxicity as MPTP in primates and rodents (Irwin and Langston 1985; Heikkila et al, 1985a).

Specific inhibition of monoamine oxidase B by deprenyl was shown to block the neurotoxic effects of MPTP on rodent striatum, while monoamine oxidase A inhibitors were not effective (Heikkila et al, 1984, 1985b). Monoamine oxidase B was shown to be present in rat striatal astrocytes (Levitt et al, 1982) and serotonergic nuclei and in primate serotonergic neurons (Westlund et al, 1985) using immunohistochemical methods. Neither study found monoamine oxidase B in dopaminergic or catecholaminergic nuclei. Nakamura and Vincent used MPTP as the substrate for monoamine oxidase B in rat brain sections and showed that oxidation to MPP⁺ occurred in only a few sites in the rat brain, in noradrenergic and serotonergic neurons of the brain stem and histamine neurons of the hypothalamus. MPTP toxicity in the nigra was therefore due to the oxidation of MPTP to MPP⁺ in cell groups, such as striatal astrocytes or brain stem neurons, with close proximity to dopaminergic terminals. The selective vulnerability of the nigro-striatal projection appeared to result from uptake of MPP⁺ into dopaminergic nerve terminals of projection neurons of the substantia nigra and to a lesser extent the reticular formation and ventral tegmental area (Nakamura and Vincent 1986). Monoamine oxidase B inhibition
by deprenyl did not abate the toxicity of intraventricular MPP⁺ in mice, 
confirmation that it was the toxic agent (Bradbury et al, 1985).

Studies of dopamine uptake blockade in striatal slices and in vivo in rats and 
mice demonstrated that uptake of MPP⁺ into striatal dopaminergic terminals was 
a critical step in the destruction of the nigrostriatal pathway (Mayer et al, 1986). 
Inhibitors of dopamine uptake, including GBR 13069, nomiphensine, mazindol 
and cocaine, reduced the uptake of MPP⁺ into mouse striatal slices, to varying 
degrees. MPP⁺ was found to be an effective releaser of pre-loaded ³H-dopamine 
but this release was blocked by mazindol. Pretreatment with dopamine uptake 
inhibitors also blocked toxic effects of MPTP in vivo in mice (Mayer et al, 1986; 
Ricaurte et al, 1985). These findings were supported by studies of dopamine 
uptake and MPTP metabolism in rat mesencephalic dopamine neurons in culture 
in which the toxicity of MPP⁺ was confirmed and was shown to be inhibited by 
mazindol (Sanchez-Ramos et al, 1986).

Subsequent primate studies have extensively documented the effects of MPP⁺ 
on the nigrostriatal dopaminergic system and their close similarity to the 
destructive effects of PD. German and colleagues, for instance (German et al, 
1988), showed that extensive nigral cell loss followed MPTP administration, 
primarily in the ventro-lateral nigra. Patterns of dopamine loss in the striatum 
paralleled the loss of midbrain neurons and their striatal projections. The 
largest reduction of striatal dopamine was seen in the caudate nucleus (>90%) 
with a slightly smaller reduction in the putamen (80-90%), in contrast to early
findings from human brain in PD. Striatal tyrosine hydroxylase (TH) immunoreactivity demonstrated greatest loss of dopamine innervation to the dorsal and lateral neostriatum, the ventromedial striatum and nucleus accumbens being relatively less affected, as in PD. The primate MPTP intoxication model is not precisely the same as Parkinson’s disease since the basal forebrain and the cortex are unaffected in most cases. It was thought, however, that the selective toxicity of MPTP to the substantia nigra might also be the result of the acute nature of the insult in experimental animals and that chronic toxic action might eventually involve the VTA and other regions. In addition, studies of the effects of MPTP in older primates showed a more widespread toxicity, involving the locus ceruleus, the dorsal raphe and basal forebrain, suggesting that MPTP might be more toxic to older animals and show a pattern of damage closer to the human disease (Forno et al, 1986, 1988).

1.2.4.3 Dopaminergic activity

Considerable interest has been focussed whether dopamine, the major nigral neurotransmitter, might itself be neurotoxic in some circumstances, rendering the nigra selectively vulnerable to neurodegeneration in PD. Catalysis of dopamine by monoamine oxidases generates hydrogen peroxide (Cohen 1983) and recent findings suggest that peroxidation of dopamine potentiates the formation of dopaminochrome, which can be shown to be toxic to neuroblastoma cells in culture (Galzigna et al, 2000). In addition, dopamine has been shown to undergo auto-oxidation, due to its quinone structure (Bindoli et al, 1992), which may also generate free radicals. Dopamine has also been
shown to be neurotoxic to cultured cells, such as chick post-mitotic sympathetic neurons and rat phaeochromocytoma cells. Culture of PC12 cells, for instance, in the presence of dopamine-melanin (produced by chemical oxidation of dopamine) and dopamine alone, produced a dose-dependent drop-out of viable cells over a period of hours. This toxic effect was enhanced by the inclusion of ferric iron in the culture medium. Anti-oxidants protected cell against the toxic effects of dopamine but not of dopamine-melanin. Examination of extracted DNA by gel electrophoresis showed a pattern of fragmentation consistent with cell death by apoptosis (see below, para 1.3.1), which was confirmed by terminal deoxynucleotide transferase-mediated biotin 3’ end-labelling (TUNEL) histology for DNA strand breaks (Offen et al, 1997). It was thought that the apoptotic effect might be induced by generation or sequestration of free radicals by melanin.

The neurotoxin 6-hydroxydopamine (6OHDA) is able to selectively destroy the nigrostriatal pathway following systemic or intracranial injection in rats, which is used as an experimental model of cell loss in PD. This effect is thought to be mediated by the production of the superoxide radical, hydrogen peroxide and the hydroxyl radical, thus it is possible also that reactive metabolites of dopamine may be toxic to nigral neurons. Taken together, the findings from post-mortem studies of the nigra and from cell culture and experimental studies indicate that dopamine and its metabolites could contribute to the susceptibility of nigral neurons to damage in PD by an oxidative stress mechanism (Fahn and Cohen 1992; Offen et al, 1997). In addition, by analogy with the effect of MPTP, a
nigral neurotoxin might be taken up more readily by active neurons, through the
dopamine uptake system, rendering these neurons more vulnerable to toxic
insult.

1.2.5 MPP⁺ and mitochondrial activity

The means by which MPP⁺ brings about nigral cell death may be either its
function as a mitochondrial toxin or by induction of oxidative stress
(Johannessen et al, 1986; Jenner et al, 1992a, 1992b) or a combination of these
effects. MPP⁺ appears to bind to the inner mitochondrial membrane close to the
rotenone-sensitive complex I site, resulting in inhibition of oxidation of NAD-
linked substrates, specifically by reduction of the activity of mitochondrial
complex I (NADH-ubiquinone CoQ reductase). This complex of enzymes
catalyses electron and hydrogen transfer between NADH, generated by Krebs
cycle enzymes from pyruvate, glutamate and malates, and ubiquinone. Complex
I is the first of four inner mitochondrial membrane complexes which comprise the
electron transport chain. Complex II catalyses transfer of electrons between
succinic acid and ubiquinone and works in parallel to complex I, with succinate
as substrate. Complex III, ubiquinone-cytochrome C reductase and complex IV,
cytochrome C oxidase, complete the transfer of electrons and complex IV passes
electrons from reduced cytochrome C to molecular oxygen to produce water.
The process can be summarised:

\[
\text{NADH} + H^+ + 3\text{ADP} + 3\text{Pi} + \frac{1}{2} O_2 \rightarrow \text{NAD}^+ + 3\text{ATP} = H_2 O
\]

Electron transfer between the four complexes pumps hydrogen ions across the
inner mitochondrial membrane from the mitochondrial matrix to the inter-
membrane space. The accumulation of electrons and hydrogen in the inter-
membrane space generates a proton gradient which in turn provides the energy
to drive the synthesis of ATP by the ATP synthetase complex (complex V)
which is also located in the inner mitochondrial membrane (Jung and Brierley
1985).

Studies of isolated mitochondria from rat brain or liver have demonstrated the
effect of MPP⁺ on mitochondrial function. Incubation of isolated rat brain
mitochondria with pyruvate or malate as substrates combined with MPP⁺
resulted in a concentration- and time-dependent decrease in oxygen utilisation in
comparison with control incubations. No effect of MPP⁺ was seen if succinate
was the substrate, demonstrating that the active inhibition by MPP⁺ selectively
affected NAD-linked substrate utilisation by complex I (Ramsay et al, 1986;
Vyas et al, 1986) and that complex II was unaffected. The decrease in oxygen
utilisation was accompanied by accumulation of reduced pyridine nucleotide
(NADH) from the Krebs cycle and an increased production of lactate from
glucose indicating reduced complex I activity and overall reduction of oxidative
phosphorylation respectively. Similarly, in isolated hepatocytes, ADP-stimulated
mitochondrial respiration was reduced by incubation in either MPTP or MPP⁺
with a rapid, dose-dependent depletion of ATP which resulted in cell death
(DiMonte et al, 1986). MPP⁺ is actively accumulated by mitochondria and
concentrated 50-100x, allowing it to reach toxic levels in mitochondria while
cellular levels remain low. Ramsay (Ramsay et al, 1986) identified energy-
dependent accumulation of MPP\(^+\) in isolated rat liver mitochondria, which led to a 40-fold concentration of MPP\(^+\) from the incubation medium. The reduction of ADP-stimulated respiration by MPP\(^+\) was abolished in sonicated mitochondria, in which the mitochondrial membrane was inverted and if mitochondria were incubated in the presence of uncouplers of the mitochondrial uptake system, e.g. dinitrophenol, indicating that the effect of MPP\(^+\) was mediated by energy-dependent accumulation. A number of structural analogs of MPP\(^+\) have been investigated for their capacity to inhibit mitochondrial respiration and the oxidation of NAD\(^+\)-linked substrates. Although many compounds reduced oxidation of NADH in mitochondrial membrane preparations, only compounds which could be concentrated by the MPP\(^+\) carrier effectively inhibited respiration in intact mitochondria (Singer et al, 1988; Ramsay et al, 1989).

1.2.6 Impairment of energy metabolism and cell death

Depletion of cellular ATP by MPP\(^+\) might induce the death of nigral neurons by a number of complex and interlinked events, including glutamate excitotoxicity (for review see e.g. Flint Beal 1992). Depolarisation of neuronal membranes, following depletion of ATP could lead to NMDA receptor activation by removal of the Mg\(^{2+}\) blockade (Zeevalk et al, 1992). The activation of the NMDA receptor could lead to increased calcium entry and this effect might be augmented by reduced re-uptake of transmitter glutamate by glia (Novelli et al, 1988). Calcium buffering in the cell is ATP-dependent, thus the extrusion of excess calcium from cells or its storage in the endoplasmic reticulum could both be compromised as well as the control of calcium fluxes through the inner
mitochondrial membrane. This could lead to loss of calcium influx into mitochondria and changes in mitochondrial osmotic pressure and further mitochondrial damage.

In addition to inhibiting mitochondrial respiration, MPP⁺ may also induce oxidative stress in dopamine neurons, although no evidence of oxidative stress was seen in the isolated hepatocyte model of MPP⁺ toxicity (Di Monte et al, 1986; Ramsay et al, 1986). However, the neurotoxicity of MPTP has been shown to involve generation of hydroxyl free radicals from released dopamine (Chiueh et al, 1993) and also hydrogen peroxide and hydroxyl radicals from the interaction between MPP⁺ and NADH dehydrogenase (complex I) via the intermediate product of MPTP oxidation, MPDP (Adams et al, 1993). Increased production of superoxide ions, which are generated during normal respiration, has been shown in brain mitochondrial preparations in the presence of the complex I inhibitor, rotenone (Zoccarato et al, 1988). Inhibition of complex I disrupts the flow of electrons between mitochondrial complexes and could result in the leakage of electrons and leading to increased O₂⁻ production (Jenner et al, 1992). Mitochondrial complex I appears to be particularly susceptible to inhibition by free radicals, thus it has been suggested that a self-amplifying cycle of complex I deficiency and progressive cellular damage can be set up following the initial toxic insult (Schapira et al, 1998). The finding (Shapira et al, 1990a, 1990b) that complex I enzyme activity was decreased in nigral homogenates in PD (see below, Chapter 4) has provided strong support for the possibility that degeneration in the nigra could be related to the effects of
endogenous or exogenous neurotoxins.

1.3 Apoptosis: a possible mechanism of cell death in Parkinson’s disease

1.3.1 Programmed cell death: evidence in PD nigra

Programmed cell death (PCD) or apoptosis serves to remove redundant or damaged cells from a variety of systems including the nervous system. PCD is active in the developing nervous system and acts as a regulator of neuronal numbers in e.g. cerebellum. Apoptosis, a morphologically distinct form of PCD, is also thought to serve to remove damaged cells from the CNS and is characterised by nuclear fragmentation, clumping of chromatin, and the disintegration of the cell into apoptotic bodies, containing structurally intact intracellular organelles such as mitochondria, bound to nuclear and cellular membranes (Kerr 1972, 1987). Apoptosis is morphologically and biochemically distinct from necrotic or inflammatory cell death.

Condensation of nuclear chromatin and the formation of apoptotic bodies are held to be the definitive histological markers of apoptosis and represent the final stages of destruction of the cell (Wylie et al, 1980). Apoptotic cell death is not accompanied by any inflammatory reaction and the paucity of such necrotic changes in the substantia nigra has led to the suggestion that apoptosis may be the mechanism by which dopaminergic nigral neurons die in PD. Apoptotic cell death is preceded in many instances by fragmentation of DNA by Ca$^{2+}$ Mg$^{2+}$-dependent DNAses into 180-200 base-pair (bp) fragments, or multiples of these, with endonuclease activation occurring early in the process of cell death.
(Wylie et al, 1980; Arends et al, 1990). The characteristic “ladder” pattern which develops on gel electrophoresis of fragmented DNA is considered evidence for programmed cell death (Wylie et al, 1980). This DNA pattern of laddering on gel electrophoresis has been demonstrated in animal brain in experimental models of anoxia and disease (see e.g. Heron et al, 1993; Li et al, 1995; Nitatori et al, 1995; Portera-Cailliau et al, 1995; Charriaut-Marlangue et al, 1996).

1.3.2 Cellular changes in apoptosis

Apoptosis can be divided into three phases: an induction phase, whose precise course depends on the death-inducing signal, an effector phase during which the cellular changes become irreversible and the cell becomes committed to die and a degradation phase during which the cells acquire the biochemical and morphological features associated with apoptosis. Much attention has been directed towards understanding which part of the process is the so-called central executioner and two possible mechanisms have been identified. Mitochondrial involvement and the opening of the mitochondrial permeability transition pore has been proposed as the central mechanism for cell death (Kroemer 1995) alternatively, it has been suggested that the initiation of a cascade of the cysteine proteases (caspases) is the critical step (Martin and Green, 1995).

1.3.3 Caspase activation

Caspases are usually necessary for apoptotic cell death. These proteins are expressed in an inactive form which is activated by cleavage, usually by other caspases, during the apoptotic process. Once activated caspases rapidly cleave intracellular proteins and the inactive form of caspase-activated DNAase giving rise to the activated enzyme which is responsible for the characteristic DNA fragmentation (Enari et al, 1998). Caspase inhibitors can inhibit both developmental cell death and cell death induced by transgenic expression of death genes in C elegans and Drosophila (Raff 1998). In mammals, inhibition of caspase function can prevent cell death induced in cell lines by a number of factors including withdrawal of neurotrophic support. Nevertheless, the
commitment of the individual cell to die may not necessarily correspond to the activation of caspases since inhibition of caspases does not always prevent cell death even though it may prevent apoptosis. Caspase-independent cell death has been demonstrated in a number of models of apoptosis, including death of target cells of cytotoxic T cells (Green and Kroemer 1998).

Initiation of cell death in many instances requires the activation of so-called upstream caspases by a membrane signalling event, although other stimuli such as cellular damage may also initiate apoptosis. Pro-apoptotic signal transduction involves proteins of the tumour necrosis factor (TNF) family and can be prevented by inhibition of upstream caspases (Green and Kroemer 1998). The effector or downstream caspases are responsible for cleavage of important proteins, including actin, lamin and other substrates, which may give rise to the morphological changes seen in apoptosis and lead to cell degradation and death. In mammalian cells, apoptosis depends on the formation and action of a complex of molecules, the apoptosome, comprising cytochrome C with haem, the caspase-activating factor, Apaf 1 and caspase 9. This complex cleaves and activates caspase 3 (Li et al, 1997; Zou et al, 1997) which appears to be required for the death of neurons. In caspase 3 knock-out (CPP32-/-) mice, apoptosis has been shown to be reduced in the brain, leading to hyperplasia and the premature death of the animal. In contrast, cells of the immune system were unaffected and thymocytes remained normally responsive to apoptotic stimuli, showing that caspase 3 was not necessary for apoptotic death in these non-neural cells. These findings suggested that the activation of specific caspases might be rate-limiting
to apoptotic pathways in particular cell populations (Kuida et al, 1996).

1.3.4 The role of mitochondria in apoptosis

Recently it has become clear that compromised mitochondrial respiration can directly activate the cellular pathways which lead to apoptotic cell death. Failure of proton pumping through the inner mitochondrial membrane and increased free radical accumulation leads to changes in the permeability of the inner mitochondrial membrane (Halestrup et al, 1997, Halestrup et al, 1998). Opening and closing of inner membrane pores during Na⁺ Ca²⁺ exchange is thought to be part of the normal functioning of mitochondria and may allow mitochondria to function as excitable organelles. However, accumulation of free radicals in the mitochondrial matrix induces these pores to open in a high-conductance mode, leading to osmotic changes with mitochondrial swelling and loss of mitochondrial function. This mitochondrial permeability transition allows small molecules present in the mitochondrion as cofactors in the electron transport chain, to leak out into the cytoplasm (Zamzami et al, 1996, Kantrow et al, 1997a, Kantrow et al, 1997b). Among them, cytochrome C, the substrate for complex IV, has been shown to activate caspases (Kluck et al, 1997). MPP⁺, oxidative stress and metabolic insult have all been shown to induce apoptosis in a variety of cell systems, while this process can be blocked by agents which block the mitochondrial permeability transition pore, such as cyclosporin (Seaton et al, 1998) or which prevent the accumulation of elevated intramitochondrial calcium concentrations (Kruman et al, 1999). It is possible therefore that defective mitochondrial function in PD could activate
programmed cell death, leading to loss of nigral neurons.

The proto-oncogene Bcl-2 family are involved in the regulation of apoptosis and are closely associated with mitochondria. Bcl-2, Bcl-X<sub>L</sub>, and Bcl-2w all inhibit apoptosis; Bax, Bcl-X<sub>S</sub>, Bak etc are pro-apoptotic genes. (Shimizu et al, 1996; Kroemer 1997; Decaudin et al, 1997; Zamzami et al, 1998; Martinou et al, 1998). Bcl-2 is closely associated with mitochondrial membranes and appears to prevent signs of the opening of the permeability transition pore, such as the drop in mitochondrial membrane potential which precedes pore opening (Zamzani et al, 1995), the mitochondrial release of apoptogenic proteins (Susin et al, 1996; Kluck et al, 1997; Yang et al, 1997) and apoptosis induced by the mitochondrial hypergeneration of reactive oxygen species (Hockenbery et al, 1993; Merry and Korsmeyer 1997). Bcl-x exerts an anti-apoptotic effect by holding the caspase 3/Apaf-1 complex inactively bound to the mitochondrial membrane (Reed 1997). Bax, which is pro-apoptotic, is also bound to the mitochondrial membrane and induces the release of the apoptogenic protein, cytochrome c by association with the mitochondrial permeability transition pore (Marzo et al, 1998; Putcha et al, 1999).

It has been proposed that after activation of upstream caspases mitochondrial function becomes disrupted and, as a result, apoptotic factors, including cytochrome C are released from the mitochondrial matrix into the cytoplasm with downstream activation of caspases occurring as a result of mitochondrial involvement (Green & Kroemer 1998). The opening of the mitochondrial
permeability transition pore has been implicated in cell death in a number of cell
types and may constitute the central common point of a number of apoptotic
pathways activated by a variety of second messengers, including Ca \(^{2+}\), reactive
oxygen species and changes in cellular redox potentials, induced by the
transcription factor, p53. Mitochondria are thought, therefore, to play an
important part in determining whether the cell becomes committed to the death
process and the known compromise of mitochondrial complex I activity in the
nigra in PD (Schapira et al 1990 a, b) is strongly suggestive of an important role
for mitochondria in nigral neurodegeneration in PD and a possible link with
active processes of cell death.

1.4 Genetic factors in Parkinson's disease

It is not considered likely that the initiation of the changes which lead to Lewy
body formation, nigral degeneration and the movement and cognitive disorders
of idiopathic parkinsonism could be attributed to a single gene. Nevertheless,
there is strong evidence for inheritable factors playing a role in the development
of PD. PD is more common among the relatives of patients with the disease
than in a matched control population (for review see, e.g. Gasser 1998). In
studies of patients attending movement disorder clinics, the relative risk has
been reported variously to be increased by a factor between 2 and 14 (Marder et
al, 1996). However, combining estimates from clinic- and community-based
studies, the increased relative risk is likely to be between 2 and 3 for first degree
relatives of PD patients, a figure consistent with findings for the increased risk
among relatives of patients with Alzheimer's disease (Farrer et al, 1989). Twin
studies have generally reported low concordance rates in twin pairs but examination of clinically unaffected twins by positron emission tomography (PET) scanning has shown that subclinical nigrostriatal dysfunction is much higher among the twins of PD patients than had been evident on clinical examination (Burn et al, 1992). These findings point to a familial component in the development of PD but suggest that the underlying nigro-striatal changes may be more common than the clinically overt disease (Piccini et al, 1997). In family studies the relative risk was found to rise with the numbers of previously affected family members, a pattern characteristic of multifactorial etiology with an inheritable component (Lazzarini et al, 1994).

1.4.1 Parkin

Evidence for direct involvement of genes in the development of PD has come from a few kindreds with familial parkinsonian syndromes. Some have a phenotype compatible with a diagnosis of parkinsonism, with Lewy body deposition, others have atypical syndromes and have been described as familial parkinsonism-plus syndromes. Among the latter group, autosomal recessive juvenile Parkinsonism (AR-JP) has been ascribed to a gene locus on chromosome 6q25.2-27 and subsequently to mutations and deletions in the “parkin” gene. These individuals manifest L-DOPA-responsive parkinsonism, associated with nigral degeneration but differ from typical sporadic Parkinson’s disease in that LB are not usually present. A number of mutations and deletions in the so-called “parkin” gene have been described in AR-JP (e.g. Hattori et al, 1998; Kitada et al, 1998) and the presence of deletions is thought to result in the
formation of truncated parkin protein. In a European study, nineteen different rearrangements of exons and sixteen different point mutations in this gene were found to be associated with familial early-onset parkinsonism (49% of cases) and with isolated parkinsonism at age 20 years or younger (77% of cases) ( Lucking et al, 2000). Nevertheless, altered parkin gene expression has been not been demonstrated in studies of sporadic PD, indicating that the gene is unlikely to be implicated in the idiopathic form of the disease ( Shimura et al, 2000).

1.4.2 UCH-L1

Ubiquitin carboxyterminal hydrolase L1 (UCH-L1) is a component of the ubiquitin-proteasome pathway and is also implicated in the development of the PD phenotype. A missense mutation in the gene was identified in two affected individuals in a German family with typical late-onset Parkinson’s disease ( Leroy et al, 1998). This mutation was not observed in a large group of unaffected individuals. However, studies of other families with late-onset L-DOPA-responsive parkinsonism failed to establish UCH-L1 as a candidate gene ( Harhangi et al, 1999).

1.4.3 Alpha synuclein

Alpha synuclein is associated with autosomal dominantly-inherited PD and the gene has been mapped to chromosome 4 in humans. In one large Mediterranean (the Contursi) kindred, dominantly-inherited, early-onset, but typical, PD with Lewy body deposition, was shown to segregate with markers on
chromosome 4q21 (Polymeropoulos et al, 1996), known as the Park1 locus.

Subsequently a point mutation on exon 4 of the gene encoding alpha-synuclein, substituting threonine for alanine at position 53 (Ala53Thr) was identified by Polymeropoulos and co-workers (Polymeropoulos et al, 1997) in the Contursi and three Greek kindreds. In the Contursi kindred 85% of individuals with the genotype manifested early-onset, L-DOPA-responsive PD with nigral degeneration and Lewy body pathology while the mutation was not found in unrelated families from the same region. A second point mutation, Ala39Pro, has subsequently been identified three subjects in one German kindred (Kruger et al, 1998).

The observation that alpha synuclein was a major component of LB (Spillantini et al, 1997; Mezey et al, 1998) suggested a molecular mechanism for the pathogenesis of idiopathic PD and the development of Lewy body pathology. In familial cases with alpha-synuclein mutations, abnormalities of alpha synuclein expression and altered physico-chemical properties might predispose vulnerable neurons to the formation of insoluble aggregates and result in Lewy body formation (see below, Chapter 6). However, neither alpha-synuclein mutation has so far been found in sporadic PD cases. The Ala53T mutation gives rise to an novel restriction site for Tsp45 I, which can be used to screen DNA for the presence of the mutation. Warner and Schapira studied 70 PD cases and 100 controls for the mutation (Warner and Schapira 1998); Chan and co-workers (Chan et al, 1998a, 1998b) screened 100 young-onset patients and 25 pathologically-proven late onset cases and Zareparsi and colleagues examined
65 cases from 40 separate kindred (Zareparsi et al, 1998). The Tsp45 I restriction site could not be demonstrated in any of these studies, indicating that the mutation was not present. In addition, two studies of familial cases (Parsian et al, 1998; Jenco et al, 1998) using similar methods, were unable to demonstrate that the alpha synuclein gene was responsible for PD in these families. Linkage analysis has also failed to demonstrate positive evidence for alpha synuclein involvement in multi-case families (Gasser et al, 1997) and in several kindreds with familial PD (Vaughan et al, 1998). None of these findings rules out the possibility that other alpha synuclein mutations occur in sporadic PD but it is generally accepted that they are not involved and are a rare cause of the dominantly inherited forms of the disease.

1.4.4 Protein degradation mechanisms and PD

UCH-L1 and parkin are both components of the ubiquitin-proteasome degradation pathway. UCH-L1 is known to cleave ubiquitin polymers and to hydrolyse bonds between ubiquitin and small molecules; parkin binds ubiquitinated proteins before degradation. Studies of brain homogenates in AR-JP cases showed an absence of this protein, suggesting that the truncated form was unstable and that the loss of function associated with its absence was a primary cause of neuronal degeneration in this form of familial PD (Shimura et al, 1999). Expressed in E. coli, the mutated form of the UCH-L1 protein showed reduced enzymic activity. Genes for UCH-L1 and parkin and alpha synuclein are strongly expressed in the nigra, indicating an important role for them in the biology of dopamine neurons. It is also thought that dysfunction of the ubiquitin-
proteasome protein degradation pathway plays a part in the formation of neuronal inclusions and in cell death in PD (McNaught and Jenner, 2001).

1.5 Post-mortem studies of brain

Post-mortem studies present many technical and scientific problems but are essential to understanding the normal function of the brain and the pathophysiology of disease. The valuable insights which can be gained from experimental approaches, such as the study of animal models of neurodegenerative disease or of isolated neural cells in culture needs to be evaluated in human brain studied post-mortem. The use of molecular biological techniques makes it possible to address important questions about the role of genes in brain function and pathology, linking gene expression with the development of disease phenotype and the imperative to apply these techniques, in particular, in situ hybridization, to the study of human brain has generated a large body of research in recent years.

1.5.1 In situ hybridization histochemistry: methodological and theoretical considerations

In situ hybridization histochemistry (ISHH) permits the unequivocal identification in tissue sections of cells transcribing mRNA species of interest in neural development and function and can thus provide valuable information on molecular events in individual neurons. It is widely used for the study of gene expression in animal brain and in culture. Experimentally, it is of value in determining the response of neural cells to perturbation of their normal function.
It can be used to identify changes in gene expression following, for instance, lesion or treatment with neurotoxins in animal models of PD and the response of specific neural cell types to change in culture conditions or to toxicity. In post-mortem brain studies, in animals and human subjects, ISHH can be used to map the regional distribution of specific mRNAs or RNA subtypes, alone or in combination with immunohistochemistry (Harrison and Pearson 1990). Refinement of autoradiographic techniques and the use of computerised image analysis also allow ISHH to be used for quantitative and semi-quantitative studies in brain (Baskin et al, 1993).

1.5.2 Probes for ISHH

ISHH depends on the ability of complementary sequences of nucleotides to form stable hybrids in appropriate conditions with endogenous sequences on tissue sections. To identify mRNA in brain by ISHH, probes complementary to the sequence of interest can be derived from cloning techniques, i.e. cDNA (see e.g. Chan Palay et al, 1988; Kuljis et al, 1989; Javoy-Agid et al, 1990; Hill et al, 1993; Kastner et al, 1993; Zhang et al, 1993) or cRNA probes (riboprobes) (Terenghi et al, 1987; Albin et al, 1991), or short complementary DNA sequences, oligonucleotides (Mengod et al, 1990; Landeweermeyer et al, 1993; Uhl et al, 1994), can be synthesised. Hybridization of the probe with mRNA can be identified in tissue sections by labelling with isotopes such as $^{35}$S, $^{125}$I or $^{33}$P and subsequent autoradiography, or by incorporating molecules such as digoxigenin or biotin, to be visualised by chromogenic, fluorescent (Williams et al, 1990; Emson 1993; Kiyama 1991; Warford and Lauder 1991) or
bioluminescent reactions (Emson 1993).

The greater size of cDNA and cRNA probes greatly enhances their sensitivity, in that a larger number of labelling molecules can be incorporated during the synthesis of the probe and they are theoretically of greater value in identifying low-abundance mRNAs (e.g. Wilson et al, 1997). However, they are thought to have poorer penetration of tissue and, in the case of double stranded cDNA probes, the two strands may reanneal with one another, reducing the probe available for hybridization to mRNA in tissue. They may also produce a less specific signal than that of oligonucleotide probes and do not permit the discrimination of splice variants in which there are large sequence overlaps and may hybridize non-specifically to non-related mRNA sequences with high homology (Warford and Lauder 1991; Emson 1993; Woodroofe et al, 1994).

The use of oligonucleotide probes has a number of advantages as a routine tool for neurobiology. Advances in understanding of human and animal genomes has generated huge numbers of sequences, offering the possibility to design oligonucleotide probes to most mRNA species. The short length of oligonucleotides offers high specificity for particular gene sequences and they can be used to identify individual mRNA transcripts in a gene family in a way that the larger size of cDNA and cRNA probes may not allow. Their short length offers good penetration of tissue sections (Stahl et al, 1993) and they can be isotopically and non-isotopically labelled (Emson 1993). The disadvantage of oligonucleotide probes is that they may not produce a high enough hybridization
signal, in comparison with cDNA and cRNA probes, to identify mRNAs with very low copy numbers. This difficulty may be overcome, at least in some instances, by the use of "cocktails" of oligonucleotides, directed at different, non-overlapping sequences in the mRNA to be examined (Stahl et al, 1993).

1.5.3 Validity of ISHH for study of cellular changes

The presence of mRNA is sufficient to indicate that a gene is being transcribed and, generally speaking, the amount of mRNA present is thought to represent the rate of transcription, although this may not always hold true. Factors which affect the stability and degradation of the mRNA may alter the relationship between transcription and the amount of mRNA in the cell. The stability of beta-tubulin mRNA, for instance, is linked to the synthesis of β-tubulin peptide (Yen et al, 1988) and the mRNA has regulated instability. mRNA may not always be present with its translation product. Study of NPY expression in cortical neurons showed that in about 10% of neurons immunopositive for NPY, the corresponding mRNA could not be identified. This finding indicated a mismatch between transcription and storage of NPY in some neurons and may represent a regulatory process (Chan Palay 1988).

An important theoretical limitation of ISHH in the study of molecular events in the cell is that changes in the level of mRNA expression may not necessarily reflect changes in rates of synthesis of the encoded peptide, although this is assumed as a general principle (Harrison and Pearson 1990). In support of this principle, there is a close relationship between total brain mRNA, for instance,
and rates of protein synthesis during development. Dunlop and colleagues measured protein synthesis rates in developing rat brain tissue by incorporation of labelled amino acids and related this to total levels of mRNA to give a value for RNA activity. RNA activity remained consistent throughout development whereas levels of mRNA and protein rose during that period. These studies showed that protein synthesis rates were determined by overall RNA concentration, rather than alteration in ribosomal translation rates (Dunlop et al, 1984). However, changes in peptide synthesis may also be affected by altered efficiency of translation or from post-transcription or post-translation controls. Ideally, changes in rates of mRNA expression should be related directly to measurements of peptide synthesis in the case of each mRNA/protein relationship but accurate in situ quantitation of peptides synthesis is very difficult. Measurement of peptide levels in brain homogenates may not address the issue of peptide synthesis in specific cellular constituents of that region. In addition, peptides are frequently not found in neuronal perikarya but may be translocated by the cell to axonal or dendritic structures, making it very difficult to identify their cellular origins in tissue sections.

Parallel changes in mRNA expression and peptide synthesis have nevertheless been demonstrated in brain and neural tissue and in culture. Experimental studies of neuronal cytoskeletal protein expression demonstrate a close correspondence between the expression of mRNA and protein. Davis and colleagues examined the migration of newly synthesised [\(^3^H\)RNA into dendrites of cultured hippocampal neurons following pulse-chase labelling with
[3H]uridine for periods up to 12 hours. With increasing time, radioactive RNA was found further along dendritic processes. Immunostaining for the neuron and dendrite-specific microtubule-associated protein MAP2 combined with autoradiography, showed that only radiolabelled dendrites were MAP2 immunopositive (Davis et al, 1987). This finding showed that a close temporal relationship existed between the transport of RNA into dendrites and dendritic expression of peptide. Studies of dorsal root ganglion axons, stimulated to regenerate by axonal crush, showed that following crush axotomy, there was a reduction of mRNA encoding low-molecular weight neurofilament (NF) protein and increase in β-tubulin mRNA (Oblinger et al, 1989). Parallel changes in NF protein in crushed axons had been demonstrated in an earlier study, in which a fall in the amount of neurofilament protein entering the cytoskeleton followed crush injury and lower incorporation of methionine into NF protein was observed (Oblinger et al, 1988). Taken together, these findings suggested that changes in NF protein synthesis followed that of the mRNA.

Induction of tyrosine hydroxylase mRNA, peptide and enzyme activity in the adrenal medulla of aged rats was studied following forskolin treatment (Tumer et al, 1997). A single dose of forskolin stimulated mRNA expression while more prolonged treatment induced parallel increases in tyrosine hydroxylase mRNA, tyrosine hydroxylase immunoreactivity and enzyme activity, indicating that for the TH gene, induction of transcription was correlated with peptide expression.
Some correspondence has been observed in human brain between mRNA levels and protein expression. In a study of Alzheimer’s disease cortex, a decrease in both total and polyadenylated RNA was observed in regions showing high concentrations of neuritic plaques and neurofibrillary tangles. This decrease was partly due to an abnormality of the alkaline ribonuclease activity but was accompanied by decreased protein synthesis in the affected regions of the brain (Sajdel-Sulkowska and Marotta 1984). In contrast, studies of synaptophysin in the temporal cortex in schizophrenia showed that synaptophysin mRNA was reduced, both at the regional and the cellular level, in CA4, CA3 neurons, subiculum and parahippocampal gyrus. Synaptophysin immunoreactivity, however, did not show any significant difference in any of the subregions of the temporal cortex (Eastwood et al, 1995). In nigral neurons a close correlation was observed between tyrosine hydroxylase mRNA expression in emulsion autoradiographs and TH immunoreactivity in a group of cases comprising both PD and control subjects (Kastner et al, 1993). Similarly, separate studies demonstrated reduction in neurofilament mRNA expression in individual substantia nigra pars compacta neurons in Parkinson’s disease, compared to control cases (Hill et al, 1993) and in immunoreactivity for neurofilament protein (Gai et al, 1994). The increase in PE mRNA demonstrated in human striatum in PD (Nisbet et al, 1995) paralleled an increase in enkephalin immunoreactivity demonstrated in rat striatum following lesion with 6-OHDA (Voorn et al, 1987) and in the cynomolgus monkey striatum following lesion with MPTP (Lavoie et al, 1991). These findings suggested a parallel between mRNA and peptide expression in striatal neurons and supported the suitability
of ISHH studies to elucidate changes in gene expression in neurodegenerative disease.

1.5.4 Examination of mRNA expression in animal models of PD

ISHH has been used extensively to study the molecular effects of altered dopamine transmission on gene expression in the striatum in rat and primate models of PD. For instance, W Scott-Young and colleagues, who pioneered the use of oligonucleotide ISHH for experimental studies of rat brain, used it to measure changes in striatal neuropeptide (proenkephalin and substance P) mRNA expression in rats following unilateral 6-OHDA lesion of midbrain dopamine neurons (Scott-Young et al, 1986). Williams and colleagues, using the methods of Young, used the dopamine antagonist haloperidol to reduce dopamine transmission in the basal ganglia and demonstrated an increase in striatal neurotensin mRNA (Williams et al, 1990). Similarly, increased expression of enkephalin, and decreased expression of substance P and pro-somatostatin mRNAs were detected in striatum using ISHH following manipulation of striatal dopamine levels with 6-hydroxydopamine (Nisenbaum et al, 1992) or with dopamine receptor antagonists (Augood et al, 1991; Gerfen et al, 1991). ISHH was also used to demonstrate that D1 and D2 receptors were present on different neuronal populations in striatum while double-labelling methods demonstrated that D2 receptors were restricted to enkephalin-positive striatal neurons (Gerfen et al, 1995). Similarly, ISHH was used to study the response of striatal neurons to lesion of the nigrostriatal pathway. Induction of immediate early genes was shown to follow unilateral dopamine depletion and
challenge with dopamine receptor agonist. The high levels Arc, zif268 and c-fos mRNAs expressed in lesioned striatum in comparison with the un-lesioned side, was evidence for a supersensitive response of dopamine receptor-bearing neurons to the ablation of dopamine input (Gerfen et al, 1995; Gerfen 2000).

1.5.5 Human brain ISHH studies

*In situ* hybridization histochemistry (ISHH) is now applied commonly to the examination of gene expression in human brain. It permits the study of the molecular neuroanatomy of the brain, while quantitative studies of mRNA expression provide a means to examine changes in gene expression which may be associated with the development of disease phenotype (for review see e.g. Harrison and Pearson, 1990). In addition to its advantages of anatomical precision and high sensitivity for the study of the brain, ISHH enables cellular changes to be investigated in brain regions in which extensive cell loss is a feature of disease. In the substantia nigra in PD, or the cerebral cortex in Alzheimer’s disease, measurement of regional changes in expression of many molecules may be confounded by reduction in cell numbers. Examination of mRNA expression in individual cells can overcome this difficulty and can dissect which changes precede cell loss and which are consequent upon it, as can comparisons between disease-affected and non-affected brain regions.

In addition, ISHH can be used to clarify any relationship between the neuropathological signs of disease and gene expression in affected regions. For instance, ISHH used to map the distribution of amyloid-beta-protein precursor
mRNA in control and Alzheimer’s disease (AD) cortex, showed that positive neurons were present in control cortex in regions in which the highest densities of neuropathological markers, neuritic plaques and neurofibrillary tangles, were seen in AD. However, high numbers of neurofibrillary tangles were associated in Alzheimer’s disease with a reduction of the numbers of positively hybridizing neurons, with a smaller reduction in cases with fewer tangles, indicating that expression of the precursor mRNA was a necessary but not sufficient condition for tangle formation (Lewis et al, 1988).

1.5.6 ISHH studies of basal ganglia

ISHH studies have provided much information on the phenotype of substantia nigra pars compacta neurons, in particular, gene expression related to dopamine transmission and have been used to gain insight into the selective vulnerability of nigral neurons in Parkinson’s disease. mRNA encoding the rate-limiting dopamine-synthetic enzyme tyrosine hydroxylase (Javoy-Agid et al, 1990; Kastner et al, 1993), as well as dopamine transporter and the synaptic vesicle amine transporter have been demonstrated in melanised nigral neurons (Bannon et al, 1992; Harrington et al, 1996) and have been shown to be strongly, though variably expressed in both melanised and non-melanised cells. Nigral neurons have also been shown to express mRNA encoding NMDA receptors (Counihan et al, 1998), and receptor mRNA for neuropeptides, such as opioids and neurotensin (Yamada et al, 1995, 1997) have been demonstrated. To elucidate the possible involvement of glutamate-mediated neurotoxicity in Parkinson’s disease, Counihan and colleagues studied expression of NMDA receptor subunit
mRNA. These were shown to be strongly expressed in nigral neurons, especially NR1 and NR2D subunits, although they found no relationship between the pattern of nigral neuronal degeneration and NMDA receptor mRNA and no differential expression of receptor subunits among substantia nigra (A9) neurons in comparison with other populations (Counihan et al, 1998). High-affinity uptake systems and enzymatic transformation are mechanisms by which the potential toxicity of glutamate to neurons is controlled. ISHH studies also demonstrated that nigral neurons express high levels of the glutamate transporter EAAT3 mRNA, suggesting that these neurons might be vulnerable to excitotoxic mechanisms and adapted to resist them (Plaitakis et al, 2000).

Following the findings in animal studies, that ablation of nigro-striatal dopamine transmission produced an increase in striatal expression of pro-enkephalin mRNA and a reduction in substance P mRNA in medium spiny neurons, Nisbet and colleagues measured these mRNAs in striatal medium spiny neurons, by ISHH. In Parkinson's disease, pro-enkephalin mRNA expression was increased in the caudate and in intermediolateral putamen in comparison with controls, in parallel with the changes in the animal model. In contrast there was no reduction of expression of substance P mRNA in human striatum. Although the model was not precisely parallel, these findings demonstrated the relevance of the animal models for study of changes in gene expression in PD (Nisbet et al, 1995).
1.5.7 Limitations of post-mortem brain studies

Application of ISHH to study of human post-mortem brain depends on the preservation of mRNA post-mortem, which is known to be highly variable between individuals. The factors contributing to this variability and to RNA degradation, are likely to be numerous and theoretically include ante-mortem changes, post-mortem delay, between death and preservation of tissue, and differential susceptibility of different mRNA species to terminal events. A number of molecules are known to be highly labile in brain and it is possible that post-mortem delay could contribute to a reduction in expression of, e.g. catecholamines, suggesting that this factor might be an important confounding variable in human studies. Noradrenaline and nor-metanephrine concentrations in brain, as well as P-creatine and ATP have been reported to be strongly influenced by the duration of post-mortem delay (Ravid 1992) The presence of pathology in neuronal populations may also predispose them to peri-mortem degradation of their molecular constituents, making it difficult to be certain that changes observed in mRNA expression in pathological states represent primary pathological events or simply indicate that the cells observed are moribund. All studies of human brain in chronic disease states raise this same difficulty of interpretation. However, parallel studies of expression of several genes, or of housekeeping genes, or of affected and unaffected cell populations all offer some insight into the effect of pathology on gene expression.

The possibility that different mRNA species could be variably preserved post-mortem also poses a theoretical limit to the reliability of ISHH studies. Post-
mortem preservation of RNA and mRNA have been extensively studied by denaturing gel electrophoresis and *in vitro* translation techniques. Early studies reported variable yields of RNA from extracts of human brain but established that 28S and 18S ribosomal RNAs were present, although there was some selective degradation of the larger RNA, and that many transcripts of mRNA were translationally active *in vitro*. Northern hybridization demonstrated relatively intact mRNA for a number of species, including many average-sized and small-sized molecules, although some random degradation to lower molecular weight products was observed. This change did not necessarily correlate with loss of rRNA, demonstrating that despite overall degradative changes, many mRNA species had relatively good post-mortem preservation (for review see Morrison 1988). Similarly, reverse-transcribed cDNAs derived from RNA isolated from human cortex showed that these were the same size profile as a cDNA transcribed from a rat cortex template. This and similar studies have demonstrated that, in spite of some inter-individual variation, much mRNA extracted from brain post-mortem is un-degraded and in its original poly-adenylated form (Morrison 1988).

1.5.8 Peri-mortem factors

Nevertheless, 40-50% of brains obtained at autopsy in early studies of Alzheimer’s disease were found to have greatly reduced levels of intact mRNA and to be unsuitable for molecular biology studies (Morrison 1988). Improved brain retrieval protocols, minimising both post-mortem delay and possible pre-mortem hypoxia increased the proportion of brains which could be used for
molecular biological studies of Alzheimer’s disease. This supported the view that among the important factors influencing post-mortem mRNA preservation was the degree and duration of ante-mortem hypoxia. Harrison and colleagues found a strong negative correlation between duration of terminal coma and expression of mRNA encoding the muscarinic M1 receptor as well as the activity of glutamic acid decarboxylase (GAD) in temporal cortex homogenates from Alzheimer’s disease brain (Harrison et al, 1991). Burke and colleagues studied mRNA encoding phenylethanolamine N-methyltransferase (PNMT), β-actin and three amyloid precursor protein (APP) transcripts in neurons from the ventral medulla and demonstrated variable sensitivity of the mRNA species studied to pre- and post-mortem factors. Levels of PNMT mRNA were strongly negatively correlated with hypoxia, identified by blood gas measurements, while two APP transcripts and β-actin were affected by the interval between death and freezing and storage times (Burke et al, 1991) suggesting that different mRNA species might be variably affected by ante- and post-mortem variables.

Experimental findings in primate brain and observations on human brain supported the importance of agonal status and ante-mortem events to preservation of brain molecules. Middle cerebral artery occlusion in baboons was used to determine the effects of anoxia on the activity of GAD, uptake of GABA into synaptosomes and the expression of proteins. In ischaemic cortex, GABA uptake was reduced by more than 50% in comparison with unaffected cortex and GAD activity was reduced by about 25%, demonstrating the sensitivity of these markers to cerebral anoxia and suggesting that reduced brain
oxygenation in patients dying in coma might account for the reduction in GAD activity observed in the cortex of these individuals (Bowen et al, 1976). Similar effects have been observed on enzyme systems in human brain. A comparison between neurologically normal subjects dying following an agonal terminal phase and those dying suddenly demonstrated a correlation between a severe terminal phase and tissue pH. The activity of the metabolic enzyme, phosphofructokinase, the concentration of several amino acids and neurotensin, and the activity of GAD were all influenced by severity of terminal phase (Perry et al, 1982). Yates and colleagues measured lactate concentrations, as an index of hypoxia, and pH values in agonal and sudden death controls. Lactate concentrations in the agonal controls were higher, and pH values correspondingly lower, in the frontal cortex and caudate nucleus, whereas higher pH values and lower lactate concentrations were observed in the same regions of controls dying suddenly. Lactate concentrations and tissue pH were significantly correlated in both brain regions. The activities of GAD, phosphate-activated glutaminase (PAG), synthetic enzymes of GABA and glutamate respectively, were both found to be influenced by pH and lactate concentrations in tissue (Yates et al, 1990). GAD activity had also previously been shown to be negatively correlated with the duration of ante-mortem hypoxia (Reinikainen et al, 1988).

1.5.9 Lactate acidosis

The value for post-mortem brain tissue pH is strongly influenced by lactic acidosis, arising from ante-mortem hypoxia and anaerobic cellular respiration.
The titration curve produced by adding lactic acid to mouse brain showed that the reduction in pH seen in human brains post mortem could be largely accounted for by the presence of accumulated lactate (Hardy et al, 1985). Concentrations of lactate in human brain (up to 25 mM), however, were higher than those measured in mouse brain immediately after decapitation (12-16 mM). The immediate post-mortem rise in lactate concentration in mouse reflected the anaerobic metabolism of glucose and glycogen stores but the difference between the human and mouse values indicated that much of the accumulated lactate in human brain resulted from anaerobic metabolism ante-mortem. It was thought possible that there was no post-mortem contribution to lactate concentration in humans and that lactate levels reflected the rise due ante-mortem anaerobic glycolysis. Depletion of glucose and glycogen stores ante-mortem thus could prevent the post-mortem rise seen in experimental animals (Hardy et al, 1985).

Hardy and colleagues measured tissue pH in brain tissue homogenised in water from a series of cases classified into four groups: i) violent fast death, due to shooting or blunt trauma  ii) fast death, sudden death due to natural causes such as myocardial infarction iii) intermediate death, of individuals who were ill but not expected to die and iv) slow death, death with a protracted terminal phase. Brain pH was significantly higher in groups i) and ii) confirming that long terminal illness is associated with brain acidosis. pH values were unaffected by post-mortem delay, age or other factors and correlated well with pH values in blood and CSF. pH values for the medulla correlated highly with values for the cortex, suggesting that change in pH occurred over the whole brain. The authors
concluded that brain pH reflected ante-mortem hypoxia and could be used as a biochemical index for agonal state (Hardy et al, 1985). Similarly, measurement of brain pH, whether by CSF or homogenised tissue has been suggested as routine assessment of agonal state for all brain banks (Ravid 1992).

1.5.10 Post-mortem interval and preservation of RNA

No correlation was found in Hardy’s or other studies between brain pH, lactate concentrations or any of the biochemical indices examined and the duration of post-mortem delay (Hardy et al, 1985; Perry et al, 1982; Yates et al, 1990). In a series of 14 studies reviewed by Barton and colleagues, of total and polyadenylated mRNA extracted from post-mortem brain, there was no decline in RNA value with increasing post-mortem interval. Similarly, a study of polyadenylated mRNA in Alzheimer’s disease and control visual cortex showed that there was little effect of post-mortem interval up to 90 hours post mortem and none at all in the first 36 hours (Harrison et al, 1991). Other studies of specific mRNAs, have reported that there was no effect of post-mortem interval on the expression of a variety of transcripts in brain. In general, it has been agreed that post-mortem interval is less important than agonal state in determining post-mortem preservation of many molecules (for review see Barton et al, 1993).

1.6 Rationale for the present studies

The studies described below have been directed towards the application of ISHH to the study of gene expression in Parkinson’s disease, using the unique
collection of well-characterised human brain available at the Queen Square Brain Bank (formerly the Parkinson's Disease Society Brain Bank). The aims were:

i) to identify appropriate preservation protocols for the use of ISHH on human brain which would not preclude its use for other research techniques,

ii) to overcome, where possible, the difficulties inherent in the study of post-mortem tissue - individual variability and the confounding effects of perimortem factors.

iii) to develop protocols which would permit the routine use of semi-quantitative ISHH and to use these for comparison of mRNA expression between PD and control material

Oligonucleotide probes were chosen for their specificity and were used to map gene expression and measure changes in PD cases. The results presented here are all based on the development of the routine protocols and the approach to matching case-control studies described in Chapter 2.
CHAPTER 2

DEVELOPMENT OF A PROTOCOL FOR ROUTINE SEMI-QUANTITATIVE IN SITU HYBRIDIZATION HISTOCHEMISTRY ON HUMAN BRAIN

This section describes the development of protocols for the routine use of in situ hybridization histochemistry to permit the study of gene expression in human post-mortem brain and the use of semi-quantitative techniques for the examination of changes in gene expression in Parkinson’s disease. The assessment of tissue pH, a marker for ante-mortem hypoxia, as an indicator of post-mortem mRNA preservation and studies of the long-term stability of brain RNA are also described.

The results of the methodological study and the examination of tissue pH have been published (see Published papers and abstracts arising from this thesis, Appendix 1).

2.1 Introduction

2.1.1 Optimisation of ISHH protocols

There is a bewildering variety of published protocols for ISHH in studies of brain based largely on the methods of Lewis (Lewis et al, 1985) or Young (Scott-Young et al, 1986) and employing the different probe types described above. Human brain tissue for ISHH has been preserved by immersion in formalin, either long-term (Kuljis et al, 1989; Hill et al, 1993; Bergeron et al, 1994) or short-term, followed by cryoprotection in sucrose and rapid freezing (e.g. Terenghi et al, 1987; Chan Palay 1988; Javoy-Agid et al, 1990; Bannon et al, 1992; Kastner et
al, 1993; Zhang et al, 1993; Uhl et al, 1994) or by rapid freezing alone (Brene et al, 1989; Albin et al, 1991; Strada et al, 1992; Landewehrmeier et al, 1993). A large number of preparative techniques are described for tissue sections, including treatment with proteinases, (e.g. Kuljis et al, 1989; Bannon et al, 1992: Bergeron et al, 1994; Uhl et al, 1994) detergents, (e.g. Kuljis et al, 1989; Hill et al, 1993) acetylating (Young et al, 1986; Albin et al, 1991; Bannon et al, 1992; Bergeron et al, 1994) and delipidating (Young et al, 1986; Brene et al, 1989) reagents often with a "pre-hybridization" step (Javoy-Agid et al, 1990; Bannon et al, 1992; Hill et al, 1993), where tissue is equilibrated with hybridization buffer before the addition of probe. Nevertheless, development of ISHH protocols for animal brain (Wisden and Morris, 1994) and cell cultures (Singer et al, 1987) have demonstrated that ISHH protocols employing oligonucleotide probes can be simplified to make routine use of the technique feasible; little comparable study has been directed to simplifying ISHH protocols for use on human brain.

2.1.2 Preservation of human tissue post-mortem

Tissue preservation techniques are critical to successful hybridization on human tissue sections. Good histology is essential, since without it the anatomical specificity of ISHH is lost. Storage methods, also, must ensure the long-term stability of tissue morphology and mRNA, while fixation techniques must be compatible with RNA retention in situ.

Immersion of fresh tissue in 10% formalin is the method most commonly employed for storing human brain for neuropathological examination, with subsequent preparation of wax-embedded blocks for histological
evaluation and there is a wealth of such material for study. However, preliminary studies using oligonucleotide probes showed that this material gave inconsistent ISHH results with low levels of hybridization signal which were insufficiently clear or reliable for quantitation. In contrast, those obtained with rapidly-frozen tissue, a preservation method commonly employed for ISHH studies of animal CNS but less commonly for human studies (Brene et al., 1989; Albin et al., 1991; Landwehrmeyer et al., 1993), were more consistent and reproducible and gave higher hybridization signal. In general, frozen tissue has poorer histological preservation than tissue fixed in formalin (Harrison and Pearson 1990). Nevertheless, rapid freezing of human brain offers practical advantages for tissue collection and preservation and rapidly-frozen tissue is suitable for a wider range of research approaches than tissue preserved for long periods in formalin.

2.1.3 Matching of cases for quantitative studies

Evidence from both human and experimental studies indicates that mRNA and peptides vary somewhat in their response to the effects of a number of peri-mortem variables. Of these, agonal status is the most important determinant of preservation of molecules post-mortem. In the absence of detailed clinical information, sufficient to determine the severity of the agonal phase, measurement of brain pH offers the best indicator presently available for agonal status.
2.1.4 Long-term stability of mRNA

There is relatively little published data from studies of the long-term stability of brain mRNA in frozen tissue. Human brain tissue, in particular control brain, is often difficult to obtain, and it may take time to build up sufficient material for study. The long-term stability of mRNA in frozen human brain is an important issue if case-control study groups cannot easily be matched closely for storage time of tissue. Harrison and colleagues in a study of the relative importance of pre-and post-mortem factors to mRNA preservation, reported that measurement of the mRNA species they studied was unaffected by storage time up to 33 months (Harrison et al, 1995). Mengod and colleagues (Mengod et al, 1992) demonstrated that hypothalamic neuropeptide mRNAs measured by quantitative ISHH in frozen sections were comparable in recently obtained tissue and in tissue stored for up to 72 months. Similarly, Leonard and colleagues also compared β-actin mRNA expression, measured by northern hybridization in frozen brain tissue stored for short periods (weeks) with tissue stored up to 60 months before analysis and showed it to be quantitatively unchanged (Leonard et al, 1993).

2.2 Study design

Rapidly-frozen human brain was assessed for its histological preservation and its suitability for ISHH studies. The results were broadly compared the those obtained with formalin-fixed tissue, using microscopy and semi-quantitative analysis of film and emulsion autoradiographs. A number of
commonly-used procedures in published ISHH protocols were evaluated with respect to their benefits, their effects on histological preservation and their applicability to the combined use of ISHH and immunohistochemistry. These studies were directed towards developing a simplified protocol for routine use of ISHH to study gene expression in human neurodegenerative disease.

The value of measurement of pH in brain homogenates as an index of mRNA preservation was also studied. Tissue pH was measured in homogenates of cerebellum in a large series of cases. pH values were examined with respect to cause of death and other peri-mortem factors. Northern analysis was used to examine the relationship between brain pH and the integrity of mRNA extracted from tissue. Quantitative ISHH examination was performed with two probes, to fructose-1-6-bisphosphate aldolase (aldolase-C) and β-tubulin mRNA, and the relationship between ISHH signal and pH was determined in film and emulsion autoradiographs.

The influence of freezing temperature and length of storage on the stability of mRNA were assessed using northern hybridization and ISHH. The profiles of extracted RNA were examined on northern blots and were compared in tissue stored for varying periods of time and between tissue samples stored after freezing at -70 °C and at -20 °C. Transfer membranes were also probed with a [35S]-labelled probe to
mitochondrially-encoded ND1 mRNA (see below, Chapter 4), to examine the integrity of this mRNA following freezing and prolonged storage.

The results from hybridization of nigral neurons with probes to TH, aldolase C, β-tubulin and the nuclear encoded NDUFV1 subunit of mitochondrial complex I (see below, Chapter 4) as well as hybridization of cerebellar Purkinje neurons with a probe to the ND1 subunit of mitochondrial complex I were examined by regression analysis for a relationship between hybridization signal and months in freezer storage at -70 °C.

2.3 Materials and methods

2.3.1 Tissue

Human brains were obtained through the Parkinson's Disease Society Brain Research Centre donor bequest scheme or following routine hospital post-mortem. Additional control samples were obtained from the Medical Research Council Alzheimer’s Disease Brain Bank, Institute of Psychiatry, London. Brains were collected within 50 hours of death for these studies and the fresh brain was transported to the laboratory in a cooled container. Brains were divided mid-sagittally. The half intended for rapid freezing, was dissected into blocks or coronal slices, about 1 cm thick, coronally for the cerebral hemispheres, para-sagittally for the cerebellum and perpendicular to the long axis for the brain stem. The
remaining half was fixed in 10% formalin for six weeks for before the preparation of wax-embedded sections for neuropathological evaluation.

Tissue blocks and slices were rapidly frozen from the fresh state by being apposed to polished brass plates, pre-cooled to -70°C (Kanazawa 1983); tissue blocks were then covered with aluminium foil and a thin layer of granular dry ice and the covered brass plates were returned to the freezer at -70°C for up to 30 minutes to ensure complete freezing. Once frozen, tissue blocks were removed from the brass plates into self-sealing plastic bags and were stored at -70°C before use.

Cerebellar tissue pH was measured in samples of the cerebellar hemisphere lateral to the dentate nucleus. The cerebellum was chosen for pH determination because it is not thought to be affected in Parkinson’s disease and because the structure of the cerebellar folia made it relatively easy to ensure consistent proportions of white and grey matter in the samples taken. Tissue samples were homogenised from the fresh or frozen state in 10 volumes of distilled water at neutral pH and pH values were determined in duplicate and at room temperature using a standard Corning electrode.

2.3.2 Neuropathology

In the studies reported here, all cases underwent full neuropathological evaluation (Hughes et al, 1992). The brain was examined by eye and
microscopically. Representative sections were cut from tissue blocks comprising the midbrain, pons, cerebellum, striatum, basal forebrain and cortex. Sections were stained with haematoxylin and eosin, Luxol Fast Blue B, Bielshowsky silver impregnation and immunohistochemistry for ubiquitin, tau, glial fibrillary acidic protein (GFAP), β-amyloid and alpha synuclein. All PD cases had marked depigmentation of the midbrain and dorsal pons, with cell loss evident in the substantia nigra pars compacta and locus ceruleus. Lewy bodies, identified by ubiquitin immunohistochemistry, were evident in surviving neurons of the nigra, in the locus ceruleus, in the brain stem, frontal, temporal and insular regions of the cortex and in the basal forebrain. Multiple system atrophy (MSA) was identified by the presence of glial cytoplasmic inclusion bodies in the striatum and other regions. Progressive supranuclear palsy (PSP) was identified by the presence of widespread intraneuronal globose tangle formation, visualised by tau immunohistochemistry. Control brains were similarly screened to exclude any undiagnosed neuropathology.

2.3.3 Preparartion of tissue sections

Glass slides were pre-coated twice by immersion in 0.25% gelatin, 0.025% chrome alum in RNAase-free (i.e. DEPC-treated) water (Young et al, 1986). 12 μm cryostat sections were cut from frozen blocks (allowed to warm to between -12 and -15 °C) from the regions to be examined and were thaw-mounted onto gelled slides and briefly air-dried.
Once dry, tissue sections were stored at -70°C before use in containers double-wrapped in self-sealing plastic bags, to prevent dehydration.

Tissue sections (12 μm) from formalin-fixed, wax-embedded material, used for comparison, were cut from wax blocks in RNAase-free conditions and mounted on pre-coated slides, by drying on a hotplate at 40 °C for 30 minutes. Slides for examination were stored at room temperature.

2.3.4 Cases

Tissue sections were selected for comparison between different protocols, either at random from available material (flash-frozen or formalin fixed and wax-embedded), using groups of four to eight subjects for each comparison, or, where comparisons were made directly on the same individual or individuals, using duplicate serial sections from subjects known to have good mRNA preservation (see below). Post-mortem details of these subjects examined are shown in Table 2.1.

For studies of brain pH, a series comprising 52 individuals, 20 with PD or other parkinsonian syndromes and 31 control subjects, aged between 62 and 88 (mean 75.8, SD 8.6) years of age. Post-mortem delay in this group was up to 51 hours; material with a longer interval was not included in the
study. Clinical details are listed in Table 2.2.

For studies of mRNA stability during freezing and long-term storage, blocks from the cortex and cerebellum from two individuals, one control, one PSP were frozen at -20 °C, in addition to the normal freezing procedure and were both stored for a period of approximately 36 months at -70 °C. RNA was extracted from blocks frozen at -20 °C and parallel samples frozen at -70 °C, for comparison by northern analysis. In order to examine the long-term addition stability of mRNA, individuals were selected so as to compare long, medium and short duration of storage following routine freezing at -70 °C. RNA was extracted from 4 control and 4 PD samples of pons, with storage times between 2 and 9 years and from 6 control and 6 PD samples cerebellum stored for periods between 2 and 9 years. RNA extractions were performed at the same time for each group.

2.3.5 Preparative steps for in situ hybridization

All solutions were prepared in baked, RNAase-free glassware and sterile plastic using sterile or diethylpyrocarbonate (DEPC)-treated water. In the simplified protocol, unfixed, frozen tissue sections were allowed to warm to room temperature (10 minutes), fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 154 mM sodium chloride, 0.89 mM potassium dihydrogen orthophosphate, 5.7 mM di-sodium hydrogen orthophosphate) pH 7.4 for 5 minutes at room temperature
Table 2.1

Clinical and post-mortem details of cases used for quantitative assessment of preparative protocols.

<table>
<thead>
<tr>
<th>Case</th>
<th>age (years)</th>
<th>sex</th>
<th>post-mortem delay (hours)</th>
<th>brain tissue pH</th>
<th>neuropathological evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>m</td>
<td>33</td>
<td>6.79</td>
<td>control</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>m</td>
<td>16</td>
<td>6.71</td>
<td>control</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>m</td>
<td>17</td>
<td>6.74</td>
<td>control</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>f</td>
<td>18</td>
<td>6.86</td>
<td>control</td>
</tr>
<tr>
<td>5</td>
<td>77</td>
<td>m</td>
<td>27.5</td>
<td>6.49</td>
<td>control</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>f</td>
<td>18</td>
<td>6.7</td>
<td>control</td>
</tr>
<tr>
<td>7</td>
<td>67</td>
<td>m</td>
<td>22</td>
<td>6.55</td>
<td>control</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
<td>m</td>
<td>5.5</td>
<td>6.51</td>
<td>control</td>
</tr>
</tbody>
</table>

Cases used for assessment were selected for good mRNA preservation, on the basis of tissue pH.
Table 2.2
Clinical data and post-mortem variables in cases used for assessment of pH and mRNA preservation

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>PMD (hours)</th>
<th>Cause of death</th>
<th>pathology</th>
<th>pH value</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
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<td>PD</td>
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<td>P2</td>
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<td>81</td>
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<td>bronchopneumonia</td>
<td>Pick's disease</td>
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<td>P3</td>
<td>M</td>
<td>82</td>
<td>15</td>
<td>bronchopneumonia, ca lung</td>
<td>PD/CVD</td>
<td>6.09</td>
</tr>
<tr>
<td>P4</td>
<td>F</td>
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<td>35</td>
<td>bronchopneumonia</td>
<td>MSA</td>
<td>6.14</td>
</tr>
<tr>
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<td>M</td>
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<td>24</td>
<td>bronchopneumonia</td>
<td>PD</td>
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<td>advanced PD</td>
<td>PD</td>
<td>6.25</td>
</tr>
<tr>
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<td>30</td>
<td>MI/ischaemic heart disease</td>
<td>PD</td>
<td>6.3</td>
</tr>
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<td>bronchopneumonia</td>
<td>PD</td>
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<td>bronchopneumonia</td>
<td>PD</td>
<td>6.34</td>
</tr>
<tr>
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<td>20</td>
<td>bronchopneumonia</td>
<td>PD/CVD</td>
<td>6.35</td>
</tr>
<tr>
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<td>MI</td>
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<tr>
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<td>CVD</td>
<td>6.4</td>
</tr>
<tr>
<td>P15</td>
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<td>unknown</td>
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Control cases

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Abbreviations: COAD, chronic obstructive airways disease; CVD, cerebrovascular disease; PD, idiopathic Parkinson’s disease; MI myocardial infarction; PMD, post-mortem delay
(paraformaldehyde post-fixation), washed twice in PBS and once in physiological saline, dehydrated through graded ethanol solutions of 70%, 80% and 95% in DEPC-treated water and allowed to air-dry prior to hybridization. The total time required to prepare slides for hybridization was between 20 and 25 min.

For examination of the effects of glutaraldehyde fixation and to assess the usefulness of flash-frozen tissue for immunohistochemistry, sections were fixed in 4% paraformaldehyde in PBS, containing glutaraldehyde at concentrations between 0.1 and 1.0%, for 5 minutes on ice. Evaluation of prefixation was carried out on small blocks of tissue fixed in 4% buffered paraformaldehyde at pH 7.4 for 24-48 hours, depending on the size of the block, and then equilibrated with graded sucrose solutions: 10, 15 and 25% and then rapidly frozen, as described above. Solutions for pre-fixation were prepared in RNAase-free conditions. Glutaraldehyde-fixed or pre-fixed sections were processed for ISHH in the usual way.

2.3.6 Oligonucleotide probes

The following oligonucleotides:

i) GACACTTTTCTTGGGAACCAGGGGACCTT, complementary to bases 576 to 605 of the human sequence encoding tyrosine hydroxylase (TH) (Nagatsu, 1991)

ii) GAACATGGGACGTGAATGCTCCGAGATGCG, complementary to bases 2740 to 2769 of the human sequence encoding beta-tubulin mRNA (β-tubulin) (Lee et al, 1984):
iii) CGACGCAGGGCAGTGACAGTTGCCATGGC, complementary to bases 2837-2866 encoding human fructose-1-6-bisphosphate aldolase (aldolase-C) (Rottman et al, 1987) were used for these studies. Probes were obtained from the Medical Research Council, National Institute for Medical Research, London, Symbicon or Oswel DNA Services (Southampton, UK). Antisense sequences of TH, β-tubulin and aldolase-C were examined for homology with unrelated mRNA sequences using the NCBI BLAST e-mail server (Altschul et al, 1990). The aldolase C probe recognised rat, mouse, human and rabbit sequences of aldolase C; the β-tubulin probe similarly, recognised β-tubulin sequences from a number of species, but no other sequences with high homology. The TH probe recognised the full-length transcript of the human TH gene and had homology with rodent sequences.

For storage studies, a 40 base probe complementary to bases 3454 to 3495 of the human mitochondrial sequence encoding the ND1 sub-unit of mitochondrial complex I was additionally employed. Details of the characteristics of this probe are described below (Chapter 4).

Oligonucleotides were 3' end-labelled with $[^{35}S]$ deoxyadenosine 5'-(α-thio)-triphosphate (1500 Ci/mmol, NEN, DuPont, Europe) using terminal deoxynucleotide transferase (Promega Corporation) at 500-1000 units/ml in cacodylate buffer at 37°C for 45 - 60 minutes, following the manufacturer's protocol. Reactions were stopped by either by addition of
10 mM triethylamine in 100 mM Tris-HCl, 1 mM EDTA, pH 7.7 (Buffer A) or 10 mM Tris 1 mM EDTA buffer (TE) at pH 8.0. Unincorporated bases were separated from the labelled probe on "NENsorb" (NEN, Dupont) columns or on G50 Sephadex. NENsorb columns were used following the manufacturer's protocol. The reaction mixture was applied to the column in Buffer A, the column washed twice in Buffer A and radiolabelled nucleotides were recovered by elution in 50% ethanol in sterile water. Alternatively, G50 Sephadex was equilibrated in TE pH 8.0 and used to prepare columns 2 ml by volume in 2.5 ml sterile syringes, plugged with baked glass wool. The reaction mixture in TE pH 8.0 was applied to the column and the radiolabelled probe collected in TE in the first two fractions after the void volume of the column, in approximately 800 μl of buffer. Elution characteristics of each batch of G50 Sephadex were determined prior to probe purification. Probes purified by either method were concentrated by precipitation in 0.3M sodium acetate and cold 70% ethanol at -70°C for one hour (Maniatis et al, 1982) in the presence of 150 μg ml yeast tRNA (Gibco BRL) as carrier. Recovered oligonucleotides were re-dissolved (final concentration approximately 40 nM) in 10 mM Tris, 1 mM EDTA, pH 7.6, containing 10 mM dithiothreitol (DTT) and stored at 4°C.

2.3.7 In situ hybridization

Labelled probe was diluted in hybridization buffer (50% neutral formamide, 10% dextran sulphate, 0.6M sodium chloride, 0.06M sodium...
citrate, 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 500 ug/ml salmon sperm DNA, 250 ug/ml yeast tRNA, 50 μM unlabelled dATP) to give an approximate final concentration of 400 pM and a final specific radioactivity of 3 x 10⁶ dpm/ml. DTT was added to a final concentration of 1 mM. To determine specificity of hybridization, the hybridization buffer contained, in addition, a one hundred-fold excess of unlabelled over labelled probe (Uhl et al, 1985; Noguchi et al, 1989).

Tissue sections were overlaid with probe in hybridization buffer, protected by parafilm coverslips and incubated overnight at 37°C. Following hybridization the tissue sections were washed in 0.15M sodium chloride, 0.015M sodium citrate pH 7.0 (1 x SSC) at 55°C for 60 minutes (four 15 minute washes) and at room temperature for 60 minutes (two 30 minute washes). Slides were briefly dipped in distilled water, to remove excess salts, followed by 70% ethanol and allowed to air-dry. Following the completion of hybridization, no further precautions were taken against RNAase contamination.

2.3.8 Comparison of preparative protocols

The usefulness of acetylation and delipidation steps was examined, as follows: for assessment of the effect of acetylation on ISHH, routinely cut and fixed tissue sections were additionally immersed in 0.1M triethanolamine and 0.25% acetic anhydride in 0.9% saline pH 7.0 for 10 minutes at room temperature, washed twice in PBS and dehydrated as
usual. For assessment of the effects of delipidation, tissue sections were dehydrated to absolute alcohol and immersed in chloroform for 5 minutes, after which they were rehydrated through 100% to 95% ethanol and allowed to air-dry.

Where wax-embedded sections were to be used, these were dewaxed in xylene for 10 minutes at room temperature, rehydrated through graded alcohols and incubated in Proteinase K (Sigma), 5 µg/ml in PBS for up to 15 minutes at 37 °C (Singer et al, 1987). Sections were then dehydrated to 95% ethanol before hybridization.

2.3.9 Autoradiography

After hybridization, film autoradiographs were prepared by apposing slides to Hyperfilm (Amersham) and exposing for 10-20 days; emulsion autoradiographs were prepared by dipping slides in Ilford K5 nuclear track emulsion diluted 1:1.5 in distilled water and exposing for 4-6 weeks. At the end of the appropriate exposure period, film autoradiographs were developed using automated X-Ray film processing. Emulsion-dipped slides were developed in Ilford Phenisol (1:4 in distilled water) 3.5 minutes at 20 °C, washed in Ilfostop stop bath (diluted 1:20 in distilled water) for 1 minute and fixed in Ilford Hypam (1:4 in distilled water) 3.5 minutes. Slides were subsequently washed in distilled water (30 minutes), counterstained, dehydrated through graded ethanols, cleared and mounted in DPX mountant (Merck, UK).
2.3.10 Histological counterstaining

To assist microscopic examination, tissue section autoradiographs were counterstained in either Toluidine blue, for ISHH alone, or, following immunohistochemistry, in haematoxylin. For paraformaldehyde pre-fixed tissue sections, Toluidine blue (Gurr, BDH) 0.05% in 0.05% aqueous borax (Robinson, 1982) was used. Immersion times were varied empirically depending on the age of the staining solution. Destaining of K5 emulsion was achieved by immersion in 70% alcohol, for up to 20 minutes. Sections processed for immunohistochemistry were counterstained in Harris' haematoxylin (Gurr, BDH) (Stevens, 1982) for a few seconds and briefly destained in acid alcohol.

2.3.11 Image analysis and statistics

Comparisons between different fixation methods and pre-treatments were made on film autoradiographs and on tissue sections or emulsion autoradiographs, by eye, by microscopic examination (at 400x magnification) or using a computer assisted image analysis system (Quantimet Q570, Leica, Cambridge, UK). Quantitative data from films were obtained by densitometry. Intensity of hybridization signal was measured as mean grey value (i.e. arbitrary units, inversely proportional to signal density) or, in some instances, equivalent values for radioactive concentration were obtained using $^{14}$C microscale standards (Amersham). Film autoradiographic measurements were taken over the regions of interest from duplicate tissue sections. On emulsion autoradiographs
hybridization signal was measured as the area of the cell profile covered by silver grains (grain area, µm²) over individual cells.

Grain area was chosen as the raw data measure. The development of silver grains in photographic emulsion follows a course which comprises a lag phase during which few grains are formed, a linear phase, during which a cascade is generated, with existing silver grains potentiating the formation of grains in closely adjacent emulsion and a saturation phase, during which no new grains are formed. During the linear phase, individual silver grains form visible clumps which spread out into the neighbouring emulsion and the size of these increases with increasing radioactive dose. Preliminary experiments using rat midbrain, probed with the TH probe, showed that grain area over individual neurons increased linearly with increasing exposure time (and therefore, with increasing radioactive dose) between 10 and 25 days (data not shown). In neuronal populations with variable cell size, values for grain density, i.e. grain area adjusted for the area of cytoplasm, were used instead. Fields were chosen at random for counting, from the same region of each neuronal group studied, either using an automated microscope stage or defining the area to be assessed and working across this systematically, analysing all neurons encountered. Data from between 10 and 25 cells was pooled to give a mean value per tissue section. Duplicates for each individual were averaged and results expressed as means and standard
deviations; the statistical significance of quantitative results was
examined using regression analysis or two-tailed Student’s $t$-test.

2.3.12 Immunohistochemistry

Tyrosine hydroxylase peptide was identified in neurons using a polyclonal
rabbit antibody (PelFreeze Biologicals, Arkansas USA) coupled by a
biotinylated second antibody to streptavidin peroxidase and visualized
using diaminobenzidine (DAB) chromogen, using both paraformaldehyde
pre-fixed and glutaraldehyde post-fixed material. Post-fixed sections
were washed in PBS and both pre- and post-fixed sections were
dehydrated in graded ethanol solutions before exhaustion of endogenous
peroxidases in 0.3% hydrogen peroxide in methanol for 20 minutes. (For
comparison, wax-embedded sections prepared for routine
neuropathological examination, were dewaxed in xylene before
exhaustion of peroxidases). Following rehydration all tissue sections were
incubated in 10% normal goat serum for 10 minutes at room temperature
to block non-specific immunoreactivity, and then sequentially in TH
antibody, 1:200 overnight at room temperature, in biotinylated goat anti-
rabbit IgG (Amersham Life Sciences, U.K.), 1:100 for 30 minutes, and
streptavidin-horseradish peroxidase complex (Amersham Life Sciences
UK) for 15 minutes. Goat serum, antibodies and streptavidin complex
were all diluted in 1% normal goat serum, 1% BSA, 0.3% Triton X-100 in
PBS (diluent solution). Immunoreactivity was visualized using DAB
chromogen (0.5 mg/ml) in phosphate buffer (Sigma, UK), containing
0.03% hydrogen peroxide for 6 minutes. The reaction was terminated by washing with phosphate buffer; sections were counterstained with Harris' haematoxylin, dehydrated and mounted.

2.3.13 Extraction of RNA and Northern hybridization

Total RNA was extracted from tissue using the method of Chomczynski and Saachi (Chomczynski and Saachi 1987). Tissue was homogenised on ice in 10 volumes of 4M guanidinium isothiocyanate containing 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1M 2-mercaptoethanol (solution D). After acidification with 2M sodium acetate pH 4.0, proteins and DNA were removed from the homogenate by partition between acid guanidinium reagent and phenol with CHCl₃: isoamyl alcohol 49:1. RNA was recovered from the aqueous phase by precipitation with an equal volume of isopropanol at -20 °C for 1 hour followed by centrifugation at 10,000 g for 20 minutes. The resulting pellet was resuspended in solution D and precipitated again with an equal volume of isopropanol. The final RNA pellet was washed with 75% ethanol and air-dried. After drying it was resuspended in 10 mM Tris 1 mM EDTA pH 7.6, 0.25% sodium dodecyl sulphate (SDS) and the RNA concentration determined by spectroscopy at 260 nm.

RNA extracts were separated on agarose gels using standard methods (Maniatis 1982). Aliquots of RNA (approximately equivalent to 20 μg) were mixed with formamide (final concentration 50%), formaldehyde
(final concentration 4.5%) in 1 x MOPS buffer (22 mM 3-[N-morpholino]propanesulphonic acid, 50 mM sodium acetate, 10 mM EDTA) in a total volume of 20 µl. Samples were heated to 65 °C for 5 minutes, cooled on ice and mixed with 0.1 volumes of loading solution (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue). RNA aliquots were run on horizontal denaturing gels: 1% agarose, 6% formaldehyde in 1X MOPS buffer against RNA standards (0.24-9.5 kb, Gibco BRL or 0.2 to 10.0 kb, Sigma, UK) for up to 7 hours at approximately 40 volts. RNA was subsequently blotted onto nylon membranes (Hybond, Amersham UK) and stained with 0.05% methylene blue in 0.5M sodium acetate to visualise RNA and standards. Gels were examined and photographed under visible light.

For northern hybridization, oligonucleotide probes were 3' end-labelled with [³²P]dATP (NEN DuPont, 6000 Ci/m mol) as described above, for [³⁵S] labelling of probes. Transfer membranes were pre-hybridized in 15% deionised formamide, 7% SDS in 0.5M sodium phosphate, pH 6.8 for 30 minutes at 50 °C and hybridized overnight at the same temperature and in the same buffer containing [³²P]-labelled oligonucleotide diluted to approximately 1.5 x 10⁶ dpm/ml. Membranes were then washed in 1 X SSC containing 0.1% SDS at 50 °C for 30 minutes, followed by 0.2 X SSC containing 0.1% SDS at 50 °C for 30 minutes. The washed membranes were then exposed either to Kodak X-omat Xar-5 X-ray film or to Hyperfilm (Amersham) and developed using an automated
processor. In the study of freezing methods and long-term mRNA stability, transfer membranes were hybridized with probe to mitochondrial complex I mRNA, labelled with $[^{35}\text{S}]$-dATP.

2.4 Results

2.4.1 Tissue morphology: effects of different preservation and fixation protocols

Using the method described above, flash-frozen tissue was well preserved with excellent histology, even after long periods of storage at -70°C. Macroscopic features, major nuclei and white matter tracts were clearly identifiable in flash-frozen tissue blocks and sections and there was little discernible distortion or cracking. Microscopic examination of post-fixed, Toluidine blue-stained 12 μm cryostat sections showed that brain microanatomy was similarly well-preserved, with individual neurons, major dendrites and intracellular structures easily distinguishable in all the brain regions examined, as shown in Figure 2.1A-C. Following brief post-fixation in 4% paraformaldehyde, flash-frozen tissue retained good morphology throughout the ISHH procedure and following prolonged and rigorous washing (see Figure 2.2).

The effects of different fixation protocols examined are shown in Table 2.3. Paraformaldehyde post-fixation of tissue sections was the best method of preserving flash-frozen tissue for ISHH. Paraformaldehyde pre-fixation for short periods and subsequent flash-freezing of fresh
Figure 2.1

Histological integrity of flash-frozen tissue

Preservation of structure in flash frozen tissue blocks at regional and light microscope levels.

A: Cryostat section from temporal cortex, stained with 0.05% Toluidine blue in 0.05% borax, showing preservation of cortical ribbon, major white matter tracts and hippocampal formation (arrow).
   Scale bar 0.33 cm

B: Cryostat section through hippocampal formation, stained with Toluidine blue as above, showing dentate gyrus (arrow) surrounding numerous well-preserved hippocampal pyramidal neurons. Scale bar 250 μm

C: Cryostat section showing neurons of the trochlear (IVth cranial nerve) nucleus, with excellent preservation of sub-cellular structures, Nissl substance, nuclei and nucleoli, as well as dendrites. Glial nuclei are also seen on this section. Stained in Toluidine blue, as above.
   Scale bar 55 μm
Table 2.3
Effects of preparative protocols on ISHH

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<td>preservation of tissue structure and immunoreactivity</td>
<td>slightly improved morphology</td>
<td>inconsistent hybridization signal and background</td>
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<td>improved preservation of immunoreactivity</td>
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<td>reduced non-specific binding to white matter</td>
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<td>improved preservation of immunoreactivity</td>
<td>small reduction in ISH in emulsion ARG</td>
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Commonly-used preparative steps were examined for their effect on ISHH on human brain tissue; results obtained are shown in column 2 & 3. The effects were assessed by comparison with simplified, routine ISHH method following film and/or emulsion autoradiography (using 35S-labelled oligonucleotide probes). The quantitative effects of glutaraldehyde fixation are shown in Table 2.4

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tissue, although it preserved the morphology of individual neurons, resulted in some damage to large neurons visible at x400 magnification in the regions examined, especially substantia nigra, in spite of cryoprotection in 15% sucrose, which was otherwise effective in preventing most fixation and freezing artefact. Higher sucrose concentrations (20 and 25%) were tested but did not result in any further improvement in morphology.

2.4.2 In situ hybridization histochemistry

Using the simplified hybridization protocol described above with paraformaldehyde post-fixation, excellent ISHH results were routinely obtained with flash-frozen brain. Hybridization signal from oligonucleotide probes was strong and well-localized to appropriate brain regions, in film autoradiographs and to cells in emulsion autoradiographs. Examination of brain stem film autoradiographs showed that TH oligonucleotide probe, assumed to recognise dopamine-synthesizing neurons, hybridized to neurons of the human substantia nigra (A9) and extra-nigral neurons in the mesencephalic tegmentum, the ventral tegmental area (A10) and the retro-rubral field (A8) (see Figure 2.2A). Brain stem emulsion autoradiographs were examined at x500 magnification. Silver grains were present over both melanized and non-melanized cytoplasm in mesencephalic, dopaminergic neurons and could be easily distinguished from neuromelanin (Fig 2.2 B, D). Silver grains were also detected over proximal dendrites of these neurons, as has been
observed previously (Dumas et al, 1990). Some non-melanised neurons were also labelled (Figure 2.2D). Little formation of grains was seen over the nigral neuropil in these preparations or over non-dopaminergic neurons, e.g. in red nucleus or cranial nerve nuclei.

Similarly good results were obtained with other oligonucleotide probes e.g. to the "housekeeping" genes, aldolase-C and β-tubulin in all brain regions studied. Aldolase C hybridization signal, for instance, was seen in neurons of the pons, in Purkinje neurons of the cerebellum, where very strong signal was obtained with little or no background over the granule cell layer or molecular layer (Figure 2.2E), and in neurons of the cortex. Strong β-tubulin hybridization was observed in the red nucleus, cranial nerve nuclei (Figure 2.2F) and in neurons of the cortex and cerebellar granule cell layer.

2.4.3 Specificity of hybridization

Evidence of specificity was provided by competition with unlabelled probe. The inclusion of a 100-fold excess of unlabelled TH probe in hybridization buffer abolished darkening of film autoradiographs in the substantia nigra pars compacta and VTA, which indicated specific hybridization but did not affect the signal seen over fibre-rich areas of the brain stem, e.g. cerebral peduncle and red nucleus (not shown). This labelling was less intense than hybridization signal over the substantia nigra pars compacta, less granular in appearance, and was assumed to be a
Figure 2.2

*In situ* hybridization on flash-frozen tissue

A: Film autoradiograph (reversed image) of hybridization of tyrosine hydroxylase (TH) probe with dopamine neurons in a midbrain hemisection. Hybridization signal is seen over the dorsal (d) and ventral (v) tiers of the nigra and with neurons of the VTA, close to the midline. cp = cerebral peduncle. Scale bar 0.25 cm

B: Emulsion autoradiograph of substantia nigra cryostat section hybridized with TH probe, showing silver grain formation above individual melanised neurons. Counterstain, Toluidine blue; scale bar 30 \( \mu m \)

C: Absence of hybridization signal over neurons in the presence of a 100-fold excess of unlabelled TH probe. Counterstain, Toluidine blue; scale bar 30 \( \mu m \)

D: Hybridization of TH probe with melanised nigral neurons in the presence of a 100-fold excess of an unrelated probe sequence, showing hybridization over individual neurons. Counterstain Toluidine blue; scale bar 30 \( \mu m \)

E: Hybridization of aldolase C probe with cerebellar Purkinje neurons (reverse image of film autoradiograph); scale bar 0.5 cm

F: Emulsion autoradiograph of neurons of the third nerve nucleus hybridized with \( \beta \)-tubulin probe; insert shows hybridization in the presence of a 100-fold excess of unlabelled probe. Counterstain, Toluidine blue; scale bar 75 \( \mu m \)
chemographic artefact. Hybridization signal in emulsion autoradiographs was reduced to background levels, comparable with grain formation over neuropil, for all probes studied. Grain formation over dopaminergic neurons of the midbrain, hybridized with TH probe was reduced to background values by the inclusion of excess unlabelled probe (Figure 2.2 C, 2.2F inset) while hybridization signal was unaffected in parallel hybridizations in the presence of unlabelled probe of an unrelated sequence (see Figure 2.2D ) (Uhl et al, 1985).

The specificity of hybridization of aldolase-C, β-tubulin and tyrosine hydroxylase probes was examined in by Northern hybridization to extracted brain mRNA. On film autoradiographs, the aldolase C probe hybridised with a single band between 1.6 and 1.7 kb, consistent with the known size of the mRNA species (see Figure 2.13). The β-tubulin probe hybridised with two bands corresponding to 1.8 and 2.7 kb, as previously reported (Slaughter et al, 1989) (see Figure 2.13). The TH probe hybridized with a single band at 1.8 kb (Nagatsu 1991).

2.4.4 Effect of fixation protocols on ISHH

Film autoradiography of brainstem sections hybridized with TH probe demonstrated that hybridization signal was low in conventionally pre-fixed, wax-embedded tissue (Figure 2.3B). Background in these sections was often variable and hybridization signal difficult to distinguish. Comparison by film densitometry of autoradiographs from pre-fixed,
Figure 2.3

Effect of fixation method on hybridization

A: Film autoradiographs from flash-frozen control midbrain cryostat hemisphere hybridised with TH probe following simplified protocol for flash-frozen tissue, showing signal over substantia nigra (arrow).
   Scale bar 0.3 cm

B: Film autoradiograph of midbrain from tissue fixed in 10% formalin and wax-embedded, from the same individual as A. Signal over substantia nigra (arrows) is not so strong and is more variable than in the flash-frozen section, edge effects are also visible. Scale bar 0.38 cm

C: Hybridization of TH probe with melanised nigral neurons in tissue pre-fixed in 4% paraformaldehyde, equilibrated with 15% sucrose and flash-frozen; background is higher than in flash-frozen, post-fixed tissue (Figure 2.2 B) and grain formation over neurons is reduced.
   Scale bar 50 μm.
wax-embedded and flash-frozen sections (in duplicate) from five control individuals with good mRNA preservation, hybridized in parallel with TH probe, showed that hybridization signal was lower in all wax-embedded sections, even after proteinase K treatment (Figure 2.3 A, B). The film density value, determined over substantia nigra in tissue hybridized with labelled probe alone, was corrected for the value for tissue hybridized in the presence of a 100-fold excess of unlabelled probe. Mean signal/noise ratios obtained were 1.35 (n=5) for wax-embedded, proteinase K-treated sections, compared with 2.02 (n=5) for flash-frozen tissue. Short-term pre-fixation in 4% paraformaldehyde also gave poorer results than post-fixation of flash-frozen tissue. Emulsion autoradiography and light microscopic examination of midbrain sections hybridized with TH probe showed that non-specific signal over neuropil was higher than in flash-frozen, post-fixed sections of tissue (Figure 2.3C) and appeared to increase with increasing concentrations of sucrose (20%, 25%) used as cryoprotectant. 15% Sucrose was the lowest concentration at which morphology was adequately preserved and acceptable ratios of specific to non-specific signal could still be achieved. Flash-frozen tissue post-fixed with glutaraldehyde, 0.1, 0.5 and 1% in 4% buffered paraformaldehyde at 0°C, also showed good histological preservation. Hybridization signal from tissue fixed at lower glutaraldehyde concentrations was generally comparable with that in tissue post-fixed with paraformaldehyde alone, but appeared slightly lower in some hybridizations. The effect of fixation with 0.5% glutaraldehyde was therefore compared with paraformaldehyde
alone using aldolase C and β-tubulin probes to hybridize duplicate
sections from cerebellum, pons and cortex from a control individual with
good mRNA preservation. Hybridization signal, measured as grain area
or grain density over individual neurons, was reduced between 5 and 40%
by glutaraldehyde fixation, depending on the neuronal group examined
(quantitative comparisons are shown in Table 2.4).

2.4.5 Effect of pre-hybridization protocols on ISHH

The effects of individual pre-hybridization steps (see Table 2.3) were
measured on paraformaldehyde post-fixed tissue, using results from the
simplified protocol as control values. Mean grain density was measured
in duplicate serial sections hybridized with aldolase-C probe over neurons
from the pons, red nucleus and fifth nerve nucleus (brain stem).
Acetylation did not improve hybridization signal or decrease background.
The values obtained from sections pre-treated by acetylation were within
a few percent of controls; signal/noise ratios, derived from comparison of
grain density values over hybridized neurons with grain density values
over neuropil, were somewhat lower than control values: 3.8 compared to
4.7 in pontine neurons (3 cases); 4.0 compared to 6.5 in fifth nerve
nucleus (one case) and 3.7 compared to 6.1 in red nucleus (one case).
Delipidation by immersion in CHCl₃ also showed no reproducible
advantage and generated large variations in grain density values, mean
values for test samples varying between 87 and 195% of control values,
depending on the neuronal group. Mean grain density test and control
Table 2.4
Effect of fixation protocols on hybridization signal

<table>
<thead>
<tr>
<th>Neuronal group</th>
<th>value</th>
<th>Probe</th>
<th>4% paraformaldehyde</th>
<th>0.5% glutaraldehyde</th>
<th>0.5% glutaraldehyde + acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortical pyramidal</td>
<td>grain</td>
<td>aldolase C</td>
<td>0.218 (100%)</td>
<td>0.191 (88%)</td>
<td>0.151 (69%)</td>
</tr>
<tr>
<td>neurons</td>
<td>density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pontine neurons</td>
<td>grain</td>
<td>aldolase C</td>
<td>0.173 (100%)</td>
<td>0.108 (62%)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>red nucleus neurons</td>
<td>grain</td>
<td>β-tubulin</td>
<td>0.255 (100%)</td>
<td>0.185 (72%)</td>
<td>0.141 (55%)</td>
</tr>
<tr>
<td></td>
<td>density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cerebellar Purkinje</td>
<td>grain</td>
<td>aldolase C</td>
<td>702 (100%)</td>
<td>673 (95%)</td>
<td>460 (65%)</td>
</tr>
<tr>
<td>neurons</td>
<td>area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean values and percent of control from flash-frozen duplicate sections of cortex pons and cerebellum post fixed in 4% paraformaldehyde at room temperature (control value) or 0.5% glutaraldehyde in 4% paraformaldehyde at 0 °C, with and without subsequent acetylation. Following fixation, tissue was dehydrated through graded alcohols and hybridized with the probe indicated.

Grain density = grain area (μm²)/cell area (μm²). Cell boundaries were difficult to detect in Purkinje neurons, therefore only grain area was measured. Ns = not scored because of poor hybridization signal.
values (respectively) in CHCl₃-treated tissue were 0.131 compared to 0.067 in the red nucleus; 0.101 compared to 0.115 in the fifth nerve nucleus and 0.091 compared to 0.089 in pontine neurons. However, the mean signal to noise ratios in the same CHCl₃-treated sections were 2.5, 8.5 and 3.7 in red nucleus, fifth nerve nucleus and pons respectively compared to 6.1, 6.5 and 5.7 in control sections.

2.4.6 Effects of fixation on tyrosine hydroxylase immunochemistry

Immunohistochemical results obtained using the flash-frozen brain stem tissue sections post-fixed with 4% paraformaldehyde, routinely prepared for ISHH, were poor in comparison with sections from conventionally-fixed, wax-embedded tissue or from tissue pre-fixed in 4% paraformaldehyde, equilibrated with sucrose and flash-frozen. In these, latter, preparations DAB chromogen was discretely located to individual neurons in the substantia nigra and numerous immunoreactive, dopaminergic fibres were clearly visible (see Figure 3.1B). In contrast, in flash-frozen midbrain sections, post-fixed in 4% paraformaldehyde, DAB chromogen could be seen in regions containing melanized, dopaminergic neurons but chromogen reaction products were diffusely located around cell perikarya and groups of neurons, rather than being precisely localized to individual cells. THIR fibres were not apparent in these sections and no TH immunoreactivity could be seen in fibres in flash-frozen, paraformaldehyde post-fixed striatum, although these were weakly stained in wax-embedded tissue sections.
Tyrosine hydroxylase immunochemistry in flash-frozen, post-fixed human
brain stem sections was greatly improved by incorporation of 0.1-1.0%
glutaraldehyde in the 4% paraformaldehyde fixative, giving results
comparable with formalin-fixed, wax-embedded or paraformaldehyde pre-
fixed tissue. TH immunoreactivity in the substantia nigra, preserved by
glutaradehyde fixation, is shown in Figure 2.4B. In the striatum, also,
dense THIR fibres, with good morphological preservation were also
demonstrated using this fixation method (Figure 2.4A). No TH
immunoreactivity was seen in either pre- or post-fixed sections when
primary antibody, biotinylated secondary antibody or streptavidin had
been omitted from the immunostaining procedure.

This fixation method was also suitable for other antigens; for instance,
GFAP immunoreactivity was also successfully demonstrated in flash-
frozen tissue following glutaraldehyde fixation of brainstem, cerebellar
and cortical tissue sections. GFAP immunoreactive cell bodies could be
detected in the deep cerebellar white matter, in pontine fibres and in the
substantia nigra (Figure 2.4C).

2.4.7 Brain tissue pH
Tissue pH values from cerebellar cortex fell between 5.86 and 6.86 with a
mean value of 6.37 (SD 0.24). pH values for all cases are shown in Table
2.2. The mean values for the pathological material was 6.32 (SD 0.19) and
for control material 6.4 (SD 0.26); these were not significantly different
Figure 2.4

Immunohistochemistry in glutaraldehyde-fixed tissue

A: Tyrosine hydroxylase immunoreactivity in flash-frozen section through putamen and globus pallidus, following fixation in 0.05% glutaraldehyde in 4% buffered paraformaldehyde, showing strong immuno-labelling of dopaminergic fibres and terminals in putamen and in fibres of the medial medullary lamina (arrowed).

Scale bar 0.2 cm.

B: Nigral neurons, showing strong staining of perikaryon and dendrites in flash-frozen nigra, fixed as in A. Counterstain, haematoxylin; scale bar 20 μm.

C: Glial fibrillary acidic protein (GFAP) immunoreactivity in astrocytes of cerebellar white matter (arrowed), fixed as in A. Counterstain, haematoxylin; scale bar 40 μm.
(Student’s t-test). Two subgroups of the cases, comprising both PD and control subjects were evaluated as examples of slow and fast death. The mean pH value for individuals dying from bronchopneumonia (assumed to be a slow death with a long agonal phase) was 6.24 (SD 0.16); in contrast individuals dying from myocardial infarction or a ruptured thoracic aneurysm (assumed to be a rapid deaths) gave a mean tissue pH value of 6.38 (SD 0.27) and 6.53 (SD 0.27) respectively. Neither group differed significantly from the pneumonia group or from the series as a whole (p > 0.05, Student’s t-test).

There was no relationship between pH value and age at death, or post-mortem delay in the complete series of cases or in the pathological subjects (Figure 2.5). Similarly, in the PD cases, there was no correlation between tissue pH value and clinical variables such as the duration of illness or L-DOPA dosage (not shown).

Quantitative analysis was carried out on a group of cases with pH values between 6.1 and 6.86, comprising 10 controls and 9 parkinsonian cases, with a range of pH values, drawn from the cases shown in Table 2.2. Hybridization with β-tubulin probe to the granule cell layer was quantified by film densitometry and aldolase C by analysis of grain area over individual Purkinje neurons. The intensity of hybridization signal on both film and emulsion autoradiographs was consistently low for subjects with
A: pH value and age at death. There was no correlation between age at death and pH value for either control or pathological groups.

B: pH value and post-mortem delay. There was no correlation between post-mortem delay and pH value for either control or pathological groups.
a low tissue pH, the highest signal being obtained from tissue with a pH value above 6.5. Results from quantitation of autoradiographic signal are shown in Table 2.5. For both probes, the range of values for the control and pathological groups overlapped with no significant difference between groups, although the highest values were in the control group.

Regression analysis showed a significant correlation between pH value and hybridization signal in both film and emulsion autoradiographs (see Table 2.5, Figure 2.6). No relationship between hybridization signal and post-mortem delay could be detected in either hybridization (not shown). Similar results were obtained in other brain regions. Hybridization signal from film autoradiographs of striatal sections hybridized with probes to mRNA encoding pre-proenkephalin and pre-protachykinin was also strongly correlated with pH, but not with post-mortem delay (data not shown). β-tubulin mRNA hybridization signal in midbrain dopaminergic neurons also correlated strongly with pH (see Table 2.5).

Northern analysis of extracted RNA from two cases with low pH (6.08, 6.21) and two with high pH (6.68, 6.79) showed that RNA in tissue samples with high pH was relatively well-preserved with 18 and 28S ribosomal bands clearly visible but, in contrast, RNA extracts from tissue with low pH were hardly visible on the stained transfer membranes (see Figure 2.7). Subsequent hybridization with 32P-labelled aldolase C and β-tubulin probes showed that mRNA from the two high pH samples was
Regression analysis of autoradiographic data

<table>
<thead>
<tr>
<th>Probe</th>
<th>Autoradiography</th>
<th>brain region</th>
<th>r</th>
<th>P&lt;</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldolase C</td>
<td>emulsion</td>
<td>cerebellum</td>
<td>0.76</td>
<td>0.001</td>
<td>19</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>film</td>
<td>cerebellum</td>
<td>0.85</td>
<td>0.001</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>emulsion</td>
<td>substantia nigra</td>
<td>0.81</td>
<td>0.005</td>
<td>11</td>
</tr>
<tr>
<td>PPE</td>
<td>film</td>
<td>striatum</td>
<td>0.7</td>
<td>0.001</td>
<td>28</td>
</tr>
<tr>
<td>PPT</td>
<td>film</td>
<td>striatum</td>
<td>0.69</td>
<td>0.001</td>
<td>30</td>
</tr>
</tbody>
</table>

Regression statistics from quantitation of film and emulsion autoradiographs. PPE, pre-proenkephalin probe; PPT, pre-protachykinin probe. Grey values were obtained from film densitometry from the following hybridizations: β-tubulin, cerebellum; PPE striatum; PPT striatum), grain areas (aldolase C, cerebellum) and grain densities (β-tubulin, substantia nigra) from grain area measurements on emulsion autoradiographs. All values were analysed with respect to tissue pH. Results from film autoradiographs from cerebellum and striatum are from different individuals.
Figure 2.6
Relationship between hybridization signal and pH value

A

Density (grey) values from densitometry of film autoradiographs of cerebellar granule layer hybridised with b-tubulin probe showing significant correlation ($r=0.85$, $p<0.001$)

B: Grain area values ($\mu m^2$) from Purkinje neurons hybridised with aldolase C probe showing significant correlation between pH and hybridization signal ($r=0.76$, $p<0.0010$)
Figure 2.7
Reduction of mRNA in low pH tissue

A: RNA preservation in tissue extracts: northern blots of tissue from control cerebellum stained with methylene blue, showing 18 and 28S ribosomal bands in high pH tissue and relatively greater degradation in low pH tissue. Position of size markers shown on right.

pH of control tissue: C7, 6.21; C31, 6.79; C3, 6.08; C27, 6.68.

B: Hybridization of RNA extracts with aldolase C probe (lanes as indicated above).

C: Hybridization with β-tubulin probe, as B.

Little or no hybridization for either aldolase C or β-tubulin is visible in lanes containing extracts from acidic samples, C7 and C3.
present, although somewhat degraded. In the two low pH samples, little or no hybridization could be detected for either probe (Figure 2.7).

2.4.8 Effects of freezing technique and long-term storage

The appearance of RNA on methylene blue-stained transfer membranes did not indicate any preferential degradation in samples frozen at -20°C and stored for 36 months in comparison with the samples routinely frozen at -70°C. Both slowly and rapidly-frozen tissue showed RNA lanes with clear 18 and 28S ribosomal bands (Figure 2.8 A) and a comparable degree of RNA degradation. With respect to the length of storage time, RNA extracted from tissue routinely flash-frozen at -70°C showed no preferential degradation in tissue stored for 108 months compared to tissue with shorter storage periods either in cerebellum (Figure 2.8B) or pons (Figure 2.8C) or in PD or control cases.

Examination of mitochondrial ND1 mRNA expression in these extracts confirmed that preservation of this species was not affected by freezing technique, nor by freezer storage time. Although the signal produced by mRNA extracted from the PSP tissue was lower than that seen in the control case (subjectively assessed), there was no difference in signal between the samples frozen at different temperatures either in cortex or in cerebellum (Figure 2.9 A, B). Similarly, ND1 mRNA extracted from pons and cerebellum showed no reduction with length of storage time.
Figure 2.8

RNA stability in frozen tissue

A: Northern blots from RNA extracts of cortex, stained with methylene blue. Lanes 1-2 contain extracts from tissue frozen at -20 °C, lanes 3-4 from the same tissue frozen at -70 °C. Lanes 1 and 3 contain extracts from one control case; lanes 2 and 4 from one PSP case. All lanes show some degradation, but there is no consistent pattern suggesting that RNA in tissue frozen at -20 °C is less stable.

B: RNA extracts from control cerebellum stored for different periods of time at -70 °C. Storage time of tissue samples in lanes: 1, 106 months; 2, 108 months; 3, 80 months; 4, 70 months; 5, 15 months; 6, 28 months. There appears to be no change with respect to storage time.

C: RNA extracts from pons stored for varying periods of time at -70 °C. Lanes 1-4 control samples; lanes 5-8, PD samples. Storage time of tissue in lanes: 1, 99 months; 2, 85 months; 3, 15 months; 4, 22 months; 5, 108 months; 6, 103 months; 7, 19 months; 8, 20 months. There appears to be no change with respect to storage time and no effect of pathology.

Position of size largest and smallest markers shown on right of all panels; O = origin.
Representative autoradiographs from these experiments are shown in Figure 2.9 C-E.

2.4.9 Hybridization signal and storage time

Regression analysis of hybridization signal in nigra and Purkinje neurons revealed no relationship between mean grain density values (i.e. grain density per cell in each case) and duration of storage. TH, aldolase C, β-tubulin and NDUFV1 mRNA signal in the nigra gave regression values of 0.15, 0.04, 0.15 and 0.1 respectively, showing no relationship between months in storage and signal level. The absence of an effect of storage on ND1 mRNA in cerebellar extracts, seen on northern hybridization of cerebellar extracts was confirmed by regression analysis of ND1 signal over Purkinje neurons in controls (r = 0.16) which showed no deterioration of signal with time. Scatterplots of signal values against time in storage for these probes is shown in Figures 2.10 and 2.11.

2.5 Discussion

These studies were directed towards developing simple ISHH protocols for routine use with flash-frozen tissue and to avoid possible additional sources of variation, human tissue being inherently heterogeneous with respect to mRNA preservation due to a variety of pre and post-mortem factors (Barton et al, 1993). As an alternative to formalin preservation, flash-freezing tissue has inherent advantages for research, in that it permits a wide range of techniques to be applied to the study of post-
Figure 2.9

Preservation of ND1 mRNA

Representative autoradiographs from northern blots probed with ND1 probe.

A: Extracts of cerebellum frozen at -20 °C (lanes 1 and 2) and at -70 °C (lanes 3 and 4) from the subjects shown in Figure 2.8, showing hybridization of ND1 probe with two bands at 1.0 and 3.0 kb approximately. There appears to be no consistent difference due to freezing method.

B: Extracts of cortex frozen at -20 °C (lanes 1 and 2) and at -70 °C (lanes 3 and 4) from the subjects examined in Figure 2.8A, showing hybridization of ND1 probe with one band at approximately 1.0 kb. There appears to be no consistent difference due to freezing method but tissue from case 2 appears to express less cortical ND1 mRNA than that from case 1.

C: Extracts from control cerebellum, probed with ND1 probe, showing hybridization to two bands, as above. Lanes 1-6 contain tissue extracts as shown in Figure 2.8B with storage times: 106, 108, 80, 70, 15, 28 months. There was no consistent difference with respect to length of time in freezer storage, between 15 and 108 months.

D: Extracts from control pons probed with ND1 probe, showing hybridization to a single band at approximately 1.0 kb. Tissue freezer storage times are as in lanes 1-4 in Figure 2.8C: 99, 85, 15 and 22 months. There was no consistent difference with respect to freezer storage time.

E: Extracts from PD pons probed with ND1 probe, showing hybridization to a single band at approximately 1.0 kb. Tissue freezer storage times are as in lanes 5-8 in Figure 2.8C: 108, 103, 19 and 20 months. There was no consistent difference with respect to freezer storage time.

The position of size markers is shown to the right of panels. O = origin.
Figure 2.10
Stability of hybridization signal in stored tissue

A

Grain density values per cell were calculated from pooled data from individual neurons in each case. No relationship was found between hybridization signal and length of time in freezer storage at -70 °C (examined by regression analysis).

A: Grain density values per cell in cases examined for TH mRNA expression

B: Grain density values per cell in cases examined for aldolase C mRNA expression
Grain density values per cell from pooled data from individual neurons in each case; control and PD values combined. There was not relationship between hybridization signal and storage time at -70 °C.

A: Grain density values per cell in cases examined for β-tubulin mRNA expression

B: Grain density values per cell in cases examined for NDUFV1 mRNA expression
mortem brain including those, like ISHH, which are sensitive to fomalisation.

2.5.1 Preservation of anatomical structure in flash-frozen tissue

Histological integrity was an important consideration for these studies. Brain tissue flash-frozen by the method described above had excellent histology, rivalling that of conventionally fixed, wax-embedded material. Only slight cracking and distortion was produced in tissue blocks by this method of flash-freezing, even of relatively large regions such as striatum or temporal cortex, thus avoiding the reported disadvantages of freezing in liquid nitrogen (e.g. Bancroft, 1982). Anatomical orientation was facilitated by the ease of preparation of large tissue sections (up to 15 cm²). The excellent histology of this material was preserved during storage at -70 °C, with no deterioration seen over periods of several years, in spite of repeated increases in temperature necessary for the preparation of cryostat sections.

2.5.2 Effects of formalin

Poor results were obtained in ISHH studies employing archival tissue which had been fixed long-term in 10% formalin and subsequently wax-embedded. On film autoradiographs of tissue sections cut from this material, specific hybridization signal was extremely difficult to detect even with proteinase pre-treatment and very low in comparison both to background and to signal in flash-frozen tissue. Prolonged contact of
human brain tissue with formaldehyde is held to cause non-specific changes, both in ISHH signal and in antigenicity (Pollock and Wood, 1988; Wisden and Morris, 1994; Lucassen et al, 1995) and similar effects of formalin fixation have been noted by other authors. Systematic evaluation of formalin fixation of human cortex demonstrated reduction of total mRNA signal after only a few hours of exposure, probably reflecting the effect of greater cross-linking on the accessibility of tissue, even to small probes (Dr P. Harrison, personal communication). Lucassen and colleagues (Lucassen et al, 1995) have also reported lower hybridization signal for vasopressin mRNA in human suprachiasmatic nucleus and paraventricular nucleus in formalin-fixed, wax-embedded human tissue, although the authors still found such material useful for study of this abundant mRNA. The effects of wax-embedding were not examined in the present studies but it seems likely that deterioration in hybridization signal in tissue prepared for neuropathological examination might result from a combination of factors including exposure to non-polar solvents and heating, which has been reported to alter antigenicity (Escolar et al, 1988) or decrease ISHH signal (Wisden and Morris, 1994).

Treatment with proteinases is thought to assist the penetration of probe into tissue and is recommended by some authors (e.g. Angerer et al, 1987; Brigati et al, 1983; Herrington et al, 1990) as a method of increasing ISHH signal in tissue which has undergone fixation and embedding. However, incorporation of proteinase pre-treatments into the pre-
hybridization protocol did not eliminate the problems encountered using fixed material. Film autoradiography demonstrated that proteinase treatment improved hybridization somewhat but also generated considerable intra-section background variation which obscured specific signal. It also seems likely that the rigorous drying of conventionally-prepared material may alter the physical properties of tissue and prevent penetration both of probes and antibodies. Recent studies have demonstrated that formalin-fixed, wax-embedded material may be treated by autoclaving and is suitable for ISHH (including quantitative studies) (Oliver et al, 1997). Similarly, ISHH studies of the expression of synaptophysin mRNA demonstrated that this could be readily detected in autoclaved sections using oligonucleotide probes and that the pattern of hybridization signal correlated strongly with that previously demonstrated with flash-frozen tissue (Eastwood et al, 1999). This suggests that the incorporation of water, under pressure into wax embedded sections may facilitate the entry of probes into the tissue. Similar results can be obtained for immunohistochemistry using wax-embedded sections (personal observations). However, although it seems that mRNA can be retrieved from fixed, wax-embedded tissue and in spite of the value of this approach for immunohistochemistry, paraformaldehyde pre-fixation has drawbacks for brain bank tissue collection because pre-fixation limits the range of uses to which tissue can subsequently be put (e.g. biochemical studies).
Exposure of tissue to formaldehyde had deleterious effects on other histological techniques studied. Immunohistochemical detection of TH in the cortex and in nigro-striatal pathways was difficult or impossible in the formalin-fixed, wax-embedded material studied here. Appreciably better results were obtained with flash-frozen tissue post-fixed in paraformaldehyde/glutaraldehyde fixative. In preliminary experiments aimed at developing terminal deoxynucleotide transferase-mediated dUTP-biotin 3' end-labelling (TUNEL) histochemistry to study DNA fragmentation in substantia nigra neurons, it was not possible to demonstrate TUNEL labelling in formalinised and wax-embedded human ileum, used as a reference tissue for apoptotic cell death (see below, Chapter 5). In contrast, short-term fixation of rat ileum (48 hours) in buffered paraformaldehyde, with wax embedding gave results consistent with the expected pattern of TUNEL labelling, indicating the apoptotic death of intestinal cells. Flash-frozen rat ileum gave the same results as the short-term paraformaldehyde-fixed ileum, thus flash-frozen human nigra was subsequently used for the apoptotic studies described below. It seems possible that the problems encountered with TUNEL histochemistry are similar to those of ISHH and that autoclaved, wax-embedded brain tissue might also be used for TUNEL studies.

2.5.3 ISHH protocols

The simplified protocol described above, employing flash-frozen tissue, fixed after freezing and sectioning in buffered 4% paraformaldehyde gave
excellent results for ISHH using oligonucleotide probes for a large number of mRNA species (see also Nisbet et al, 1994, 1995; Eve et al, 1997, 1998) with good hybridization signal and low non-specific labelling of tissue, demonstrated by competition studies. In comparison with this protocol, other fixation methods gave poorer or more variable results. Light post fixation and dehydration of flash-frozen tissue were the only necessary preparative steps for successful and reliable ISHH. These findings are consistent with earlier studies of animal (Wisden and Morris, 1994) or human (Strada et al, 1992) CNS or of cell or chromosome preparations (Singer et al, 1987). There was no demonstrable advantage resulting from the inclusion of acetylation, delipidation or proteinase or detergent treatments in the standard ISHH protocol. In practice, procedures theoretically designed to increase tissue penetrability and ISHH signal such as delipidation and proteinases, generated variability in hybridization signal and autoradiographic background and compromised tissue morphology. Use of simple pre-hybridization protocols gave low and even autoradiographic background, assisting successful quantitation studies and permitting relatively large brain regions e.g. whole human striatum and large areas of cortex to be successfully hybridized. The applicability of ISHH to large structures and regions is of particular importance to the study of human tissue.

The oligonucleotide probes employed for these studies gave consistently good results using the labelling methods described above and employing
the same simple preparative protocols and washing steps for all the probes used. With respect to the quantitation methods used here, the relatively low degree of isotopic labelling of oligonucleotides, combined with the short path length of the isotope used, $[^{35}S]$, produced a manageable degree of grain formation in emulsion autoradiographs, which was particularly useful for quantitation of signal.

2.5.4 Immunohistochemistry

For immunohistochemistry, however, post-fixation of flash-frozen tissue in 4% paraformaldehyde did not preserve TH antigenicity sufficiently to allow unambiguous identification of dopaminergic cells, for instance. In comparison, pre-fixation in 4% buffered paraformaldehyde for short periods (24-48 hr), was effective in preserving TH antigenicity in midbrain dopaminergic neurons and gave results comparable in this region to those obtained with conventionally-fixed tissue. Fixation of tissue for short periods prior to freezing is widely employed for immunohistochemical studies of human brain and for combined labelling by in situ hybridization and immunohistochemistry (e.g. Chan Palay, 1988; Javoy-Agid et al, 1990).

However, TH immunoreactivity could be preserved in flash-frozen tissue by fixation in 0.5% glutaraldehyde in 4% buffered paraformaldehyde. This gave good immunohistochemical results for a small number of peptides examined, including tyrosine hydroxylase, rivalling TH
immunohistochemistry on conventionally-fixed, wax-embedded sections. In addition, in striatum, TH-positive fibres, which could not be easily detected in other preparations were readily identifiable, suggesting that the improved detection of THIR could be related to greater cross-linking in glutaraldehyde-treated tissue (Singer et al, 1987), preventing the leaching out of soluble antigen. In support of this view, immunoreactivity for ubiquitin, localized in Lewy inclusion bodies in cortical neurons, could be demonstrated in sections of human cortex post-fixed in 4% paraformaldehyde alone (not shown). By manipulation of fixation conditions, flash-frozen tissue could be successfully employed for immunohistochemistry.

2.5.5 Studies of peri-mortem factors: tissue pH

The range of pH values observed here was consistent with previous studies of brain pH. Hardy’s study reported values for the medulla oblongata between pH 5.8 and 7.5. Values above pH 7.0, in his study, were associated with violent death, and those below 6.5 with slow death, i.e., following a prolonged agonal phase, or intermediate death i.e. following admission to hospital but without a prolonged agonal phase (Hardy et al, 1985). Perry and colleagues’ study of post-mortem preservation of amino acids and enzyme activities reported pH values between 5.7 and 6.7 (Perry et al, 1982). Values in the present series were close to these and to Hardy’s “slow” and “intermediate” death categories. Similarly, Harrison and colleagues, in a later study, recorded brain pH
values, measured in cerebellum or neocortex, between 5.7 and 7.02 with a mean value of 6.46, very close to that of the present study (Harrison et al, 1995).

In contrast to earlier studies, the values recorded in the present series did not closely reflect the recorded manner of death. There was no relationship between mode of death and pH values. The "slow" death group, i.e. that dying from bronchopneumonia, which implies a long agonal phase did not have a different mean pH value to those assumed to have had a shorter agonal phase, having died from ruptured aortic aneurysm or myocardial infarction, although the lowest mean value was in the bronchopneumonia group and the highest in the ruptured thoracic aneurysm group. It is possible that with a larger series, a correlation with mode of death might have been shown. However, it is also likely that the absence of a relationship reflects the paucity of clinical information on the terminal phases of the individuals studied here and that the stated cause of death did not adequately reflect the severity of agonal status or that factors which were not included in the clinical assessment may have influenced tissue acidosis.

Brain pH did not appear to be influenced by brain disease, although the highest values in the series appeared in the control group. The lack of a significant difference between values in the two groups suggested that individuals dying with PD do not necessarily have higher levels of brain
hypoxia than controls. It was not possible in this series to draw conclusions about the cases with other movement disorders, in this series since the numbers were too small. However, a recent comparison of the cases in the Queen Square Brain Bank showed that the mean pH value for MSA cases was 6.29 (n=27) compared to 6.46 (n=29) for control cases and was significantly different (p = 0.01, Student's t-test), suggesting that hypoxia may have been a more important feature of the terminal phase in this disease.

In the series described above, pH was not related to post-mortem interval, up to 48 hours, or to the age of the subjects. The finding with respect to post-mortem interval is consistent with previous studies of rat and human tissue (Ravid 1992; Harrison et al, 1995). A relationship between age and pH, however, has been reported in one study in a series of cases with an age range from 20 to 90 years. The authors commented that this trend might be partly explained by slower modes of death in the older individuals (Harrison et al, 1995). The difference between Harrison's study and the study described here, which are comparable, may explained by the present series comprising a rather narrower age range, effectively from 63 to 91 years, with no cases below 40 years of age, thus making it difficult to identify an age-related trend.
2.5.6 Effect of peri-mortem factors on mRNA

The effect of pH on mRNA preservation was consistent between control and pathology groups. Hybridization signal, measured by film densitometry or assay of grain area, was strongly negatively correlated with acidic tissue pH for all five probes and in all regions examined. This was consistent for both control and pathological cases. The absence of a correlation between post-mortem delay and hybridization signal was also observed for both groups independently. These findings support the suitability of post-mortem human tissue for analysis of mRNA expression and demonstrate that the presence of brain pathology does not necessarily compromise post-mortem mRNA preservation.

A subsequent study of mRNA and protein preservation in post-mortem human cortex supports these findings (Harrison et al, 1995). This also showed that brain pH, measured by the same methods as described here, was a correlated with mRNA abundance in post-mortem brain. Not all mRNA species, however, were similarly sensitive, but significant correlations with pH were found for cyclophilin, synaptophysin, GAP 43 and 5-HT$_{2A}$R mRNAs in some brain regions and demonstrated that more acidotic brains had less mRNA signal. The authors also found hybridization signal of cyclophilin, synaptophysin and 5-HT$_{2A}$R mRNA to be negatively correlated with increasing post-mortem interval. Nevertheless, findings with respect to post-mortem delay and its effect on mRNA preservation vary considerably. Barton and colleagues (Barton et
al, 1993) reviewed the findings from fourteen separate studies of total and poly (A)' mRNA and found a small decline recorded in one study. A series of twenty studies of preservation of individual mRNAs recorded an effect of post-mortem delay in two. These inconsistent findings support the view that some mRNA species may be preferentially affected by post-mortem delay but most evidence suggests that it is a less important determinant of mRNA preservation than agonal state.

The use of oligonucleotide probes for identification of mRNA in situ or on transfer membranes raises the issue that degraded mRNA could be identified (Barton et al, 1993). In fact, in extracts of RNA from the cases used for Northern analysis in the present study, no difference was seen between the high and low pH cases with respect to total RNA concentration, measured by spectroscopy. Examination of the hybridized transfer membranes showed that hybridization signal was reduced or absent in the two low pH cases, indicating extensive degradation. The two high pH cases were less degraded but with a smear in front of the main bands which showed that the probe identified less than full length transcripts. Since this degradation is likely to have occurred ante-mortem, however, matching of groups for pH is expected to take account of the extent of this partial degradation.

The reason for the sensitivity of mRNA to agonal state and brain pH is not known. Lactate accumulation and the resulting fall in brain pH may
activate acid ribonucleases, resulting in degradation of mRNA but the susceptibility of individual species may depend on intrinsic factors such as regulatory sequences or the distribution of mRNA between translationally active or inactive pools (Barton et al, 1993). If ante-mortem hypoxia does activate acid ribonucleases then the post-mortem stability of mRNA is surprising. It has been suggested that catabolism of RNA is energy-dependent and ceases after death as a result of a rapid fall in ATP concentration (Morrison 1981) or that either ribonuclease inhibitors persist post-mortem longer than ribonucleases or that RNA is less susceptible to RNAase digestion once translation has ceased (Barton et al, 1993).

2.5.7 RNA preservation following freezing and long-term storage

Only two cases were examined to test the effects of freezing technique and the results of these experiments need to be viewed with caution. However, the preliminary results suggest that some mRNA species may be unaffected by freezing technique and the longer time taken for tissue to become fully frozen at -20°C. This is consistent with the findings from studies of post-mortem delay, which demonstrated that mRNA is generally stable between death and the freezing of tissue. It may also indicate that the disintegration of slowly frozen tissue, caused by ice crystal formation, does not activate enzymatic degradation which could persist at low temperatures. Northern hybridization for ND1 mRNA showed that this appeared to remain stable for up to 36 months.
following freezing at -20 °C. Similarly, while only one mRNA species, has been studied by northern hybridization and the result can only be regarded as indicative, these preliminary results also suggest that mRNA may remain stable for protracted periods of storage at -70 to -80°C. This possibility is supported by the finding that hybridization signal in individual neurons was not affected by storage times, for periods of several years.

These findings are consistent with those of earlier studies which also demonstrated the relatively long-term stability of frozen mRNA (Mengod et al, 1992, Leonard et al, 1993, Harrison et al, 1995). However, studies of in vitro translation suggest that even when mRNAs are detectable in tissue and appear unchanged in abundance, some degradation may still occur during storage. The use of oligonucleotide probes for detection of mRNA may mask this effect and the results of the present study do not indicate that mRNA has not undergone any degradation, only that the transcript remains detectable by in situ hybridization and hybridization signal is not related to time in storage. Some mRNAs have been demonstrated to be adversely affected by storage. For instance, Leonard and colleagues reported that while the amount of β-actin mRNA was stable during freezer storage, mRNA from tissue samples stored up to 60 months was less effective as a substrate for in vitro translation than material stored for 1-2 months. Similarly, some degradation of mRNA encoding phenylethanolamine N-methyl transferase, amyloid precursor protein and
actin mRNAs has been demonstrated in individual tissue samples stored for up to one year (Burke et al, 1991). Expression of mRNA encoding the NMDA receptor GluR1, measured by quantitative ISHH has been shown to be modestly affected by freezer storage time (Eastwood et al, 1995). It is likely therefore that individual mRNA species vary in their sensitivity to long-term storage and should be individually assessed for some quantitative studies.

2.6 Summary

Flash-frozen human tissue sections were found to be suitable for routine ISHH studies of human post-mortem brain using oligonucleotide probes. Following brief post-freezing fixation and dehydration, excellent histological resolution and signal/noise ratios were obtained with a high degree of reproducibility, permitting semi-quantitative studies to be carried out successfully (see e.g. Nisbet et al, 1995). For routine ISHH, no other pre-treatment of tissue (such as acetylation or delipidation) was necessary. mRNA was affected by ante-mortem hypoxia but appeared stable post-mortem, both immediately before freezing and subsequently over long periods of storage. With appropriate fixation techniques, flash-frozen tissue was also suitable for immunohistochemistry, demonstrating its suitability for other molecular biological techniques.

In the semi-quantitative ISHH studies described below, flash-frozen tissue sections were used routinely and prepared using the simplified preparative
procedure described above, for hybridization with $^{35}$S-labelled oligonucleotide probes and preparation of emulsion autoradiographs for quantitation. All case-control series examined for quantitative studies were matched for age and as closely as possible for tissue pH. In the absence of a study of the effect of post-mortem delay for each mRNA examined, tissue was additionally matched for this factor.
CHAPTER 3

EXPRESSION OF TYROSINE HYDROXYLASE AND HOUSEKEEPING GENES IN SURVIVING SUBSTANTIA NIGRA NEURONS IN PARKINSON'S DISEASE

In the studies described in this section, TH mRNA expression was examined as an index of neuronal dopaminergic activity in substantia nigra subregions, to clarify whether ventrolateral neurons had intrinsically higher rates of TH mRNA expression than neurons of the dorsal tier which might expose the ventral tier to higher levels of dopamine-induced oxidative stress or neurotoxicity. Control and PD cases were compared to determine whether there was any evidence of increased TH gene expression in PD. Expression of other genes was examined in parallel with the studies of tyrosine hydroxylase in order to address the possibility that surviving neurons in PD might be in a pre-morbid condition with abnormal mRNA transcription rates, or that mRNA expression in general could be influenced by therapeutically-administered L-DOPA.

The results of some parts of these studies have been published, see Appendix 1.

3.1 Introduction

3.1.1 The dorsal and ventral nigral tiers

The neurons of the SN pars compacta (SNpc), that is, the melanised dopaminergic neurons of the midbrain, comprise two roughly parallel mediolaterally-orientated groups of neurons. The dorsal tier is composed of loosely grouped, generally small, fusiform neurons, which are continuous
medially with the neurons of the A10 area (ventral tegmental area); the ventrolateral tier is composed of tightly-packed clusters of much larger, polygonal cells, with some groups protruding in finger-like projections into the underlying pars reticulata. SNr neurons are topographically closely related to the neurons of the ventral pars compacta but are part of the output pathway of the basal ganglia. The two tiers of the pars compacta are most easily distinguished at the level of the exit of the third nerve from the brain stem and the dorsal tier becomes more prominent in the caudal portions of the nigra (van Domberg and ten Donkelaar 1991). In the rat, neurons of the ventral tier have been shown to have a complex arrangement of dendrites, which extend ventro-laterally into the underlying neuropil of the pars reticulata or mediolaterally into the approximately horizontal plane of the pars compacta and occasionally, into the dorsal tier, while neurons of the dorsal tier have dendritic fields which are only mediolaterally orientated (Fallon and Loughlin 1995) and communicate with other neurons of the dorsal tier.

The two tiers are differently innervated. Striatonigral projections largely innervate the ventral subregions of the SN and utilise GABA, substance P and dynorphin but the striatum does not project strongly to the dorsal tier. In addition to striatal inputs, the ventral nigra has excitatory inputs from the glutamatergic subthalamic nucleus and glutamatergic and cholinergic projections from the pedunculopontine tegmental nucleus and the cortex. Thus the ventral tier neurons and neurons of the pars reticulata are under the influence of striatonigral and excitatory projections. Dorsal tier neurons, in contrast,
receive direct limbic inputs from the frontal and cingulate cortices and indirect input from limbic projections to the striatum and from the amygdala (Fallon and Loughlin 1995). In addition, in the rat nigra, collateral fibres from projection neurons of the SNr have been demonstrated which have inhibitory synapses on dopaminergic SNpc neurons (Tepper et al, 1995). It is likely, therefore that there is GABAergic inhibition of pars compacta neurons through ventrolateral dendritic fields and inhibitory synapses while the presence of pars compacta dendrites in the pars reticulata permits dopaminergic modification of GABAergic output.

3.1.2 Dopamine synthesis and metabolism

Tyrosine hydroxylase (TH) is the rate-limiting enzyme for dopamine biosynthesis. Its substrates are tyrosine and molecular oxygen and it catalyses the addition of a hydroxyl group to the meta position of tyrosine giving rise to 3,4-dihydroxy-L-phenylalanine (L-DOPA) in the presence of iron and biopterin as co-factors. L-DOPA is converted by DOPA decarboxylase (aromatic amino acid decarboxylase, AADC) which removes a carboxyl group to form dopamine. In other catecholaminergic neurons, dopamine may be further converted to noradrenaline by dopamine β-hydroxylase and to adrenaline by phenylethanolamine N-methyltransferase. In dopaminergic terminals TH is present with its biopterin co-factor, allowing dopamine to be synthesised in locally. Biopterin, however, is present at sub-saturating concentrations, which may play a part in regulating synthesis of dopamine in these terminals.
Dopamine is inactivated either by oxidative deamination by monoamine oxidase B, giving rise to 3,4-dihydroxyphenylacetic acid (DOPAC) which is then methylated by catechol-O-methyltransferase (COMT) to homovanillic acid (HVA), or can be directly methylated to 3-O-methyldopamine by COMT, which then acts as a substrate for MAO-B. Both pathways generate HVA as an end product, which is the major metabolite of dopamine and measurements of HVA in cerebrospinal fluid are used, for instance, as an index of brain dopaminergic activity, mainly of the striatum.

3.1.3 Compensatory mechanisms for reduced dopamine innervation

The presence of compensatory mechanisms in PD is indicated by the natural history of the disease, since over 50% of nigral neurons and over 80% of striatal dopamine are lost before the motor signs of the disease appear (Agid et al, 1987; Marsden 1990), indicating a prolonged asymptomatic stage, during which plasticity of the nigro-striatal dopamine system is able to compensate for the loss of neurons. The sort of compensatory mechanisms which might be involved, increased dopamine turnover and synthesis and increased activity of TH, are indicated by post-mortem and experimental studies.

Early studies of dopamine metabolism in PD demonstrated that the ratio of homovanillic acid to dopamine in striatum was strikingly increased, indicating increased turnover of dopamine in remaining nigro-striatal terminals (see above, para 1.2.2) (Hornykiewicz and Kish 1986). It was proposed, therefore, that in established PD, increased turnover of dopamine in surviving nigral neurons
served as a means of compensation for reduced dopamine innervation of the striatum. Similarly, increased tyrosine hydroxylase activity, leading to increased dopamine synthesis in remaining nigro-striatal terminals, was also proposed as a compensatory mechanism, based on the finding that although dopamine synthetic enzymes were also reduced in the nigra and striatum in PD (Lloyd and Hornykiewicz 1970, Lloyd et al, 1975; Nagatsu et al, 1977), TH activity was not as badly affected as that of AADC or indeed, levels of striatal dopamine. Increased TH activity in the striatum in PD was also thought to provide a molecular basis for efficacy of L-DOPA treatment (Lloyd et al, 1975). Post-synaptic mechanisms, such as dopamine receptor super-sensitivity and increased striatal dopamine receptor numbers, particularly in the heavily affected putamen, were also thought to contribute to a complex of compensatory changes in PD (Hornykiewicz and Kish, 1986).

There are a number of potentially confounding factors which need to be taken into account in these studies. Therapeutic L-DOPA administration could theoretically influence a number of pre- and post-synaptic mechanisms or increased catabolism of dopamine could occur in PD through the activity of AADC, which has been shown to be present in striatal glia (Tashiro et al, 1989). Post-mortem diffusion of dopamine from storage sites and its consequent availability for enzymic degradation might also have contributed to the increase in HVA/dopamine ratio observed in PD. Nevertheless, evidence for compensatory increases in dopamine synthesis has been shown in the 6-OHDA-lesioned rat model of PD. In unilaterally-lesioned rats, dopamine levels in the
striatum were reduced by about 90% before asymmetrical motor responses (the experimental parallel to the human disease) were demonstrable, which closely parallels the degree of pre-symptomatic loss in postulated in human subjects. Following systemic administration of radiolabelled tyrosine the ratio of labelled to total dopamine was higher in the lesioned than in the un-lesioned striatum (Agid et al, 1973). Partial lesions of the nigra, using 6-OHDA resulted in a proportional reduction of dopamine concentrations and TH activity in striatum. When more than two-thirds of nigral neurons were destroyed, dopamine synthesis and turnover was accelerated in striatum and nigra, as shown by increased levels of DOPAC and HVA (Hefti et al, 1980; Melamed et al, 1982). Dopamine release from nigro-striatal terminals was also shown to be regulated in the lesioned nigro-striatal projection; levels of 3-methoxytyramine, an intermediate metabolite of dopamine, produced by extra-neuronal activity of COMT, remained constant until lesions exceeded 80% of normal dopamine input, showing that striatal dopamine release could be preserved at normal levels until the greater part of the projection was destroyed (Altar and Marien 1989)

3.1.4 TH expression in substantia nigra

Study of the expression of the rate-limiting dopamine-synthetic enzyme, TH by in situ hybridization histochemistry (ISHH) of mRNA or peptide by TH immunohistochemistry may provide insight into the activity of dopamine synthetic pathways at the cellular level and can directly address the question whether altered TH gene expression is involved in either in neurodegeneration or in compensatory mechanisms in PD. Use of semi-quantitative ISHH techniques
enables measurements to be made at the level of individual neurons and thus to avoid the difficulties inherent in examination of dopamine markers in the degenerating substantia nigra.

ISHH studies of TH mRNA expression in surviving nigral neurons have, nevertheless, produced conflicting findings. TH mRNA expression in nigral neurons in PD was reported to be reduced at the cellular level in comparison with controls (Javoy-Agid et al, 1990) and a subsequent study showed both reduced mRNA and TH protein in surviving nigral neurons. Values for both mRNA and TH protein were variable among control neurons, but the values were significantly correlated, suggesting a close correspondence between mRNA and peptide expression (Kastner et al, 1993). In support of this finding, another dopaminergic marker - expression of the dopamine transporter (DAT) mRNA, has been reported to be reduced in melanised nigral neurons in Parkinson's disease (Uhl et al, 1994; Harrington et al, 1996; Counihan and Penney 1998). In contrast, Joyce and colleagues, using immunohistochemistry to measure levels of TH protein and ISHH to identify and quantify TH mRNA in nigral neurons, reported that mean TH mRNA expression in individual ventral tier neurons in PD, was significantly elevated in comparison with control neurons, suggesting up-regulation of TH synthesis in PD (Joyce et al, 1997).

3.1.5 Nigral cell loss and dopaminergic activity

Findings from post-mortem studies of oxidative stress and the effects of MPTP on nigral neurons suggest that dopaminergic activity may be a factor in neuronal
vulnerability (see above, Chapter 1). Dopamine metabolites and neuromelanin may both play a part in exposing dopaminergic neurons to increased concentrations of oxygen or other free radicals which may be beyond the anti-oxidative capacity of the cell to regulate. The neurotoxic metabolite of MPTP, MPP⁺ is taken up into neurons via the dopamine re-uptake site, suggesting that active dopaminergic neurons would be most vulnerable to similar neurotoxins. The pattern of cell loss produced by intoxication by MPTP in humans and primates is very similar to that seen in PD (German et al, 1988) thus it is possible that the affected neurons are more active in terms of dopamine transmission than those which are relatively unaffected.

3.2 Study rationale and design

Tyrosine hydroxylase, aldolase C, β-tubulin and phosphofructo-2-kinase/fructose 2,6 bisphosphatase (PFKFB3) gene expression was studied by semi-quantitative ISHH in neurons of the substantia nigra (A9), comparing matched groups of PD and control cases. Comparison of TH, aldolase C and β-tubulin mRNA expression in dorsal and ventral nigral neuronal tiers was carried out in controls, in those individuals where the two tiers could be reliably distinguished on film autoradiographs from the same hybridization, to assess intrinsic differences in gene expression. TH mRNA expression was examined in control and PD groups to address the possibility that TH gene expression might be up-regulated in PD, as a compensatory mechanism. TH immunohistochemistry was carried out on control sections for subjective comparison with TH mRNA expression.
3.3 Materials and methods

3.3.1 Probes

The sequences and characteristics for TH, aldolase C and β-tubulin probes are detailed above (Chapter I). For phosphofructokinase studies, a combination of two probes probe complementary to bases 634 to 657 and 1315-1338 of the human phosphofructo-2-kinase/ fructose 2,6,bisphosphatase (PFKFB3) gene (AF109735 Genbank database, Manzano et al,1998), sequences: GGGCATCTC CTCTGCACCTTTATC and GGGCATCTCCTCTGCACTCTTATC were used. Both probes recognised the human sequence of the PFKFB3 gene and had 87 and 80% homology, respectively with the rat and murine sequences. These probes were not examined by northern analysis.

3.3.2 Cases

Clinical and post-mortem details of all the experimental groups are shown in Table 3.1. Case-control study groups were matched for age, post-mortem delay and tissue pH. For analysis of TH mRNA expression, 12 PD and 8 control cases were examined and, in addition, two control subjects both aged 40 years, with post-mortem delays of 15 and 23 hours and pH values of 6.61 and 6.58 respectively. Disease duration in the PD patients ranged from 4 to 40 years, with a mean duration of 15 years. L-DOPA dosage in the PD group (most recently reported dosage in the year before death) was between 200 and 2800 mg/24 hours, with a mean value of 812 mg/24 hours. Aldolase C expression was studied in a matched series of 11 PD and 10 control cases, β-tubulin mRNA expression in 6 PD and 6 control cases in duplicate hybridizations and PFKFB3
Table 3.1
Clinical details for groups: TH and housekeeping gene expression

<table>
<thead>
<tr>
<th>Probe</th>
<th>number</th>
<th>age (years)</th>
<th>pmd (hours)</th>
<th>pH</th>
<th>duration (years)</th>
<th>L-DOPA (mg/24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROLS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH</td>
<td>8 (5M,3F)</td>
<td>79.6 (69-90)</td>
<td>24 (5.5-34)</td>
<td>6.37 (6.21-6.55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aldolase C</td>
<td>10 (7M,3F)</td>
<td>75.5 (63-89)</td>
<td>21.8 (5.5-30)</td>
<td>6.52 (6.23-6.71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td>6 (5M, 1F)</td>
<td>74.6 (67-84)</td>
<td>23.5 (5.5-34)</td>
<td>6.45 (6.26-6.55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFKFB3</td>
<td>4 (3M, 1F)</td>
<td>79 (53-91)</td>
<td>38.5 (23.5-53)</td>
<td>6.59 (6.5-6.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD CASES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH</td>
<td>12 (7M, 5F)</td>
<td>76.8 (76-88)</td>
<td>18.3 (8.5-30)</td>
<td>6.43 (6.25-6.6)</td>
<td>15 (4-40)</td>
<td>812 (200-2800)</td>
</tr>
<tr>
<td>aldolase C</td>
<td>11 (7M, 4F)</td>
<td>76.6 (67-88)</td>
<td>18.7 (4.8-30)</td>
<td>6.43 (6.3-6.6)</td>
<td>16 (5-20)</td>
<td>822 (300-2800)</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>6 (3M,3F)</td>
<td>79 (72-88)</td>
<td>16.5 (4.8-30)</td>
<td>6.48 (6.3-6.6)</td>
<td>18.3 (5-16)</td>
<td>466 (300-1000)</td>
</tr>
<tr>
<td>PFKFB3</td>
<td>8 (5M,3F)</td>
<td>78 (68-88)</td>
<td>20.3 (7-34)</td>
<td>6.5 (6.3-6.7)</td>
<td>16.25 (5-22)</td>
<td>637 (250-1150)</td>
</tr>
</tbody>
</table>

Matched groups for study of TH, aldolase C, β-tubulin and PFKFB3 probes, showing values for age, pm delay and pH, with minimum and maximum values. There was no significant difference between post-mortem variables for any probe group (Student's t-test).
mRNA expression was examined in 4 control and 8 PD cases. There were no significant differences between case and control groups for any post-mortem factor in any hybridization (examined by Student’s t-test).

3.3.3 Preparation of midbrain tissue sections

For the study of the substantia nigra, midbrain blocks were cut for flash-freezing following the anatomical orientation of van Demberg and ten Donkelaar (van Demberg and ten Donkelaar, 1991). The brain stem was cut at right angles to its long axis just posterior to the mamillary body ventrally and the posterior commissure dorsally, thus separating it from the brain. A rostral nigral block was obtained by making a parallel cut at the level of the third nerve exit from the brainstem, where dorsal and ventral nigral neuronal tiers are most easily distinguished; a caudal nigral block was obtained by a further, parallel cut at the level of the rostral pons. The resulting blocks were frozen as described above, apposing the third nerve face to the cold plate to provide a flat, well-orientated plane for sectioning. 12 μm cryostat sections were cut from the third nerve face, usually from the rostral block and thaw-mounted onto glass slides, as described above and air-dried. Tissue sections were stored at -70°C before hybridization; sections for each hybridization were prepared in the same batch. A small number of 25 μm-thick sections were cut in order to determine whether variation in TH mRNA expression was affected by section thickness.

3.3.4 ISHH

Probes were labelled with [35S]-dATP as described above (Chapter 2) and were

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separated from unincorporated radionucleotides by ion exchange on NENsorb columns or by gel filtration on G50 Sephadex. ISHH was carried out as using the simplified pre-hybridization protocol described above (Chapter 2) and hybridizing with radiolabelled probes at an approximate concentration of 400 pM and specific radioactivity between 6 and 18 x10^6 dpm pmol^{-1}. Film autoradiographs and emulsion autoradiographs were prepared as described above (para 2.3.9). Tissue sections from control and parkinsonian brains were hybridized as single batches for each probe and exposed for the same time to film or emulsion; exposure times were in the linear range for response of film or emulsion to radioactivity. Duplicate sections were hybridized with radiolabelled probe in the presence of a (nominal) 100-fold excess of unlabelled probe for calculation of background values and to determine the specificity of hybridization signal.

3.3.5 Image analysis and statistics
Following development, counterstaining and mounting, the position of nigral subregions in tissue sections was determined by examination of the autoradiographic image on X-ray film (See e.g. Figure 2.2) and under low-power magnification. The medial nigral sub-regions to be assayed were outlined on the slide; neurons of the VTA (A10) and the pars lateralis (A8) were not included in the quantitation. Quantitation of hybridization signal was carried out on emulsion autoradiographs using the Quantimet Q570 image analysis system (Leica, Cambridge). Captured lightfield images, magnified x 400, were transformed to darkfield for detection of silver grains and subsequently analysed.
to determine the area of silver grains covering non-melanized cytoplasm.

Individual neurons were not selected for counting; fields within the delimited region of the substantia nigra were chosen by moving the objective along the long axis of the marked region in one direction, to avoid double-counting, and all neurons encountered were analysed. Results were expressed as grain density, i.e. area of grains/area of cytoplasm, in order to standardize for variability in size and orientation of nigral neuronal profiles. Neuronal plasma membrane and Nissl substance were clearly distinguishable in Toluidine blue-stained tissue, making the determination of neuronal area reliable. Grains were measured if they fell within the neuronal profile. Only profiles containing a visible nucleus or showing the proximal portions of neuronal processes were scored, in order to avoid assaying fragments of degenerating neurons. Where nigral neuronal tiers could be distinguished from one another on film autoradiographs, grain density measurements were made in both regions in emulsion autoradiographs.

Neuromelanin was used as a marker for dopaminergic neurons. Only melanised cells were scored for aldolase C, β-tubulin and PFKFB3 hybridizations. The TH probe, however, labelled both melanised and non-melanised cells, therefore both were scored for assessment of TH hybridization.

Grain density values were obtained from between 25 and 40 neurons in control tissue sections and from all surviving neurons in PD cases, up to 35 neurons per case. The data were not analysed until the image analysis was complete for each
hybridization. Grain density values per cell (i.e. the mean value for the total number of neurons counted) were calculated for individual, nigral region or cell type and were compared using unpaired Student’s $t$-test. Background values were calculated from grain density over melanised neurons in parallel hybridizations using 100-fold excess of unlabelled probe.

Pooled grain density values, from groups, nigral sub-regions, or cell type were also used to generate frequency histograms grouping grain concentrations in multiples of 0.02 (from 0 to 0.60 of the cell body covered) against percent of cells counted. These histograms were used for comparison of grain density frequency between case-control groups or between nigral sub-regions. The statistical significance of differences between frequency histograms was examined in cumulative frequency curves from the same histograms compared using the Kolmogorov-Smirnov, non-parametric test (McCarthy et al, 1995).

3.4 Results

3.4.1 Distribution of hybridization signal

TH mRNA was detected in midbrain neurons, on film and in emulsion autoradiographs, in both tiers of the substantia nigra pars compacta (SNC) (see Figure 2.2), in the substantia nigra pars lateralis (A8) the ventral tegmental area (A10) and in scattered neurons of the midbrain tegmentum. Hybridization signal in emulsion autoradiographs was localised to individual melanised and non-melanised neurons. In melanised neurons, silver grains were seen over cytoplasm and neuromelanin. TH mRNA expression was highly heterogeneous
Figure 3.1

Heterogeneity of TH expression in nigral neurons

A: TH hybridization in surviving melanised nigral neurons in PD, showing heterogeneity of mRNA expression. Heavily and lightly labelled neurons are visible in this field. Scale bar 25 µm.

B: TH immunoreactivity in control nigral neurons in flash-frozen tissue fixed in 4% paraformaldehyde containing 0.5% glutaraldehyde, and immunostained as described above (para 2.3.12) showing variability in peptide expression between individual neurons. Scale bar 25 µm.
in individual neurons. Neurons with grain formation comparable with levels in
the neuropil (i.e. about 2% of the cell surface covered) were found closely
adjacent to heavily labelled neurons in all control subjects, including the two 40
year-old controls. Similar heterogeneity of expression was seen in the 25 μm
sections (not shown).

Aldolase C mRNA was identified in melanised neurons of the SNpc although it
was not so strongly expressed as in other brain stem neurons, e.g. neurons of the
third nerve nucleus or the red nucleus. Similarly, β-tubulin was expressed in
melanized neurons, although it also appeared not to be so strongly expressed as
in, for example, magnocellular neurons of the red nucleus. PFKFB3 mRNA was
expressed in scattered melanised neurons of the substantia nigra, although a
proportion of nigral neurons appeared to be unlabelled, suggesting that this
mRNA was not uniformly expressed. Hybridization signal was also seen around
the nuclei of some nigral glia, which were stained by Toluidine blue on these
preparations and appeared as slightly ovoid small bodies distributed throughout
the nigral neuropil.

In PD cases TH mRNA expression was demonstrable in surviving nigral neurons
in PD (see Figure 3.1), although far fewer neurons were present than in controls.
Subjective assessment of the level of hybridization signal indicated that this was
broadly comparable, with a similar degree of heterogeneity, to that in control
subjects. Aldolase C and β-tubulin and PFKFB3 mRNA were also expressed in
surviving nigral neurons in PD subjects.
Figure 3.2
TH mRNA grain density values in melanised and non-melanised nigral neurons

Grain density values per cell from pooled data from PD and control subjects in melanised (closed circles) and non-melanised (open circles) nigral neurons. Mean values per group were not significantly different (Student’s t-test).
3.4.2 Quantitation of TH mRNA hybridization signal

TH mRNA grain densities (per cell) in melanised neurons from individual cases varied between 0.057 to 0.163 of the cell surface covered in control nigra and 0.079 to 0.129 in PD nigra (see Figure 3.2); mean values for groups were 0.098 (SE .010, n = 8 cases) and 0.101 (SE .005, n = 12 cases) in control and PD groups respectively and were not significantly different (p = .748, unpaired Student’s t-test). TH mRNA grain density values per cell for individual cases did not correlate with disease duration or L-DOPA dosage in the PD group (see Figure 3.3). Comparison of frequency histograms for TH mRNA in melanised neurons showed a similar pattern of expression in PD and control neurons (Figure 3.4) and cumulative frequency analysis confirmed that these were not significantly different (p >0.05, Kolmogorov-Smirnov test).

Mean grain density values in non-melanised neurons were 0.099 (SE .012, n = 7 cases) and 0.127 (SE .015, n = 9 cases) in control and PD nigra respectively (see Figure 3.2). These did not differ significantly from one another (p = 0.06, unpaired Student’s t-test) or from mean values in melanised neurons.

Cumulative grain density frequencies in PD non-melanised neurons, however, were significantly elevated in comparison with melanised neurons (p <0.05, Kolmogorov-Smirnov test) although control melanised and non-melanised neurons were comparable. Frequency histograms of TH mRNA in melanised and non-melanised neurons in PD are shown in Figure 3.5.

No significant difference in TH mRNA expression was seen in the nigral
A: Grain density per cell in PD cases and disease duration. There was no correlation with duration ($r = 0.3$)

B: Grain density values per cell in PD cases and L-DOPA dose/24 hours recorded in the last year of life. There was no correlation with L-DOPA dose ($r = 0.02$).
Figure 3.4
TH mRNA expression in PD and control nigral neurons.

A

B

Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 275 control melanised nigral neurons and 312 PD melanised nigral neurons. Analysis of the cumulative frequency curves showed that the two populations are not significantly different (p > 0.05 Kolmogorov-Smirnov test).
Figure 3.5
TH mRNA expression in melanised and non-melanised neurons in PD nigra

A

B

Grain density frequency histograms (A) derived from pooled data from 312 melanised and 57 non-melanised neurons in PD nigra. Cumulative frequency curves (B) derived from frequency data show that grain density frequencies in non-melanised neurons are significantly higher than in melanised neurons (p<0.05 Kolmogorov-Smirnov test).
Grain density frequencies (A) derived from pooled data from dorsal and ventral neuronal tiers in control nigra. Cumulative frequency curves (B) show that grain density frequencies are not significantly different (p > 0.05, Kolmogorov-Smirnov test).
subregions studied. Mean grain density values in ventral and dorsal tiers were 0.108 (SE .009) and 0.123 (SE .020) respectively in control subjects (n=5 cases, p = .51) and 0.114 (SE .015) and 0.129 (SE .020) respectively in PD subjects (n=4 cases, p = .24). Grain density frequencies were not different between nigral tiers, either for control or PD subjects (Figure 3.6).

Young controls

Grain density values per cell from the two young control subjects were 0.128 (SE .012) and 0.082 (SE .008) in melanised and 0.131 (SE .021) and 0.127 (SE .020) in non-melanised neurons and fell within the range of values for the aged control group. Cumulative grain density frequencies for these two subjects were also not significantly different from the older group (p>0.05, Kolmogorov-Smirnov test).

3.4.3 TH immunohistochemistry

TH antibody labelled nigral neurons heterogeneously, the intensity of DAB chromogen development varying between individual, even closely adjacent, cells in both control and PD tissue (Figure 3.1B). Intensity of TH immunoreactivity was not quantified, however.

3.4.4 Aldolase-C mRNA expression

Mean grain density values for aldolase C mRNA in melanised neurons were 0.11 (SE .008, n = 10 cases) in controls and 0.106 (SE .004, n = 11 cases) in the PD group; these were not significantly different, (p = .835 unpaired Student’s t-test).
Aldolase C mRNA expression in nigral neurons

A

Frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 172 PD and 255 control neurons. Cumulative frequency curves show that grain density frequency in the two populations is not significantly different (Kolmogorov-Smirnov test).
Figure 3.8

Aldolase C mRNA expression in dorsal and ventral nigral tiers

A

Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 110 dorsal tier and 134 ventral tier neurons. Cumulative frequency curves show that grain density frequency in the two populations is not significantly different (p > 0.05, Kolmogorov-Smirnov test).
Mean grain density values for ventral 0.106 (SE .01, n = 8 cases) and dorsal tiers in control cases 0.113 (SE .01, n = 7 cases) were also not significantly different. Pooled grain density values from PD and control groups (Figure 3.7), or from dorsal and ventral neuronal tiers (Figure 3.8), generated similar histograms; grain density cumulative frequencies confirmed that grain density frequencies were not significantly different between groups or between ventral and dorsal tiers in control subjects (p> 0.05, Kolmogorov-Smirnov test).

3.4.5 β-tubulin mRNA expression

Mean grain density values for β-tubulin mRNA in melanised neurons from control and PD subjects were 0.132 (SE .004, n = 6 cases) and 0.129 (SE .01, n = 6 cases) respectively and were not significantly different (p = 2.36, unpaired Student’s t-test). There was no significant difference between mean values for dorsal and ventral nigral tiers, 0.135 (SE .007) and 0.137 (SE .004) respectively in control subjects (n = 6 cases) ( p = 0.86, Student’s t-test). Grain density frequency histograms and cumulative frequencies were not significantly different between the two groups (Figure 3.9) or between ventral and dorsal tiers in control subjects (Figure 3.10) (p > 0.05, Kolmogorov-Smirnov test).

3.4.6 PFKFB3 mRNA expression

In this hybridization, duplicate serial sections hybridized in the presence of a one hundred-fold excess of unlabelled probe were assayed “blind” in parallel with the test sections. Nigral neurons were not heavily labelled by this probe mixture and differences between labelled neurons and background values (i.e. cold
Figure 3.9
β-tubulin mRNA expression in nigral neurons

A

Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from IPD (n=161) and control (n=199) neurons. Cumulative frequency curves show that expression in the two populations is not significantly different (Kolmogorov-Smirnov test).
Figure 3.10

β-tubulin mRNA expression in dorsal and ventral nigral tiers

A

Grain density frequency histograms (A) and cumulative frequency curves (B) from pooled data from 81 dorsal tier and 96 ventral tier neurons. Analysis of cumulative frequency curves showed that the two populations were not significantly different (p > 0.05 Kolmogorov-Smirnov test).
Figure 3.11
PKFB3 mRNA expression in nigral neurons.

Grain density frequency histograms (A) and cumulative frequency curves (B) from pooled grain density values from 88 PD and 114 control nigral neurons. Analysis of cumulative frequency curves showed that PD and control values were not significantly different (p > 0.05, Kolmogorov-Smirnov test).
competition values) were small. Grain density cumulative frequencies from test and parallel cold competed sections were therefore compared using the Kolmogorov-Smirnov test. Cumulative frequency curves for the two were significantly different ($p<0.05$), with higher values seen in the test sections. Comparison of cumulative frequency curves for PD and control cases showed no significant difference for grain density between groups (Figure 3.11).

3.5 Discussion

3.5.1 Cell loss and dopaminergic activity

Systematic studies of nigral cell numbers and patterns of neuronal depletion in PD have indicated that the ventral nigral neuronal tier is more vulnerable to cell loss in the early course of the disease than the dorsal nigral subregions, although both are badly affected at end stage. Study of the reduction of dopaminergic markers in the striatum supports this, in that the pattern of dopamine terminal loss is greatest in the projection fields of the ventro-lateral nigral regions, i.e. in the dorsal putamen. There is experimental evidence that neuronal activity is a contributory factor to the higher rate of cell loss observed in ventral tier neurons, including the pattern of cell death induced in primates by MPTP which is similar to that seen in PD. Thus MPTP might be expected to affect neurons with greater rates of dopamine uptake and release preferentially. Alternatively, increased dopaminergic activity, either endogenous or as a compensatory response to dopaminergic cell loss, could theoretically expose nigral neurons to increased toxicity from endogenous dopamine, induced by increased oxidative stress (see above, Chapter 1). Increased dopamine turnover, for instance, induced in mice
by reserpine treatment, resulted in an increase in striatal oxidised glutathione, showing a link between dopamine metabolism and a change in the redox status of the cell (Spina and Cohen 1989).

The findings presented here indicate that increased expression of the TH gene does not play a part in the pathogenesis of PD. TH mRNA expression in melanised neurons in the ventral nigral tier in control material was similar to that seen in dorsal tier neurons and indicates that there are no constitutively different patterns of TH gene expression in the ventral nigra. In view of the close correlation between expression of TH mRNA and TH peptide observed in the human nigra (Kastner et al, 1993) and in other systems, such as cultured PC18 cells (Tank et al, 1986a, 1986b) and rat olfactory bulb (Stone et al, 1990), it seems unlikely that increased TH enzyme activity and increased dopamine synthesis is a factor in the preferential vulnerability of ventrolateral neurons.

3.5.2 Possible compensatory mechanisms in the nigra in PD

Similarly, the absence of an increase in TH mRNA expression in melanised nigral neurons in the PD individuals in the current study, suggests that increased dopamine synthesis in PD, inferred from changes in striatal dopamine metabolites, is unlikely to be the result of up-regulation of TH gene expression in this population of neurons. TH mRNA values were not correlated either with L-DOPA dosage or with disease duration, thus it is unlikely also that absence of up-regulation is due to L-DOPA treatment. However, increased dopamine synthesis can take place without alteration in the expression of TH gene. For
instance, up-regulation of TH activity \textit{in vivo} in the absence of altered gene transcription, was seen in rats unilaterally lesioned with 6OHDA. At nine months after lesioning, dopamine and its metabolites were decreased in the denervated striatum compared with the intact side while the DOPAC:DA ratio in was elevated, indicating increased dopamine release from remaining terminals. In parallel with striatal dopamine depletion, 80\% of neurons were lost from the lesioned nigra and TH mRNA expression was around 5\% of the total on the un-lesioned side. Striatal dopamine in the denervated striatum, however, was around 30\% of the total on the intact side, suggesting a more efficient use of TH mRNA by surviving neurons in the lesioned nigra (Passinetti et al, 1989).

The activity of TH can be modified by intracellular mechanisms such as the calcium-calmodulin system and cyclic AMP. Specific inhibition of calcium-calmodulin resulted in a dose-dependent decrease of DOPA formation in striatal slices while incubation in the presence of dibutyryl cyclic AMP increased DOPA formation. These alterations in TH activity were due to variation in the levels of two forms of the enzyme present in striatal tissue, one with high affinity for a synthetic co-factor, 6-methyl 5,6,7,8-tetrahydropterin (6MPH4) and one with low affinity. TH activity in the rat striatum was modulated by transformation between the high and low affinity forms by a calcium-calmodulin-dependent system and a cyclic AMP-dependent system (Hirata and Nagatsu 1985). Similarly, in synaptosomal preparations, activation of protein kinase C increased the activity of tyrosine hydroxylase by an increase in its affinity for 6MPH4 cofactor (Olani and Olianas, 1987). Cyclic GMP is also involved in regulating
the activity of TH; in rat PC12 cells, treatment with sodium nitroprusside, an
activator of guanylate cyclase, increased tyrosine hydroxylase activity (Roskoski
and Roskoski, 1987). Taken together, these results suggest that, both cyclic
AMP and cyclic GMP second messenger systems are able to regulate TH
activity, through the action of their protein kinases.

Tyrosine hydroxylase can be phosphorylated at four possible serine sites, 8, 19,
31 and 40 (Haycock et al, 1990) and the phosphorylation state of the enzyme can
be modified by factors such as glutamate, or activation of dopamine D2
receptors (Cho et al, 1997). Glutamate reduced the increase in activity of TH
and synthesis of dopamine produced by activators of adenylate cyclase in striatal
slices, through phosphorylation of Ser(40) (Lindgren et al, 2000). Stimulation
of afferent dopaminergic fibres in vivo also produced increased phosphorylation
of serines 19, 31, while depolarization of synaptosomes by elevated K+ produced
increased phosphorylation at Ser19, phorbol esters increased phosphorylation at
Ser 31 and cyclic AMP-linked agents at Ser40, showing that three separate
transduction systems are able mediate the regulation of TH in terminal structures
(Haycock et al, 1991). Thus, activity of TH and synthesis of dopamine may be
regulated without changes in gene transcription and the preferential vulnerability
of ventral tier neurons may be related to dopaminergic activity in terminals but
not to the intrinsic capacity of these neurons for dopamine synthesis. It is of
interest in that neurons of the dorsal and ventral tiers had similar expression of
TH mRNA even though their anatomical position, their terminal fields and input
pathways are distinct. This may possibly reflect the fact that dopamine synthesis
can be differentially regulated by modulation of TH activity. Similarly, no variation in gene expression between the dorsal and ventral nigral tiers was seen in the aldolase C or β-tubulin hybridizations, suggesting that the two populations are closely similar and supporting the findings on TH mRNA expression.

3.5.3 TH mRNA expression in melanised neurons

Previous studies of TH mRNA and peptide expression in nigral neurons have suggested that TH mRNA expression is lower in PD subjects than in controls, implying that dopamine synthesis is generally reduced in individual nigral neurons in PD, as indicated by some animal studies (Passinetti et al, 1989, Jackson-Lewis et al, 1995). Studies of small numbers of controls and PD cases, matched for clinical signs of agonal status, reported reduced expression of TH mRNA per cell using similar techniques to those described here (Javoy-Agid et al, 1990, Kastner et al, 1993). Reduced TH mRNA expression was indicated by a leftward shift of the grain density frequency in histograms derived from pooled data. In contrast, Joyce and colleagues (Joyce et al, 1997) reported a small increase, around 20%, of TH mRNA expression in surviving melanised neurons using comparable assay methods and comparing both pooled data and mean grain density per individual case, in a similar number of cases.

The results of the present study do not agree with previous findings, TH mRNA expression in the nigra in the PD cases studied here was comparable with that of controls. There was no significant difference in TH mRNA expression, measured as grain density values in melanised neurons, between individual case values.
(i.e., values per cell) in PD and control groups or in frequency distribution of pooled grain density values from control and PD groups. The discrepancy between this and the earlier studies of human post-mortem substantia nigra is difficult to explain but may relate to the larger numbers of individuals in the current study or to the matching of post-mortem tissue pH values, controlling for variation in mRNA preservation between individual subjects. Furthermore, individual melanised neurons showed considerable variation of TH mRNA expression, ranging from background values to above one-quarter of the cell surface covered. The number of melanised neurons not expressing detectable levels of TH mRNA in PD cases in the present study (23%) was slightly greater than in controls (18%). This is a somewhat higher value than that of Javoy-Agid and colleagues and Kastner and colleagues, who reported around 10% of neurons with undetectable levels of TH mRNA expression in the nigra. Nevertheless, it is possible, that inter-neuronal variation may account for the small increase reported in PD by Joyce and colleagues (Joyce et al, 1997), who used an automated counting system to select neurons above background for assay. Cells with low levels of expression, therefore, may not have been compared and this may have skewed the analysis towards a higher value (but see below, Chapter 7).

3.5.4 Non-melanised neurons in PD

Comparison of cellular TH mRNA expression showed a small increase in PD non-melanised neurons compared with melanised neurons, which was not observed in control neurons. This finding suggests that a sub-population of
dopaminergic neurons may be up-regulated with respect to dopamine synthesis in PD. The absence of up-regulation in melanised neurons suggests that this mechanism is unlikely to contribute to the continuing vulnerability of melanised cells to the disease process. The number of non-melanised neurons examined in this study is rather small but a later ISHH study of TH mRNA expression in VTA (A10) neurons in PD showed both that a larger proportion of TH positive neurons were un-pigmented, around 35% compared to 10% among neurons of the substantia nigra (A9). TH mRNA expression was higher in this groups of neurons then in melanised neurons or in melanised and non-melanised neurons in the control group (Tong et al, 2000). As in the present study, there was no difference in TH mRNA expression in VTA melanised neurons between the control and PD groups but the increase in expression of TH mRNA in non-melanised neurons of the VTA was proportionately larger than that observed in the nigra. This increased expression of TH mRNA may represent a compensatory increase in VTA neurons and suggests that this group may have a greater capacity for plasticity which could play a role in the greater resistance of VTA neurons to the disease.

3.5.5 Normal levels of gene expression in PD

Expression of mRNA encoding the three housekeeping genes was unaffected in the PD cases studied here. Like TH mRNA, aldolase C, β-tubulin and PFKFB3 mRNA values were all comparable in control and PD groups, although all three genes were expressed at lower levels than TH and, in the case of aldolase C (see below, Chapter 4) and β-tubulin, lower than in other brain stem neuronal
populations. The absence of any change in expression of TH mRNA is supported by the absence of an effect on these three genes and also by the lack of a significant correlation between disease duration and TH mRNA expression in individual cases. Moreover, the frequency distributions of TH mRNA hybridization signal in PD cases in this study do not suggest the existence of two populations of neurons, one moribund and one upregulated (see Figures 3.4 and 3.5). The absence of any gross change in expression of the other mRNA species in PD nigra shows that surviving nigral neurons retain their capacity to synthesise at least some peptides normally and strongly suggests that other aspects of cellular function may be preserved. It is of interest that the frequency distribution of aldolase C and β-tubulin hybridization signal in nigral neurons were less broad than was observed for TH mRNA expression, suggesting a much smaller degree of variation. This may reflect the possibility that TH mRNA, in common with other markers of synaptic activity is much more regulated than the housekeeping mRNA species. The absence of any change in the two glycolytic enzyme mRNAs suggests that ATP generation from cytosolic pathways is unaltered at the level of gene expression in PD.

3.5.6 Heterogeneity of TH mRNA expression

The heterogeneity of TH mRNA expression observed in these studies among closely adjacent nigral neurons, shown in the broad profile of the histogram of grain density frequency, is consistent with findings in other studies (Javoy-Agid et al, 1990). It has been suggested that the presence of neurons with very low levels of TH expression in PD may be indicative of neuronal morbidity, however, in this
study, melanised neurons with little or no TH mRNA expression were also seen frequently in control cases, including the two young controls. This was not an artefact due to variation in the sectioning plane of nigral neuronal profiles because the same pattern was seen in 25 μm sections in which a larger proportion of each profile would be present. Similarly, in both controls and PD cases, the intensity of TH immunoreactivity also varied between closely adjacent neurons in the same manner as mRNA signal (see e.g. Figure 3.1). This variability has been reported by other groups (Javoy-Agid et al, 1993, Kastner et al, 1993) who have proposed either that regulation of TH expression in PD could be effected through trans-neuronal regulation of dopaminergic activity by striatal neurons or that a low level of TH transcripts could reflect an increased turnover rate of the mRNA (Javoy-Agid 1990).

Similar variation in expression between closely adjacent neurons has been observed for other mRNAs, and their encoded peptides e.g. neuropeptide Y (Terenghi et al, 1987) and subregional variation in gene expression occurs in midbrain neurons. Yamada and colleagues observed heterogeneity of neurotensin receptor mRNA expression in post-mortem human and in rat midbrain. Neurotensin receptor mRNA was high in neurons of the nigra and VTA but mRNA signal was unevenly distributed between neurons of the dorsal and ventral tiers and VTA, with the highest levels of signal in the ventral tier and nucleus paranigralis (VTA) (Yamada et al, 1995). ISHH studies of the expression of TH and DAT mRNA in rat and mouse brain similarly showed that hybridization signal was not evenly distributed between subdivisions of the
midbrain dopaminergic population. Although the highest values for DAT and TH mRNA expression were found in medially located midbrain neurons, DAT mRNA expression in the VTA was lower than in medial regions of the nigra, while the VTA had the highest values for TH mRNA (Weiss-Wunder and Chesselet, 1991; Blanchard et al, 1994). This suggested that TH and DAT expression were able to be highly differentially regulated in subregional populations of dopamine neurons and the higher rates of TH mRNA expression were suggested to be related to the higher degree of colateralization and rates of burst-firing, which are seen in the medial nigra and VTA, compared to the lateral subregions. The authors proposed, therefore, that differential rates of TH expression could reflect the higher physiological demand on these neurons, with the need of greater transport rates of TH to terminal fields (Weiss-Wunder and Chesselet, 1991). However, although data from rat and mouse studies indicates pronounced regional variability in expression of dopaminergic markers, regulatory mechanisms would have to operate at the level of the individual neuron to produce the pattern of heterogeneity seen in the present studies.

With respect to the possible variation in demand on individual neurons, studies of dopaminergic neuronal firing in the rat nigra showed around one-third of neurons were silent in all preparations examined, including free-moving rats (Bunney et al, 1991). These non-firing neurons were thought to be inactive as a result of membrane hyperpolarisation, which could be reversed by systemic administration of dopamine antagonists or by excitation by cholecystokinin or glutamic acid. It is possible that similar firing patterns obtain in dopaminergic
neurons in the human nigra and that reduced TH mRNA expression (and, hence, dopamine synthesis) observed in some nigral neurons in PD is part of the normal spectrum of neuronal activity. The apparent heterogeneity of nigral TH expression in individual neurons, which was common to all the cases studied here, may indicate that the human nigra has a reserve capacity for dopamine synthesis which is able to compensate for cell loss in the early stages of the disease.

However, Damier and colleagues have recently proposed a compartmental analysis of nigral subregions based on the distribution of calbindin immunoreactivity in fibres in the nigral neuropil (Damier et al, 1999, I). Calbindin immunoreactive fibres are considered to represent the main striato-nigral projection, since this pattern of immunoreactivity is destroyed in Huntington’s disease and calbindin immunoreactive medium spiny projection neurons have been identified in human striatum (Kiyama et al, 1990). A matrix of calbindin-positive fibres was demonstrated in human nigral neuropil which ran through the full rostro-caudal extent of the midbrain, in a pattern similar to that seen in primates. Within this calbindin-rich matrix, the authors identified five separate, calbindin-poor islands, which were found reproducibly both in control and PD midbrain, which they termed “nigrosomes”. The distribution of TH immunoreactive (THIR) neurons in the midbrain both within and outside of the calbindin matrix was also reproducible between the 7 subjects studied. THIR neurons were found dorsal and dorso-lateral to the calbindin-rich matrix were designated as pars dorsalis and pars lateralis neurons. Within the region
defined by calbindin immunoreactivity, 60% of THIR neurons were found distributed through the matrix and the remaining 40% were densely-packed in the enclosed nigrosomes. The authors noted that distribution of THIR neurons and calbindin immunoreactivity differed in the human brain and those of rat and primates. In the rat most dopamine neurons were to be found dorsal to the calbindin-rich regions, in monkey brain some densely-packed dopamine neurons were interspersed into the calbindin-rich region but the pattern of invagination of dopamine neurons of the human midbrain into the calbindin matrix was much more complex than that seen in animal brain. It seemed likely that the striatonigral afferents projected more strongly to neurons of the calbindin matrix than those of the nigrosomes, although there was no other evidence to support this assumption (Damier et al, 1999, 1). Given the evidently high, regional variation in gene expression seen in studies of midbrain neurons it is possible that a high degree of variation between neurons which appear to be closely adjacent may, nevertheless, reflect their membership of different compartments of a complex nigral organisation.

3.5.7 TH mRNA in young controls

Although only two young control subjects were available for study and the result should be treated with caution, there was no significant difference in neuronal TH mRNA expression between these and the aged control group, suggesting that age alone may not lead to a decline in dopaminergic activity. This is congruent with studies which have indicated that melanised cell numbers may not decline with age in human subjects (van Domberg and ten Donkelaar 1991; Pakkenberg
et al, 1991; Muthane et al, 1997). Similarly, the heterogeneity of TH mRNA expression observed in the aged control group was also present in the two young subjects, indicating that this was not an effect of ageing.

3.6 Summary

There was no evidence for involvement of TH gene expression in determining the pattern of nigral neurodegeneration in PD and no upregulation of TH mRNA expression was found in melanised neurons in the nigral subregions examined. Non-melanised neurons did, however, appear to be upregulated with respect to TH mRNA expression in PD and this finding is supported by later studies of TH mRNA in neurons of the VTA. There was no effect of L-DOPA treatment on TH, β-tubulin or aldolase C mRNA expression and no evidence that expression of these genes was related to duration of illness. The normal rates of aldolase C, β-tubulin and PFKFB3 expression suggest that there is no general downregulation of peptide synthesis in surviving nigral neurons. This, and the absence of an effect of disease duration on TH mRNA expression, supports the view that the surviving nigral population in PD has some normal functions preserved. The heterogeneity of TH expression observed here may have physiological or anatomical significance.
CHAPTER: 4
EXPRESSION OF ND1 SUBUNIT mRNA IN DOPAMINERGIC NEURONS

The aim of these studies was to identify the midbrain cell types which expressed mRNA encoding the ND1 subunit of mitochondrial complex I and to determine whether the reduction in complex I activity observed in nigral homogenates in PD could be related to mitochondrial gene expression. The expression of nuclear-encoded NDUFV1 subunit mRNA was also examined. Expression of mRNA encoding the mitochondrial ND1 gene and the glycolytic enzyme, aldolase C were compared in different midbrain and brainstem neuronal populations, to determine whether expression of metabolic genes in nigral neurons differed from that in other groups.

4.1 Introduction

4.1.1 Complex I and PD

It is now well-established that mitochondrial respiration is compromised in substantia nigra in PD, through a specific reduction of the activity of mitochondrial complex I (Schapira et al, 1990a, Schapira et al, 1990b). Schapira and colleagues undertook extensive studies of the activity of subunits of the mitochondrial respiratory chain in tissue homogenates of post-mortem human brain and demonstrated reduced activity of complex I in PD substantia nigra in three separate studies. A study of 9 controls and 9 PD cases showed a 39% reduction of the activity of NADH cytochrome c reductase, which measures the combined activity of complexes I-III. Subsequently a selective
reduction of NADH-ubiquinone reductase (complex I) was demonstrated in PD nigral homogenates, which was confirmed by the observation of a reduced ratio of the activity of NADH cytochrome C reductase to the activity of succinate cytochrome C reductase, measures of the activity of complexes I-III and complex II respectively. The mitochondrial deficit did not appear to be related to reduction in cell numbers or reduced mitochondrial mass in PD nigra, since correction for citrate synthase activity, a marker for mitochondrial matrix, did not abolish it (Schapira et al, 1990a). There was no corresponding reduction in other brain regions examined. The activities of NADH ubiquinone reductases (complex I), succinate cytochrome c reductases (complex III) and cytochrome oxidase (complex IV) were measured in the caudate nucleus, medial and lateral globus pallidus, the cerebral cortex and cerebellum in a series of 6 controls, 7 PD cases and 7 MSA cases. Reduction of complex I activity was found in the substantia nigra but could not be demonstrated in the other regions; the activities of complex III and complex IV (cytochrome oxidase) were unaffected in all regions in PD. In addition to being region-specific, the change also appeared to be specific for PD, since MSA cases showed no change in nigral complex I activity, although levels of cytochrome oxidase activity and citrate synthase activity were lower, a reflection of neuronal and mitochondrial loss (Schapira et al, 1990b). In L-DOPA-treated MSA cases, with striato-nigral degeneration, nigral complex I activity was comparable with that of controls demonstrating that treatment with L-DOPA did not by itself induce the deficit (Cooper et al, 1995). A subsequent study with a larger number of controls and PD cases also demonstrated a reduction in complex I activity of 37% (Mann et al, 1992). The
close parallels between the deficit in mitochondrial respiratory activity in PD and the effects of MPP\(^+\) in humans and primates, i.e. that the effect in brain appeared to be confined to the nigro-striatal system, and the specificity of the damage to complex I, which left other mitochondrial complexes unaffected, suggested that the reduced complex I activity observed in PD nigra could be related to the primary disease process and might be the result of some endogenous or exogenous toxin with similar properties to MPP\(^+\) (Schapira 1994).

4.1.2 Possible nigral neurotoxins

The ability of MPTP to cause degeneration of nigral neurons has focussed attention on possible role of endogenous or exogenous nigral toxins in the aetiology of Parkinson’s disease. A large number compounds with structural resemblance to MPP\(^+\) has been screened for possible neurotoxicity, many of which occur naturally in plants and some foods. Tetrahydroisouquinolines (TIQs) and \(\beta\)-carbolines are thought to be able to act as substrates for reactions which could generate possible nigral toxins. TIQs are formed by condensation of dopamine and aldehyde and an enzyme catalysing this reaction has recently been demonstrated in human brain (Naoi et al, 1996a). \(N\)-methylation of both TIQs and \(\beta\)-carbolines gives rise to \(N\)-methylpyridinium analogues of MPP\(^+\) and these derivatives are selectively toxic to the dopamine system in rats (Marayuma et al, 1996; Naoi et al, 1996b). They also inhibit mitochondrial complex I activity with variable potency (Suizuki et al, 1988, Albores et al, 1990). \(N\)-methylated \(\beta\)-carboline derivatives have been reported in Parkinson’s disease in CSF.
(Matsubara et al, 1995) and, similarly, *N*-methyl tetrahydroisoquinoline has been reported to be accumulated in the nigrostriatal system and CSF of Parkinson’s disease patients (Maruyama et al, 1996, Maruyama et al, 1997). There exists, therefore, a strong theoretical possibility that either endogenous or exogenous neural toxins may be involved in neurodegeneration in Parkinson’s disease and that dopamine may be implicated in their synthesis.

4.1.3 Cellular location of the nigral complex I deficiency

The cellular location of the loss of complex I activity has not been determined. Complex I activity might, in principle, be reduced in nigral glia or in dopaminergic or non-dopaminergic neurons in the substantia nigra pars reticulata. The numbers of dopamine neurons in the nigra are low in comparison to glia and the reported reduction might be due to changes in glial respiration as much as to dopaminergic cell loss (Schapira et al, 1994). In considering that some part of the pathogenesis of PD might arise from the action of an MPP⁺-like neurotoxin, the identity of the cells contributing to the nigral metabolic deficit is important. There is evidence for this change being localised to dopamine neurons. Hattori and colleagues in an immunohistochemical study of human post-mortem midbrain, demonstrated reduced expression of complex 1 subunit proteins in melanised neurons in the substantia nigra in PD (Hattori et al, 1991). In addition, *in situ* hybridization studies of nigral expression of mRNA encoding the ND1 subunit of complex I, using in a small group of control subjects, have indicated that expression of this mRNA was strongest in nigral regions which show the greatest cell loss in PD,
i.e. in the ventro-lateral nigra, indicating that loss of these neurons could contribute the greater part of the reduction in enzyme activity (Ruberg et al, 1997). However, biochemical studies of complex I activity in nigral homogenates also indicated that individual nigral neurons might have compromised mitochondrial respiration (Schapira et al, 1990, 1994).

4.2 Study rationale and design

The expression of ND1 mRNA was examined and quantified in substantia nigra in matched groups of PD and neurologically-normal control cases. ND1 expression was also examined in matched case-control series in the locus ceruleus, and in pontine and cerebellar neurons, for comparison with changes in the substantia nigra. The abundance of both ND1 and aldolase C mRNA expression in nigral neurons were also compared with that in other midbrain and brainstem groups to examine the relative rates of mRNA expression in nigral neurons and as an indicator of the capacity of dopaminergic neurons to generate ATP by cytosolic respiration.

The majority of respiratory chain subunits are encoded by nuclear DNA, rather than the mitochondrial genome (Taanman J-W 1999). The expression of the nuclear-encoded 51 kDa NDUFV1 subunit of mitochondrial complex I was therefore studied in nigral neurons as an indication of whether down-regulation of nuclear ND1 gene expression might also occur in PD.
4.3 Materials and Methods

4.3.1 Probes

For the study of mitochondrial ND1, a 42-base oligonucleotide probe was used. The probe sequence:

TGTAGAGTTCAGGGAGAGTCGTCATATGTGGTTCTAGGA, was complementary to bases 3454 to 3495 of the human mitochondrial sequence encoding the ND1 sub-unit (Genbank database) and had homology only with the human ND1 sequence when examined by BLAST (Altschul et al, 1990).

For examination of the nuclear-encoded subunit, a 30 base oligonucleotide was used. The sequence AATCAGTTCTTCAAGGGCACACATCTC, recognised bases 4481-4510 of the human gene encoding the 51 kDa subunit of complex I (NDUFV1; accession number AF053069, Genbank database) but had no significant homology with any other sequence.

The ND1 probe was evaluated by northern hybridisation, using the protocols described in Chapter 2. Total RNA was extracted from cerebellum, cortex and pons and hybridised with the ND1 probe, labelled in this instance with $[^{35}S]$-dATP to a final specific radioactivity of $3.85 \times 10^5$ cpm/pmol and a final concentration in hybridisation buffer of $6.91 \times 10^4$ cpm/ml. Hybridisation was carried out overnight at 50 °C and film autoradiographs prepared using Hyperfilm. The NDUFV1 probe was not evaluated by northern hybridization.

Specificity of hybridization signal and background values for the probe were determined on serial sections, hybridized in parallel with the test sections in the
presence of a 100-fold excess of unlabelled probe. The possible contribution of
probe binding to mitochondrial DNA was also examined in serial sections of
substantia nigra from 6 controls and 10 PD cases hybridized following a 30
minute incubation in RNAase (50 \(\mu\)g ml\(^{-1}\) RNAase (Sigma) in Tris EDTA
buffer (100 mM Tris, 10 mM EDTA) pH 8.0 containing 50 mM NaCl).

4.3.2 Cases

For examination of substantia nigra, 10 PD and 10 control cases were studied.
The expression of the nuclear-encoded gene was examined in 4 control and 7
PD cases. ND1 expression in pontine neurons was examined in 8 PD and 5
control cases; expression in the locus ceruleus in 13 PD and 7 control cases.
ND1 expression in cerebellar Purkinje neurons was examined in 8PD and 8
control cases. Clinical and post-mortem details are shown in Table 4.1.

4.3.3 Nigral ND1 expression

Cryostat sections from midbrain were prepared as described above (Chapter 3).
Hybridised sections were exposed to both film and nuclear emulsion for
preparation of autoradiographs. Emulsion autoradiographs were quantified as
described above (Chapter 3) for the determination of TH mRNA in the nigra,
identifying the nigral region under low power and examining all neurons
encountered along the long axis of the defined region, provided that they met the
anatomical criteria described above. Between 20 and 30 melanised neurons, in a
Table 4.1  Post-mortem details of groups studied for complex I expression

<table>
<thead>
<tr>
<th>Brain region</th>
<th>study group</th>
<th>Sex</th>
<th>Age</th>
<th>pm delay (hours)</th>
<th>pH</th>
<th>duration (years)</th>
<th>L-DOPA (mg/24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ND1) substantia nigra</td>
<td>Controls</td>
<td>1F/9M</td>
<td>74 (63-84)</td>
<td>23.6 (5.5-50)</td>
<td>6.55 (6.21-6.79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PD cases</td>
<td>5F/5M</td>
<td>77.3 (67-88)</td>
<td>16.5 (5-30)</td>
<td>6.45 (6.3-6.6)</td>
<td>16.5 (5-40)</td>
<td>1078 (400-3500)</td>
</tr>
<tr>
<td>pons</td>
<td>Controls</td>
<td>4F/1M</td>
<td>75 (53-84)</td>
<td>26.1 (18-33)</td>
<td>6.58 (6.25-6.86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PD cases</td>
<td>3F/5M</td>
<td>75.5 (68-82)</td>
<td>20.15 (7.3-34)</td>
<td>6.48 (6.25-6.7)</td>
<td>17.5 (5-28)</td>
<td>669 (200-1050)</td>
</tr>
<tr>
<td>locus ceruleus</td>
<td>Controls</td>
<td>5F/2M</td>
<td>77.4 (53-86)</td>
<td>27.8 (13.5-53)</td>
<td>6.56 (6.25-6.86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PD cases</td>
<td>5F/8M</td>
<td>78.1 (62-84)</td>
<td>19.4 (4.5-34)</td>
<td>6.42 (6.25-6.7)</td>
<td>13.2 (5-28)</td>
<td>659 (300-1050)</td>
</tr>
<tr>
<td>cerebellum</td>
<td>Controls</td>
<td>4F/4M</td>
<td>77.5 (67-86)</td>
<td>22.3 (5.5-34)</td>
<td>6.46 (6.21-6.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PD cases</td>
<td>4F/4M</td>
<td>77.6 (72-83)</td>
<td>23.9 (4.8-49.6)</td>
<td>6.44 (6.3-6.6)</td>
<td>15.4 (3-40)</td>
<td>600 (300-1200)</td>
</tr>
<tr>
<td>(NDUFV1) substantia nigra</td>
<td>Controls</td>
<td>2F/2M</td>
<td>86.3 (82-91)</td>
<td>39.4 (23.5-53)</td>
<td>6.63 (6.54-6.79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PD cases</td>
<td>3F/4M</td>
<td>77.2 (73-82)</td>
<td>18.3 (11-34)</td>
<td>6.48 (6.39-6.7)</td>
<td>15.3 (5-27)</td>
<td>590 (300-1150)</td>
</tr>
</tbody>
</table>

There was no significant difference between groups for any variable of ND1 series; the NDUFV1 series was less well-matched, mean age values were significantly different (p = 0.01).
minimum of 5 separate fields, were examined in control cases and the largest number possible in PD tissue sections (between 8 and 35 neurons per case). Measurements were made over non-melanised cytoplasm. Background values were measured over 5-10 neurons hybridised in the presence of a one hundred-fold excess of unlabelled probe. In control cases in which the ventral and dorsal nigral neuronal tiers could be distinguished on film autoradiographs, measurements were made in both regions for comparison.

4.3.4 Nigral NDUFV1 expression

Expression of the nuclear encoded gene was studied in nigral neurons using the methods described above, accumulating values for between 20 and 30 neurons per case.

4.3.5 ND1 mRNA expression in pons and locus ceruleus

For analysis of pontine neurons, between 35 and 45 neurons per case from beside the pontine midline (paramedial pontine nucleus) were selected at random, using a computerised stage. For analysis of the locus ceruleus, its position was determined under low power magnification and all visible neurons were assayed, provided they met the anatomical criteria described above (para 3.3.5). Measurements were made over non-melanised cytoplasm. To compensate for the small number of neurons present in this nucleus, measurements were made from triplicate sections in PD cases and duplicate sections in controls.
4.3.6 ND1 mRNA expression in cerebellar Purkinje neurons

Cerebellar sections were cut from parasagittal blocks from the cerebellar hemispheres at the level of the dentate nucleus. Grain density was measured in 20 neurons on each of duplicate slides from at least two separate cerebellar folia. These were identified by following the granule cell layer border and assessing all Purkinje neurons encountered.

4.3.7 Comparison of nigral and brainstem ND1 and aldolase C mRNA expression

For comparison of ND1 and aldolase C mRNA expression in other neuronal nuclei of the midbrain and brain stem, measurements were taken from extra-nigral groups of neurons, identified under low-power magnification by their location and morphology. ND1 expression was assessed in a group of 9 control cases and aldolase C mRNA expression in 6 control cases; 30 neurons were analysed in each neuronal group and grain density values per cell calculated as above.

4.4 Results

4.4.1 ND1 probe

In RNA extracts from cerebellum the probe identified two mRNA transcripts, one at approximately 1 kb and the second, slower band at approximately 3 kb; both transcripts were present in all of the cases examined (see Figure 2.9 A and C). The two bands observed were of an approximately equal size, suggesting that the two transcripts were produced in roughly similar amounts. In mRNA extracts
from cortex and pons, however, the probe identified a single band at 1 kb, consistent with the known size of this transcript (Figures 2.9B, D and E).

Specificity of hybridization signal was demonstrated for all tissue sections used. Hybridization of serial sections in the presence of a one hundred-fold excess of unlabelled probe resulted in abolition of hybridization signal both on film and emulsion autoradiographs (see Figure 4.1C). Hybridization of nigral sections following incubation in RNAase showed that hybridization signal fell to levels comparable with those obtained in the presence of an excess of unlabelled probe, indicating that little of the hybridization signal in nigral neurons could be attributed to binding of the probe to mitochondrial DNA (figure 4.1D).

4.4.2 Distribution of ND1 mRNA

Melanised neurons of the substantia nigra expressed ND1 mRNA strongly. Hybridization signal was present on both film and emulsion autoradiographs (Figure 4.1 A and B). Neuropil was frequently labelled in areas immediately adjacent to these neurons but this was only seen in the substantia nigra pars compacta and was abolished by inclusion of a 100-fold excess of unlabelled probe, as was labelling of the neuronal perikaryon (Figure 4.1). It was therefore shown to be specific and assumed to indicate binding of probe to mRNA in dendritic mitochondria. Neuropil labelling was not so strong in PD cases as a consequence of the lower cell numbers and loss of nigral dendrites. Neurons of the ventral tegmental area (A10) and of the pars lateralis (A8) were also strongly
Figure 4.1
ND1 mRNA expression in midbrain neurons

A: Film autoradiograph (reversed image) of control midbrain hemi-section hybridized with ND1 probe, showing strong hybridization signal over the substantia nigra (SN) and IIIrd nerve nucleus (III\textit{n}), with weaker signal over the red nucleus (\textit{r}). Scale bar 0.25 cm.

B: Emulsion autoradiograph showing ND1 hybridization signal over melanised nigral neurons from a control subject. Glial nuclei (arrows) in this section are not associated with high levels of signal.

Scale bar 10 \textmu m.

C: Non-specific signal in neurons hybridized in the presence of a 100-fold excess of unlabelled probe; scale bar 10 \textmu m.

D: Residual signal in control neurons following pre-treatment with RNAase; scale bar 10 \textmu m.
labelled. Hybridization signal in nigral neurons was heterogeneous, with large variations in hybridization signal between closely adjacent neurons.

ND1 mRNA was identified in other neuronal groups of the brain stem and midbrain. Non-melanised neurons of the nigra, large and medium-sized neurons of the red nucleus (see Figure 4.1A), large motor neurons of the third and fourth nerve nuclei, the mesencephalic fifth nerve nucleus and pontine neurons were all labelled, pontine neurons most heavily. Neurons of the red nucleus were the least heavily labelled. Expression of ND1 mRNA in melanised nigral neurons appeared slightly lower than that of pontine neurons (see below). In locus ceruleus neurons, ND1 mRNA was heterogeneously expressed, as in the substantia nigra, with variable levels seen in closely adjacent neurons. Cerebellar Purkinje neurons expressed ND1 mRNA strongly, as did some neurons of the molecular layer; cerebellar granule neurons did not appear to be labelled.

Strong ND1 expression was found also in other brain regions. Strongly labelled neurons were found in striatum, in both lateral and medial globus pallidus, with weaker signal in neurons of the caudate and putamen. Strong labelling was also seen in some thalamic neurons and in deep layer neurons of both temporal and frontal cortex. High levels of signal were observed in the parahippocampal gyrus and in hippocampal pyramidal neurons. In contrast, neurons of the subiculum were less heavily labelled and there were low levels of labelling in the dentate gyrus.
4.4.3 Distribution of NDUFV1 mRNA

Nigral neurons expressed low levels of this mRNA. Hybridization signal from this probe was seen in melanised neurons but was quite low in comparison with background. No glial labelling was observed. Stronger signal was seen in pontine neurons and in neurons of the colliculus.

4.4.4 Quantitation of ND1 expression in substantia nigra

Grain density values per melanised neuron were between 0.063 and 0.159, mean 0.104, in controls and 0.045 and 0.124, mean 0.078 in PD cases. Background values, i.e. hybridization in the presence of a 100-fold excess of unlabelled probe, were between 0.006 and 0.037 in controls and 0.011 and 0.019 in PD cases. There was no relationship between the grain density value per cell and disease duration or L-DOPA dosage in the PD group (Figure 4.2) when examined by regression analysis ($r = 0.36$ and 0.11 respectively).

Frequency histograms from pooled data from nigral neurons (see Figure 4.3) showed that grain density values were normally distributed in both control and PD groups but that grain density values from the PD group were significantly reduced in comparison with controls ($p < 0.001$, Kolmogorov-Smirnov test).

Ventral and dorsal nigral neuronal tiers could be distinguished in 7 control cases; the mean values for melanised neurons of the ventral and dorsal tiers were 0.106 (0.065 - 0.168) and 0.122 (0.064 - 0.168) respectively. Both histograms (Figure 4.4) showed normally-distributed values and there was no difference in
cumulative frequency between the two populations \( p > 0.05 \), Kolmogorov-Smirnov test.

### 4.4.5 Comparison with other neuronal populations

ND1 mRNA grain density values for other neuronal groups were compared with the nigra in control tissue in a separate quantitation, as described above. The grain density value per cell for nigral neurons in this series was 0.092 (0.051 - 0.118, \( n = 9 \) cases). The grain density value per cell for large to medium neurons of the red nucleus was lower 0.040 (0.019 - 0.075, \( n = 6 \) cases) than the substantia nigra; pontine neurons (mean 0.099, range 0.068 - 0.195, \( n = 4 \) cases) expressed similar levels to the substantia nigra. The mean value per cell for large motor neurons of the third, fourth and fifth cranial nerves was 0.068 (0.026 - 0.103, \( n = 6 \) cases).

### 4.4.6 Quantitation of ND1 mRNA expression in pontine neurons and locus ceruleus

Grain density per cell in pontine neurons was between 0.132 and 0.268 in controls and 0.144 and 0.27 in PD cases with mean values of 0.205 and 0.197 respectively. Grain density frequencies in these neurons were normally distributed, with a more compact curve than in the substantia nigra, indicating a less variable range of expression than in nigral neurons (Figure 4.5A). The cumulative frequencies of pontine histograms (Figure 4.5B) were not significantly different \( p > 0.05 \), Kolmogorov-Smirnov test). In melanised
Figure 4.2
Disease variables and ND1 mRNA expression

A

B

A: Disease duration (years from first report of symptoms) and ND1 mRNA values per cell from individual cases. Regression analysis showed no relationship between duration and ND1 expression ($r = 0.107$).

B: L-DOPA dosage (mg/24 hours) recorded during the final 12 months of the disease and ND1 mRNA values per cell from individual cases. Regression analysis showed no relationship between L-DOPA dose and ND1 expression ($r = 0.11$).
Figure 4.3
ND1 mRNA expression in melanised nigral neurons

A

Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 175 PD and 178 control neurons. Analysis of cumulative frequency curves showed that the two populations were significantly different (p < 0.001, Kolmogorov-Smirnov test).
Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 95 dorsal tier and 112 ventral tier melanised neurons in control cases. Analysis of cumulative frequency curves showed that grain density values in two populations were not significantly different (p > 0.05, Kolmogorov-Smirnov test).
neurons of the locus ceruleus grain density per cell was between 0.049 and 0.267 in controls and 0.102 and 0.177 in PD cases, with mean values of 0.166 and 0.149 respectively; cumulative frequencies of control and PD populations were not different (p > 0.05, Kolmogorov-Smirnov test).

4.4.7 Quantitation of ND1 mRNA expression in cerebellar Purkinje neurons

The mean grain density value in cerebellar Purkinje neurons was 0.197 (0.102 - 0.165) in controls and 0.243 (0.18 - 0.286) in PD cases. Grain density frequency histograms and cumulative frequencies for Purkinje neurons are shown in Figure 4.7. Pooled grain density values were normally distributed. Grain density values for PD neurons were increased with respect to controls and the cumulative frequency distribution of the two curves was significantly different (p<0.001, Kolmogorov Smirnov test).

4.4.8 Quantitation of NDUVF1 in nigral neurons

Mean values for PD and control groups were 0.083 and 0.085 respectively. Hybridization signal was low in comparison to background but grain density frequency histograms from duplicate serial sections hybridized in the presence of excess unlabelled probe were significantly lower than control hybridizations (p< 0.01, Kolmogorov-Smirnov test) in all cases, demonstrating that specific hybridization signal was present in nigral neurons. Comparison of PD and control populations showed that there was no significant difference in grain density frequency between the two groups (Figure 4.8).
Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 502 PD and 224 control pontine neurons. Analysis of cumulative frequency curves showed that the two populations were not significantly different \( (p < 0.05, \text{ Kolmogorov-Smirnov test}) \).
Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 242 control and 354 PD locus ceruleus neurons. Analysis of the cumulative frequency curves showed that the two populations were not significantly different (p > 0.05, Kolmogorov-Smirnov test).
Figure 4.7
ND1 mRNA expression in cerebellar Purkinje neurons.

A

Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 286 control and 315 PD Purkinje neurons, showing increased in ND1 mRNA expression in PD. Analysis of cumulative frequency curves showed that the two populations were significantly different (p < 0.001, Kolmogorov-Smirnov test).
Grain density frequency histograms (A) and cumulative frequency curves (B) derived from pooled data from 245 PD and 100 control melanised nigral neurons. Analysis of cumulative frequency curves showed that PD and control populations were not significantly different (p > 0.05, Kolmogorov-Smirnov test).
4.4.9 Quantitation of aldolase C mRNA expression in control midbrain

Melanised nigral neurons expressed low levels of aldolase C mRNA relative to other large neurons of the brain stem. Mean grain density per cell was 0.109 (0.072 - 0.141, n = 5 cases) in nigral neurons, 0.23 (n = 1) in large neurons of the red nucleus, 0.25 (0.151 - 0.25, n = 4) in cranial nerve neurons and 0.188 (0.084 - 0.282, n = 3) in pontine neurons.

4.5 Discussion

4.5.1 ND1 probe

Hybridization signal in sections hybridised with this ND1 probe were specifically labelled, as indicated by the absence of hybridization signal in the presence of a one hundred-fold excess of unlabelled probe. The two mRNA transcripts demonstrated in northern hybridizations have been described previously. Kauffman and colleagues examined mitochondrially-encoded mRNA by northern hybridization in muscle, heart and kidney from patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) and two control cases. In each tissue type examined, a probe for ND1 recognised a 1 kb band, the transcript of ND1 and a second band at 2.6 kb, representing the RNA 19 transcript encoding 16s rRNA+tRNA$^{\text{Leu}}$ + ND1. This transcript was present in control kidney and liver and in all MELAS tissue samples, in which the transcription of RNA 19 was increased. Quantitation of hybridization signal showed that in control kidney and liver, RNA 19 transcripts were about one tenth of the level of ND1 transcripts (Kaufmann et al, 1996). The transcripts recognised by this ND1 probe in cerebellar mRNA are of similar
size, although the second band appeared slightly larger than that described for kidney and liver and suggest that brain mitochondrial ND1 is transcribed in a similar, if not identical, manner to that of heart, kidney, liver and muscle but is nevertheless variable between brain regions. The absence of the second transcript in pons and cortex indicate that mitochondrial mRNA transcription is differentially regulated in brain. In contrast to the earlier findings on muscle, heart, liver and kidney, the proportions of the two cerebellar transcripts appear to be roughly equal in the two subjects examined here. This may be due to degradation of the ND1 transcript relative to the RNA19 transcript in post-mortem brain or may represent a brain-specific pattern of transcription.

4.5.2 Distribution of ND1 mRNA expression in midbrain

ND1 was strongly expressed in dopamine neurons of the SN and no evidence of glial labelling in the nigra or in surrounding regions was observed in this series of cases. Glia have fewer mitochondria than neurons and it is likely that small amounts of ND1 mRNA and complex I peptide, are present in glia which are undetectable by these methods. Complex I activity could therefore, also be reduced in glia which are a larger proportion of the total cell numbers in the nigra than dopaminergic neurons (which are thought to comprise only around 3% of the total) and could therefore be a significant source of the nigral deficit. Nigral ND1 labelling, however, was sufficiently high in dopaminergic neurons for the nigral neuronal death in PD to be reflected in a massive loss of nigral ND1 mRNA. The reduction in complex I activity in PD described by Schapira and colleagues was corrected for possible loss of mitochondrial mass by
standardisation to levels of citrate synthase, a marker for the mitochondrial matrix (Schapira et al, 1990, Cooper et al, 1995). Given that neurons have very numerous mitochondria, citrate synthase can be considered surrogate marker for mitochondrial and neuronal numbers. The measured reduction in complex I activity (37%) persisted when values were corrected for citrate synthase values (30%), suggesting that complex I activity could be reduced at the cellular level in dopamine neurons. The value from nigral homogenates, however, has to be regarded as an average of the activity in different nigral cell types.

Hattori and colleagues (Hattori et al, 1991) examined the expression of oxidative phosphorylation complexes in the nigra by immunohistochemistry and showed that in PD nigra about half the surviving neurons expressed reduced staining for complex I, judged subjectively. Reduced staining was also seen in a small proportion of melanised control neurons, although the numbers evincing reduced staining in PD cases exceeded those in control cases by a factor of ten. In oculomotor neurons, present on the same sections all cells were equally stained in both control and PD cases. Complex III and complex IV immunoreactivity appeared to be unaffected, while about half the samples studied showed some reduction of complex II immunoreactivity. Insufficient immunoreactivity was detected in glia for a judgement to be made about the involvement of glia in the nigral deficit. Although the study used subjective criteria to judge the levels of immunoreactivity, nevertheless the findings are consistent with the enzyme activity studies and point to reduced expression of complex I protein in individual neurons in PD.
The results of this study indicate that ND1 mRNA expression is reduced in PD nigra at the cellular level by a value around 25%, derived from mean values per cell in individual cases. A direct correlation between complex I mRNA expression, peptide expression and complex I enzyme activity cannot be assumed but this is consistent with the findings on peptide expression. The three sets of observations, on enzyme activity, peptide expression and mRNA expression support the view that the observed decline in nigral metabolic activity could result in some degree from reduced transcription or reduced stability of ND1 mRNA in dopamine neurons.

4.5.3 Regulation of mitochondrial gene expression

Mitochondrial mRNA expression is not thought to be much regulated; mitochondrial DNA lacks introns and regulatory sites for gene transcription. However, in these studies, expression of ND1 mRNA in large neurons of the red nucleus was low in comparison with that of nigral neurons in the same tissue section, indicating differential cellular expression of mRNA in different neuronal populations and possibly a lower intrinsic rate of ND1 transcription in red nucleus neurons. Similarly, the finding that ND1 mRNA expression was decreased in nigra in PD and increased in cerebellar Purkinje neurons in PD cases demonstrates that ND1 transcription could be relatively responsive to alteration.

Variation in transcription of mitochondrial mRNA encoding COX I and COX II subunits of cytochrome oxidase have been demonstrated in Alzheimer’s disease.
Studies of the neuronal oxidative phosphorylation pathway in Alzheimer’s disease have implicated altered complex IV (cytochrome oxidase, CO) activity in neurodegeneration and both mitochondrial and nuclear complex IV peptides have been reported to be reduced in cerebellar Purkinje cells in Alzheimer’s disease patients relative to age-matched controls (Ojaimi et al, 1999). Similarly, Kish and colleagues, demonstrated that the mitochondrially-encoded (COX I and COX II) and nuclear-encoded (COX IV, COX Vc) subunit peptides of cytochrome oxidase were all significantly reduced (between 20 and 50%) in temporal and parietal cortex in Alzheimer’s disease cases. This reduction was specific to the affected regions of cortex in AD; peptide levels in the occipital cortex were lower (12 to 17%) though not significantly so. The cortical reduction in CO subunit peptides in AD was compared with cases of Friedreich’s ataxia, where reduction of COX I and COX II levels by 20-30% could be demonstrated in the cerebellar cortex but not in the cerebral cortex, evidence for disease-specific regulation of mitochondrial proteins in affected regions of the brain (Kish et al, 1999). Expression of mitochondrial and nuclear mRNAs encoding cytochrome oxidase and NADH dehydrogenase were also examined in hippocampus, parietal lobe and cerebellum in Alzheimer’s disease. Altered expression of COX I relative to COX IV mRNAs and a decrease in ND4 and ND15 subunits of complex I were demonstrated in parietal lobe but not in cerebellum indicating that reduced expression of mRNA was implicated in alteration of oxidative metabolism in Alzheimer’s disease (Aksenov et al, 1999). The parallel between these changes and those observed in PD brain suggests that regional variation in mitochondrial mRNA transcription may be a feature of
neurodegeneration in both disease states. The cellular substrate for these changes is unknown but may involve either direct alteration of mitochondrial transcription or mRNA stability or, as proposed to explain the increase in RNA19 observed in MELAS, that the altered transcription rate results from variation of the number of templates for transcription and reflects changes in mitochondrial number (Kaufmann et al, 1996).

4.5.4 Specificity of changes in PD nigra

The studies of expression of mRNA encoding tyrosine hydroxylase and beta tubulin, described in Chapter 3, employed the same methods as those used here for analysis of gene expression but demonstrated that rates of TH, aldolase C and beta-tubulin transcription were unaltered in melanised nigral neurons in PD. Thus, the reduction in ND1 transcription is unlikely to be the direct result of degenerative changes in surviving nigral neurons or of the methods used to estimate hybridization signal. Individual values (grain area per cell) did not correlate either with levels of L-DOPA administration or disease duration, as shown in the TH studies described above, and suggesting that the decline in ND1 mRNA expression was not induced by L-DOPA treatment. This is consistent with other post-mortem studies of complex I activity, which was shown to be unaffected in the midbrain of L-DOPA-treated cases of MSA, in spite of the presence of striato-nigral degeneration. Similarly, assay of complex I activity in the striatum of L-DOPA-treated PD cases showed that it was not reduced in comparison with controls (Cooper et al, 1995). The absence of any reduction of ND1 mRNA transcription in either pontine neurons which also express high
levels of ND1 mRNA, or in neurons of the locus ceruleus is also evidence for the lack of an effect of therapeutic L-DOPA on transcription of this mitochondrial gene.

4.5.5 Nigral and brain stem ND1 mRNA expression

ND1 mRNA expression, like that of TH, was highly variable between closely adjacent neurons in control and PD cases, resulting in a histogram with a broad spread of values. In contrast, the values for ND1 expression in pontine neurons was less broadly spread. The pattern observed here for ND1 mRNA is similar to that of TH mRNA in nigral neurons (see Chapter 3) and is consistent with the immunohistochemical findings of Hattori and colleagues who observed variable ND1 peptide expression in a study of the nigra (Hattori et al, 1991). Half of surviving nigral neurons in PD cases and approximately 3% in control nigra demonstrated weak or no immunostaining with the antibodies used. In contrast, neurons of the oculomotor and red nucleus did not show the same variability. The authors concluded that this did not appear to be an artefact but might be characteristic of the substantia nigra. It is nevertheless hard to understand why these neurons should express variable amounts of complex I peptide, given that they are highly oxidation dependent and it is unlikely that individual cells would survive without complex I activity (Schapira et al, 1994).

It is possible that these changes are transient and reflect demand on individual neurons, perhaps as a response to variable input. Ca\textsuperscript{2+} entry into mitochondria as a result of accumulation in the cytoplasm during neuronal activity, can produce
lasting increases in the activity of Ca\textsuperscript{2+} -sensitive matrix tricarboxylic acid cycle enzymes, which is thought to provide a means of linking ATP production with physiological activity (Hansford 1998). The wide variation in neuronal mRNA expression was common to both control and PD populations, and although the PD curve was strongly shifted towards the negative end. Nevertheless PD cell values showed an overlap with control values which was also evident in the enzyme activity studies of Schapira and colleagues, indicating that individual neurons express a broad range of values, even in disease states.

In contrast to ND1, expression of aldolase C appeared to be relatively low in nigral neurons compared to other neuronal groups. This may indicate that melanised substantia nigra neurons have an intrinsically lower rate of cytosolic respiration but systematic quantitative study of mRNA encoding enzymes of the glycolytic pathway, peptides and glycolytic enzyme activity would be necessary to confirm this possibility. If indeed nigral neurons do have lower rates of glycolysis this might render them more vulnerable to alteration of mitochondrial respiration caused by toxins or oxidative stress.

There was no difference in complex I mRNA expression between dorsal and ventral neuronal tiers, indicating that the preferential vulnerability of the ventral tier is unlikely to be related to metabolic activity. The finding that NDUFV1 mRNA levels were unchanged indicates that expression of some nuclear- encoded genes for ND1 may be unaffected in PD and that changes could be confined to mitochondria. It is of interest that neurons of the locus ceruleus, which is also
a melanised catecholaminergic nucleus, did not appear to express lower levels of ND1 mRNA. It is unlikely, therefore, that reduced complex I expression is a primary cause of cell death, given that the locus ceruleus undergoes extensive neurodegeneration in PD, although it is also possible that the two catecholaminergic populations are simply differently affected. This finding also makes it unlikely that downregulation of ND1 expression is the result of oxidative stress related to the formation of neuromelanin (Graham 1978, 1979, Youdim et al, 1989).

4.5.6 Mitochondrial gene abnormalities and the nigral complex I deficit

The possibility that mtDNA abnormalities are responsible for the deficit in complex I activity has been extensively explored. Fusion studies, using platelet mitochondria from PD patients with a platelet complex I deficit introduced into mitochondria-depleted ρ⁰ cells showed that the resulting PD ‘cybrids’ continued to express a 25% deficit in mitochondrial complex I activity. These studies also demonstrated a strong correlation between complex I activity in platelets and that in the resulting cybrid cells (Swerdlow et al, 1996; Gu et al, 1998). These findings were direct evidence that the reduction in complex I activity in some PD cases was determined by mtDNA but specific abnormalities which could account for this have not so far been identified. Schapira and colleagues examined the complex I polypeptide profile of nigral homogenates and found no difference between control and PD nigra (Schapira et al, 1990c). Similarly, an examination of the immunohistochemical profile of respiratory complexes in post-mortem PD striatum (Mizuno et al, 1989) found no evidence for any
alteration in mitochondrial proteins which were consistent with change in the mitochondrial genome. Studies of mtDNA structure using Southern blotting (Lestienne et al, 1990; Schapira 1990c) were also not able to identify any PD-specific abnormality of mtDNA in brains of PD patients. However, use of PCR showed the presence of the so-called common 5kb deletion in brain mtDNA from both PD and elderly control subjects (Mann et al, 1992, Lestienne et al, 1991, Ikebe et al, 1990). This 5kb deletion has, however, been shown to increase in the brain with normal aging (Corral-Debrinski et al, 1992, Simonetti et al, 1992) and the presence of changes in the mitochondrial genome increases with age. Thus accumulation of these defects may be one factor in neuronal death in PD but not one specific to the disease.

The reduction of complex I mRNA expression did not progress with the course of the disease in the cases studied here. Thus, this finding would support the possibility that there is a pre-existing abnormality of mitochondrial DNA in PD. It is also possible that the absence of a correlation with disease duration reflects dependance on oxidative phosphorylation in the nigral population and that neurons with lower levels of expression of ND1 mRNA do not survive.

It has been proposed that decreased complex I activity may be secondary to oxidative damage in nigral neurons or the action of an endogenous neurotoxin. There is no direct evidence, however, that such factors are associated with a decline in mRNA expression and further studies would be necessary to clarify the role that they play. The results of the present study suggest that the reduction
in mitochondrial respiratory chain activity might be attributed in part to a
decline in transcription of ND1 mRNA but that this is not related to oxidative
stress from neuromelanin formation.

4.6 Summary
These studies offers direct evidence for high levels of expression of ND1 mRNA
in melanised nigral neurons and for a specific decrease in ND1 mRNA
expression in which may contribute to the reduction in complex 1 activity
observed in homogenates of PD nigra. This decrease was unrelated to levels of
L-DOPA administration in the subjects studied or to duration of disease. mRNA
for the nuclear-encoded NDUFV1 subunit was unaltered in PD cases suggesting
that there is no general down-regulation of mitochondrial gene expression.
Further studies would be necessary to determine whether the ND1 subunit is
specifically affected. Brain stem neurons, expressed variable amounts of ND1
mRNA. Levels in nigral neurons were high but levels of mRNA encoding
aldolase C were lower in melanised nigral neurons than in other populations,
suggesting that the metabolic characteristic of nigral neurons may be different
from those of other brain stem neurons. No reduction in ND1 mRNA expression
was seen in the other brain stem neuronal groups examined, i.e. pontine neurons
and the locus ceruleus, supporting the view that the observed deficit in ND1
enzyme activity is specific to nigral neurons in PD. However, ND1 mRNA
expression was increased in Purkinje neurons in PD, suggesting that other
metabolic alterations may take place.
Chapter 5:

STUDIES OF DNA FRAGMENTATION IN MIDBRAIN CELLS

The studies described here were undertaken to assess the applicability of terminal deoxynucleotide transferase-mediated dUTP-biotin 3’ end labelling (TUNEL) histology to identify apoptotic neurons in rapidly-frozen midbrain tissue from PD cases, and to assess whether apoptosis might be involved in the death of nigral neurons PD. The results of this study have been published (Appendix 1)

5.1 Introduction

5.1.1 Evidence for apoptotic cell death in PD nigra

Studies using TUNEL to identify apoptotic neurons in PD nigra have reported conflicting results. Mochizuki and colleagues (Mochizuki et al, 1996) found positive 3’ end-labelling of dopaminergic neurons in late-onset PD cases and Tompkins and colleagues (Tompkins et al, 1997) demonstrated TUNEL labelling of neurons, combined with morphological changes consistent with apoptosis, in both control and PD substantia nigra. Also using TUNEL labelling, Kosel and colleagues (Kosel et al, 1997) and Wullner and colleagues (Wullner et al, 1998) both demonstrated labelling of a small number of neuronal nuclei in PD and control nigra. Tatton employed a fluorescent double-labelling technique, combining TUNEL with use of the cyanin dye YOYO and demonstrated a similar level of labelling in both control and PD nigra (Tatton et al, 1998). Using only electron microscopy, Anglade and colleagues examined 3 cases of confirmed PD and demonstrated that 3-4 % of neurons had
morphological changes consistent with apoptosis (Anglade et al, 1997). In contrast, other laboratories employing TUNEL histology to detect DNA fragmentation were unable to demonstrate labelling in surviving nigral neurons in PD (Dragunov et al, 1995, Banati et al, 1998) and, more recently, Jellinger undertook an examination of PD, DLB and control nigra and found no convincing TUNEL positivity in any neuron (Jellinger 2000).

It is thought, however, that DNA fragmentation identified in situ may be a non-specific marker for apoptotic cell death (see above, para. 1.3.1). It has been reported that techniques for identifying the DNA double strand breaks, a marker of DNA fragmentation in apoptosis, may also identify single strand breaks which are typical of necrotic cell death (e.g. Charriaut-Marlangue et al, 1995, Lucassen et al, 1997). Endonuclease activation is thought to be an early component of the apoptotic process and it is not clear whether cells are committed to die once signs of DNA fragmentation appear. Studies of apoptotic cell death in the brain have, therefore, employed additional markers to confirm the presence of apoptotic cell death in PD and other neurodegenerative conditions. For instance, in Tomkins and colleagues’ study of apoptosis in nigral neurons in PD, the authors used digoxigenin-tagged dUTP, visualised with a fluorescein-labelled anti-digoxigenin antibody for in situ end labelling as an aid to identify apoptotic nigral neurons, and demonstrated DNA fragmentation in a large proportion of nigral neurons, which also showed structural changes at the light microscope level. In addition, the authors examined ultrastructural changes in neurons, and found a similarly large proportion of neurons in PD with ultrastructural changes
consistent with apoptosis. However, both ISEL positivity and morphological change were observed to a smaller degree in control nigral neurons (Tomkins et al, 1997). In contrast, studies of cortex in Alzheimer’s disease, while showing large numbers of cortical neurons undergoing DNA fragmentation, demonstrated that these neurons were only rarely positive for other markers of apoptosis, including apoptosis-specific protein (c-Jun/AP1) and caspase 3 (Stadelmann et al, 1998, 1999). Other studies of Alzheimer’s disease brain, however, reported increased Bcl2 and caspase 3 immunoreactivity in apoptotic neurons and glia, which had been identified by in situ labelling of DNA fragmentation (Masliah et al, 1998).

5.1.2 Experimental models of apoptotic cell death

Apoptosis has been observed in animal models of nigral cell death in PD, although results from different laboratories are, again, contradictory. Apoptotic neuronal death in substantia nigra pars compacta in developing rats was shown to follow the intrastriatal injection of 6-OHDA (Marti et al, 1997) and treatment of immature rat striatum with the excitotoxin, quinolinate also brought about increased dopaminergic neuronal death in the nigra with the morphological characteristics of apoptosis and DNA fragmentation (Macaya et al, 1994). However, apoptosis was not seen following intranigral injection of 6-OHDA in adult mice (Jeon et al, 1995) or in the substantia nigra in adult murine weaver mutations (Oo et al, 1995) in which dopaminergic cell degeneration occurs late (at postnatal days 24-25) indicating that the presence of apoptotic cell death in the nigra was associated with early development. Jackson-Lewis and colleagues
followed cell death in an acute MPTP mouse model of PD and were unable to identify apoptosis either by morphology (silver staining) or by in situ labelling of DNA fragmentation despite significant nigral cell death (Jackson-Lewis et al, 1995). Nevertheless, an increase in expression of the pro-apoptotic protein Bax was observed in a study of the nigra of adult mice treated with MPTP (Hassouna et al, 1996) and in a chronic low-dose MPTP mouse model, Tatton and colleagues demonstrated apoptotic nigral neuronal death using ISEL and acridine orange staining (Tatton et al, 1997).

Thus, although some histological studies have reported apoptosis in the nigra, both in PD and in control brains, and this evidence is supported to a degree by experimental findings, the precise nature of cell death in the nigra in PD is still unclear.

5.2 Study rationale and design
TdT-mediated dUTP-biotin 3' end-labelling (TUNEL) histology was employed to detect DNA fragmentation in situ in post-mortem human midbrain from a large series of parkinsonian and control cases, with the aim of determining whether apoptotic signs were more common in PD than control nigra. The presence of TUNEL labelling was assessed with respect to tissue pH, which is known to correlate with ante-mortem brain hypoxia (Hardy et al, 1985). A small group of cases with other movement disorders: multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and incidental Lewy body disease (ILBD) were included in the study, for comparison with PD.
5.3 Materials and methods

5.3.1 Cases studied

The midbrain series studied comprised 16 pathologically-verified PD cases, 14 control cases without brain pathology and 6 individuals with other parkinsonian disorders, all of which were pathologically verified: MSA, 4 cases; PSP, 1 case; ILBD, 1 case. All PD cases were verified as described above (para 2.3.2) and showed marked loss of melanised neurons of the substantia nigra, with Lewy bodies in surviving neurons. MSA was identified by the presence of widespread alpha-synuclein and ubiquitin-positive glial cytoplasmic inclusions. Significant nigral cell loss was present in all MSA cases. PSP was identified by the presence of characteristic widespread tau-positive intraneuronal tangles in cortical, subcortical and brainstem nuclei. Screening of control cases showed that none had any significant neuropathology. Clinical and post-mortem details of cases are shown in Table 5.1.

5.3.2 TUNEL histology

TUNEL labelling was carried out by adaptation of published methods (Gavrieli et al, 1992; Su et al, 1994). All reagents were prepared in sterile (autoclaved) water using sterile/baked plastic and glassware. Gloves were worn throughout the procedure to minimise contamination by DNAases (Wolfe et al, 1996). Preliminary examination of human ileum, which had undergone long-term fixation in formaldehyde established that TUNEL signal could not be detected in formalinised, wax-embedded tissue. Flash-frozen tissue sections were therefore used for these studies. 12μm cryostat sections of flash-frozen midbrain
Table 5.1
Clinical details of cases: DNA fragmentation study

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<th>diagnosis</th>
<th>duration (years)</th>
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MD, movement disorders. Diagnosis (neuropathological evaluation carried out as described in Methods) PD, idiopathic Parkinson's disease; ILBD, incidental Lewy body disease; MSA, multiple system atrophy; OPCA, olivo-ponto-cerebellar atrophy; PSP, progressive supranuclear palsy; SND, striatonigral degeneration. Cause of death (as stated on death certificate) Ca., carcinoma; LVF, left ventricular failure; IHD, ischaemic heart disease. Duration of illness, years from first diagnosis. U.k., unknown.

Mean age, PD group: 76.6 (67 to 81) years; other MD: 71 (67 to 81) years; control group: 79.5 (67 to 90) years. L-DOPA, estimated from most recent clinical notes, varied between 200 and 2800 mg/24 hours with a mean value of 740 mg/24 hours. No clinical details were available for controls.
were warmed to room temperature for 10 minutes, fixed in 1.0% glutaraldehyde in 4% paraformaldehyde in PBS (154 mM sodium chloride, 0.89 mM potassium dihydrogen orthophosphate, 5.7 mM di-sodium hydrogen orthophosphate pH 7.4) for 5 minutes at 0°C, washed in PBS and dehydrated through graded alcohols. Wax-embedded sections (from rat ileum) were de-waxed in xylene and washed in 100% ethanol. Endogenous tissue peroxidases in all preparations were exhausted by incubation in 0.3% hydrogen peroxide in methanol. All tissue sections were rehydrated though graded alcohols before TUNEL labelling; wax-embedded sections were additionally incubated with 20 μg ml⁻¹ proteinase K (Gavrieli et al, 1992) in Tris buffer.

3’ end-labelling was carried out using 1.0μM dUTP-biotin (Boehringer Corporation) and 300 units ml⁻¹ terminal deoxynucleotide transferase in cacodylate buffer (both from Promega Corporation) at 37°C, for 60 minutes. Following the labelling reaction, tissue sections were washed in 1x SSC, 15 minutes at room temperature and incubated sequentially in 2% bovine serum albumin for 10 minutes at room temperature, distilled water and PBS, followed by avidin-peroxidase complex (Amersham UK) 1: 20 in PBS for 30 minutes at 37°C. Binding of streptavidin was visualised by reaction with aminoethyl carbazole (AEC), 20μg ml⁻¹ in 0.05% v/v N’ N’ dimethyl formamide (Sigma) in acetate buffer (0.03M acetic acid, 0.07M sodium acetate, pH 5.0) containing approximately 0.03% hydrogen peroxide as catalyst) for 10 minutes at room temperature. Following conclusion of the chromogen reaction, the slides were washed in distilled water, counterstained with Mayer’s haematoxylin (Stevens
1982) and mounted in aqueous mountant.

5.3.3 Controls for TUNEL histology

Rat ileum was examined as a control for the validity of using flash-frozen tissue. Both fixed, wax-embedded and frozen sections of rat ileum stained in parallel, showed the expected pattern of labelling. Cell nuclei at the apical ends of villi were strongly labelled with AEC chromogen (Figure 5.1 A, B) and labelled nuclei were also seen in large clumps in the luminal space of the ileum, as described previously (Gavrieli et al, 1992). Fewer were seen lower down the villi or in the crypts (Figure 5.1A). Flash frozen rat ileum section were also examined as controls for the specificity of the reaction; positive and negative sections were run in parallel with the human sections for every labelling. Human sections were discarded if the rat controls were not satisfactory. Negative controls for human midbrain were examined in parallel for all cases; serial tissue sections were incubated in the absence of TdT enzyme and some in TdT in the absence of its biotin substrate. No labelling of rat ileum (Figure 5.1C) or human tissue was seen in the absence of enzyme or substrate.

5.3.4 Analysis of tissue sections

Tissue sections were examined under brightfield illumination. The extent of the nigra in each mid-brain hemi-section was delineated at low power (x 45) and outlined on the slide. The numbers of neurons containing labelled nuclei and the total number of neurons were counted at x 200 magnification throughout the nigral region using an eyepiece grid as a guide. Labelling of neuronal nuclei
was verified by examination at x400 or x1000 (oil immersion) and the percentage of labelled neurons was calculated. A randomly-selected proportion (one-third) of tissue sections were repeated to test the reproducibility of neuronal labelling. Numbers of labelled glia were also assigned a value: 0, no labelled glia; 1, between 1 and 10 glia per nigra; 2, between 10 and 100 glia per nigra; 3, more than 100.

Comparison of clinical and post-mortem data between labelled and unlabelled groups was made using Student’s t-test for unpaired samples, incorporating the Bonferroni correction for multiple groups (three) with 0.016 taken as significant.

The presence of TUNEL labelling was assessed with respect to tissue pH, measured in the lateral cerebellar hemisphere, as described above.

5.4 Results

5.4.1 TUNEL labelling of midbrain cells

In substantia nigra bright red, chromogen-positive labelling was seen in cells in the nigral region of the midbrain in all three subject groups. In melanised neurons chromogen was clearly located to nuclei and was clearly distinguishable from neuromelanin (Figure 5.1 D, E) and occasional TUNEL-positive spherical bodies were observed close to the nuclear margin (Figure 5.1E). These were assumed to indicate the formation of apoptotic bodies containing chromatin fragments and portions of the nuclear envelope (Kerr 1972). Condensed and irregularly-shaped chromogen-positive nuclei were thought to be the result of
Figure 5.1  
TUNEL labelling in rat ileum and human nigra

A:  TUNEL labelling of nuclei in flash-frozen rat ileum, luminal surface at the top, showing TUNEL-positive nuclei in the apical regions of villi (arrows). Scale bar 150 μm.

B:  High-power field from A; scale bar 80μm.

C:  Absence of TUNEL labelling in sections labelled in the absence of terminal deoxynucleotide transferase (Tdt) enzyme. Scale bar 150μm.

D:  Melanised neurons from PD substantia nigra following TUNEL histology and counterstaining with haematoxylin. TUNEL-positive nuclei are visible as red against brown neuromelanin. Neuronal cytoplasm did not appear to be stained. Unlabelled glial nuclei are also present. Scale bar 12 μm

E:  Melanised neurons from control substantia nigra following TUNEL histology and counterstaining with haematoxylin. TUNEL labelling is seen in both neuronal and glial nuclei and small round bodies close to the nuclear margin in both (arrowed) suggest the presence of apoptotic bodies. Scale bar 12 μm
clumping of nuclear chromatin, a morphological indicator of apoptosis, since normal nuclei in melanised neurons were commonly ovoid with regular margins. Extra-cellular neuromelanin was commonly encountered in the nigra in the PD and MSA cases examined, sometimes accompanied by fragments of TUNEL-positive material. Labelled glial nuclei were also to be found in all three subject groups but were particularly numerous in two out of the three MSA cases and in the ILBD case. TUNEL-positive, apoptotic-like bodies, were more common in glia than in neurons (Figure 5.1E).

5.4.2 Quantitation of TUNEL labelling

The incidence and distribution of TUNEL labelling in human midbrain is summarised in Table 5.2. In the control group, TUNEL-positive neurons were found in substantia nigra of 6 out of 14 cases and positive glia in 9 out of 14 cases, although in rather low numbers. The percentage of positive neurons in control nigra was between 0 and 10%, with a mean value of 1% for all cases. In the PD group, labelling of the nigra was more common and positive neurons were found in 10 out of 16 cases. The percentage of labelled neurons in PD cases was between 0 and 12%, with a mean of 2%. Positive glia were seen in 11 out of 16 PD cases. TUNEL-positive neurons and glia were found in 3 out of 4 MSA cases and in the ILBD case; only glia were labelled in the single case of PSP. The percentage of labelled neurons in this last group was between 0 and 19%, mean 7.65%.

Presence of TUNEL labelling was accompanied by morphological change in
nigral neuronal nuclei in 4 out of 6 control cases and 7 out of 10 PD cases. These included clumping of chromatin, irregular nuclear forms and presence of apoptotic-like bodies (Figure 5.1 D, E).

5.4.3 Extra-nigral labelling

Other regions of the midbrain and brain stem were also labelled. In the control series, labelling was found in neurons of the pons and the red nucleus and in glia of the extra-nigral mesencephalon, the superior colliculus, the pons and red nucleus. In PD cases, neurons of the pons and the superior colliculus and glia of the pons and peri-aqueductal grey matter were labelled. Labelling of neuronal and glial nuclei was seen in the red nucleus and mesencephalon of one MSA case.

5.4.4 TUNEL labelling and brain pH

The incidence of TUNEL positive cells in midbrain tissue sections was assessed with respect to brain tissue pH (see above, Chapter 2). Mean tissue pH was 6.38 for all subjects (from 5.41 to 6.89); mean values were not significantly different between subject groups (Student’s t-test). The occurrence of TUNEL labelling in control nigral neurons was strongly related to the individual tissue pH. In the control group, TUNEL labelling of neurons occurred only in tissue with pH lower than 6.38. Mean pH values for the control group with labelled neurons was significantly lower (6.28) than that without labelled neurons (6.55, p = .004, Student’s t-test.) Occasional labelled glia were found at higher values (see Table 2), although the proportion of these was very small (one or two per nigra).
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**TUNEL-positive nuclei in substantia nigra and brainstem.**

Occurrence of labelled neurons (% of total) and glia in substantia nigra and brain stem nuclei: n, neurons; g, glia. PMD, post-mortem delay (hours); ILBD, incidental Lewy body disease; MSA, multiple system atrophy; PSP, progressive supranuclear palsy. Semi-quantitative values for density of glial labelling in the nigra were derived as described in Methods (para 5.3.4). pH values for tissue were measured in the lateral cerebellum as described in Chapter 2 (para 2.3.1).

Mean pH values: IPD, 6.46; other MD, 6.13; controls, 6.39. Mean post-mortem delay values: IPD, 15.9 hours; other MD, 21.5 hours; controls, 27.1 hours.
However, there was no significant difference in mean pH with respect to labelled glia ($p = .077$); mean values were 6.36 and 6.59 for labelled and unlabelled nigra respectively. Labelling of other midbrain regions in controls was associated with low pH values. Occasional positive nuclei were seen in melanised cells in the mesencephalon and in neurons of the pons and colliculus.

In the PD group, there was no clear relationship between pH value and labelling of substantia nigra neurons or glia. Labelled neurons were found at all pH values. In 5 cases labelled neurons were seen in tissue with pH values above 6.4; in one case no TUNEL signal was detected in tissue with a pH value of 6.3. In common with the controls, however, labelling of non-nigral regions in PD midbrain and brainstem sections was detected only at tissue pH values below 6.35.

5.4.5 Post-mortem delay

There was no significant difference (Student’s $t$-test) between mean post-mortem delay of labelled and unlabelled nigra with respect to neurons or glia in either the control or PD group (see Table 2). In the PD group, there was also no significant difference in mean L-DOPA dosage or disease duration between labelled and unlabelled nigral neurons (Student’s $t$-test).

5.5 Discussion

5.5.1 TUNEL labelling in substantia nigra neurons

TUNEL-positive neurons and glia were detected in substantia nigra neurons in
all three subject groups studied. Our findings are in contrast to those of
Mochizuki (Mochizuki et al, 1996), who found no TUNEL-positive neurons in a
group of controls, although labelling was demonstrated in nigral neurons in PD,
and to Dragunow (Dragunow et al, 1995) and Banati (Banati et al, 1998) who
found no TUNEL labelling of nigral neurons in either PD or control midbrain.
Similarly, Jellinger (Jellinger 2000) found no evidence of TUNEL labelling in a
series of PD, DLBD and control cases. The findings of the present study are,
however, consistent with those of Tompkins who found labelling of melanised
substantia nigra and VTA neurons in control, PD and DLBD subjects, as well as
in nigra from Alzheimer’s disease cases and Alzheimer’s disease with concurrent
PD changes (Tompkins et al, 1997). Olanov and colleagues also found TUNEL
labelling using fluorescent dUTP with parallel chromatin condensation visualised
by the dimeric cyanin dye, YOYO, in both PD and control substantia nigra
(Olanov et al, 1998).

Examination of the occurrence of morphological signs of apoptosis in cells
in this study was limited by the use of light microscopy. Nevertheless, apoptotic-
like, TUNEL-positive spherical bodies, clumping of chromatin or irregularity of
the nuclear outline were seen in neurons and glia in more than half of cases with
TUNEL-positive nuclei in both control and PD groups (see above), although the
presence and absence of apoptotic morphology was not established for each
TUNEL-positive cell. TUNEL labelling of nuclei has been suggested to be a more
sensitive indicator of the apoptotic process than the morphological
changes which accompany its end-point (Heron et al, 1993, Li et al, 1995) but it

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may also be less specific. The TUNEL technique may also label single DNA strand breaks, characteristic of necrosis rather than apoptosis (Charriaut-Marlangue et al., 1995, see above) especially if tissue has been treated with proteinases as part of the experimental protocol (Migheli et al., 1994) (although this was not done in the present study).

Tompkins and colleagues used ISEL/TUNEL to identify neurons with apoptotic morphology and found between 0.3 and 10% of neurons affected in PD (5 cases) and between 7 and 16% in DLBD (7 cases) in comparison with 0.2 to 1.8% in control nigra (3 cases). Angalde and colleagues used electron microscopy to evaluate apoptotic-like changes and found ultrastructural features of apoptosis in 6% of 149 neurons examined in 3 PD cases (Angalde et al., 1997). The proportion of labelled neurons identified in this study is similar for PD cases. Between 0 and 12% (16 cases) of neurons were labelled in TUNEL positive nigra with a mean value for the group (including unlabelled cases) of 2%. The proportion of labelled neurons in control cases between 0 and 10% (14 cases) was higher, however, than previous studies, although the mean value for the group (1%) is comparable with that recorded in Tompkins’ study. The reported occurrence of apoptotic-like changes in PD in all is much higher than might be predicted for the disease, given the presumed slow rate of decline in nigral neuronal numbers in DLBD and PD and the short duration of apoptosis (Burke and Kholodilov, 1998). Tompkins and colleagues included both neuronal and non-neuronal figures in their estimate of numbers, giving values between 0.3 and 10% but divided the figures into early and late signs of apoptosis, i.e. cells
in which nuclear changes had become apparent but which were still intact and structures in which apoptotic bodies could be distinguished. The proportion of the total nigral population undergoing early apoptosis was lower, around 0.4 to 0.9%, which would be more consistent with the protracted course of the disease. Nevertheless the authors hypothesised that factors related to the disease process, such as the failure of phagocytic mechanisms, could account for the persistence in tissue of signs of apoptosis, such as TUNEL-positive chromatin, for some time after cell death. In the present study, only whole neurons were counted as positive and non-neuronal apoptotic figures were not included.

5.5.2 TUNEL labelling and post-mortem delay

Consistent with earlier reports, no relationship between post-mortem delay and TUNEL labelling was observed in the present study. There was no relationship observed in the four groups examined in Tomkin’s study between post-mortem delay and apoptotic signs or DNA fragmentation in midbrain melanised neurons (Tompkins et al, 1997). Studies of DNA fragmentation in Alzheimer’s disease have shown similar results. Lassman and colleagues studied DNA labelling in control and Alzheimer’s disease cortex at increasing post-mortem intervals and found no relationship with the appearance of TUNEL labelling in cortical neurons or cortical white matter (Lassmann et al, 1995). Lucassen and colleagues studied DNA fragmentation as an index of neurodegeneration in control and Alzheimer’s disease brain using ISEL (Lucassen et al, 1997). They observed variable labelling of both neurons and glia in control and Alzheimer’s disease temporal cortex and hippocampus but there was no relationship between
the density of labelled cells and the post-mortem interval in either group. These findings support the view that DNA labelling is a marker for ante-mortem changes and reflects damage to living cells.

5.5.3 Labelling of glia

The detection of glial labelling in nigra in this study, is consistent with other reports (Su et al, 1994; Mochizuki et al, 1996; Kosel et al, 1997) of TUNEL-labelled glial nuclei in the substantia nigra in PD and in the striatum in Huntington’s disease (Dragunow et al, 1995). It is possible that apoptosis may occur in glia in both PD and other movement disorders, as a compensatory mechanism to control gliosis occurring in these conditions, as has been proposed in the human brain in HIV infection (Petito et al, 1995) and in rat brain following peripheral nerve injury (Gehrmann et al, 1995).

The presence of labelled neurons in MSA and PSP cases in this study demonstrates that nigral DNA fragmentation is not specific to PD. DNA fragmentation has also been observed in other studies of surviving nigral neurons in MSA (e.g. Banati et al, 1998). However, other studies of MSA, using TdT end labelling have reported that apoptosis was present exclusively in oligodendroglia and its distribution coincided with glial pathology. Additionally, elevated Bcl-2 protein and the presence of Bax immunoreactivity showed that DNA fragmentation occurred with other markers of apoptosis (Probst-Cousin et al, 1998).
5.5.4 Methodological considerations

At least some of the variation seen in post-mortem studies of nigral apoptosis must relate to methodological factors. Long-term exposure to formaldehyde, likely to be the condition of much archival human brain material, may decrease the efficiency of the end-labelling method as has been demonstrated by the decline of TUNEL labelling in human brain material fixed for 3-5 weeks (Davidson et al, 1995). Formalin-fixed human brain may therefore not be ideal for these studies. Archival human ileum examined as a possible positive control for the labelling methods in the present study showed no TUNEL positivity. In contrast, rat ileum, fixed for a limited time (approximately 48 hours) in buffered paraformaldehyde before wax-embedding and sectioning showed strong TUNEL positive signal in luminal cells, as described by Gavrieli (Gavrieli et al, 1992). Wullner and colleagues were unable to detect TUNEL labelling in substantia nigra in studies in which standard incubation times were used for the end-labelling reaction, but demonstrated 2% of neurons labelled in PD and 1% in controls in tissue which had been incubated for prolonged periods (Wullner et al, 1999), suggesting that standard conditions could be sub-optimal for TdT activity. In addition, detection methods which rely on avidin-biotin peroxidase detection with diaminobenzindine may be unsuitable for studies of substantia nigra, given that the colour reaction product may be indistinguishable from neuromelanin in tissue treated to exhaust endogenous peroxidases (Tompkins et al, 1997). The use of flash-frozen tissue in these experiments avoided the possible difficulties associated with prolonged fixation and the colour reaction clearly distinguished reaction product from bleached neuromelanin.
5.5.5 DNA fragmentation and ante-mortem hypoxia

The presence of TUNEL-positive neurons in control substantia nigra and other midbrain regions was associated with pH values below 6.38, i.e. at a level where mRNA degradation might be expected and which indicated severe ante-mortem hypoxia. Extra-nigral TUNEL labelling was associated with low pH in all three subject groups. These findings suggest that ante-mortem hypoxia may influence DNA fragmentation. Studies of experimental ischaemia in animal brain have demonstrated apoptotic-like changes in neurons and glia in hypoxic brain regions (for review see Charriaut-Marlangue et al, 1996). In addition, TUNEL labelling of granule neurons in human cerebellum has been demonstrated to follow transient global ischaemia (Hara et al, 1995). Cellular changes and apoptosis-like cell death may, therefore, also be induced in vulnerable cell populations in human brain by ante-mortem hypoxia and peri-mortem factors may therefore account for some of the discrepancies between the different post-mortem studies cited above. The relatively more frequent labelling of nigral neurons in comparison with other mid-brain neurons may reflect their higher sensitivity to anoxic conditions.

There was no relationship between tissue pH and the incidence of TUNEL-positive neurons or glia in PD, indeed one case with a low pH value was unlabelled. Similarly, among the MSA cases, neurons and glia were labelled at pH values throughout the range. The lack of correlation between TUNEL labelling and pH in PD suggests that DNA fragmentation may indicate apoptotic cell death resulting from the primary disease process in some instances. It is
relevant, however, that recent studies of Pick’s disease show that cell death processes can be prevented by the activation of cell repair mechanisms (Gleckman et al, 1999). Activation of such compensatory mechanisms has also been demonstrated in Alzheimer’s disease cortex, where increased expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x (Smale et al, 1995; Su et al, 1996; Kitamura et al, 1999), the repair enzyme Ref-1 (Anderson et al, 1996; Cotman and Su 1996) and the apoptosis-protecting protein GADD45 (Torp et al, 1998) have been shown in injured hippocampal neurons. Activation of DNA repair mechanisms, such as Ref-1 could theoretically influence the degree of DNA fragmentation seen in individual cases in PD nigra.

5.5.6 DNA fragmentation and cell death mechanisms

It is also possible that DNA fragmentation of itself does not indicate an active apoptotic process. DNA fragmentation might also indicate a process of necrotic cell death and hypoxia and excitotoxicity may activate multiple mechanisms of cell death simultaneously (Portera-Cailliau et al, 1997). Cells rescued from apoptosis, evidenced by DNA fragmentation, by expression of the anti-apoptotic protein Bcl-2 may still go on to die by a necrotic mechanism (Raff 1998). Indeed, an apoptosis-necrosis cell-death continuum has been proposed (Portera Cailliau et al, 1997). In extensive studies of nigral expression of apoptosis-related proteins, Jellinger (2000) reported failure to detect any enhanced expression of apoptosis-related proteins Bcl-2, Bax, P53, Bcl-x or of activated and non-activated caspase 3, a serine protease specific for the death of neurons. Similarly others (Wullner et al, 1998) found no increase in expression of Bcl-2,
Bax and Bcl-x in nigral neurons in PD nor of morphological signs of classical apoptosis (Wullner et al, 1998, Kosel et al, 1996). In the light of these findings, a number of authors have proposed that DNA fragmentation may be a sub-lethal and/or reversible event in neuronal response to cell stress (Lassman et al, 1995) and the presence of DNA fragmentation in PD nigra may indicate merely that these are vulnerable or injured cells, not necessarily undergoing either active apoptosis or necrosis. This conclusion is consistent with the results of the present study where the labelling of neurons in extra-nigral regions in low pH brains and the labelling of nigral neurons in control material suggest that brain stem populations of neurons, in particular the nigra, may be strongly responsive to hypoxic conditions.

Given that ultrastructural analysis of neuronal and glial morphology was not carried out in this study, the precise relationship of DNA fragmentation to final apoptotic cell death could not be determined. It is possible that ultrastructural morphological signs could determine whether true apoptosis was identified in neurons in this analysis or the demonstration of apoptosis related proteins identified by immunohistochemistry. Nevertheless, the influence of severe ante-mortem hypoxia on the occurrence of DNA fragmentation suggests that other markers of apoptosis may be similarly affected, including expression of proteins, and poses a fundamental scientific problem for post-mortem studies of human brain cell death (Jellinger 2000). Despite the absence of a relationship between the proportion of apoptotic neurons in PD and the severity of ante-mortem hypoxia, assessed by pH measurement, other peri-mortem factors may also
influence DNA fragmentation or apoptotic morphology and account in part for
the relatively frequent occurrence of apoptotic markers. The evident effect of
hypoxia on labelling in controls and in extra-nigral midbrain in PD cases
strongly supports this possibility. The absence of DNA fragmentation in one PD
case with low pH may indicate either that other peri-mortem factors may be
involved in the initiation of DNA fragmentation or DNA repair mechanisms
may be active in some individuals.

5.6 Summary

Using the method described here, signs of DNA fragmentation were commonly
identified in the nigra, suggesting that apoptosis may be a common mode of cell
death of dopaminergic neurons. The incidence of DNA fragmentation in PD
and control groups, however, was similar, suggesting that other factors than the
disease process might be responsible for it. The involvement of apoptosis in the
disease process in PD clearly needs to be evaluated by more approaches than the
single method studied here and several studies have examined the occurrence of
morphological signs of apoptosis and the expression of apoptosis-related
proteins in Parkinson’s disease. Nevertheless the results of the present study are
consistent with previous findings and demonstrate that TUNEL labelling is
affected by ante-mortem hypoxia in controls, in other movement disorders and
in extra-nigral regions in PD. Other markers of apoptosis, including
morphological markers, may be similarly affected.

These results indicate that interpretation of the results from human post-mortem
studies of cell death in the nigra may be very difficult and that these should be approached with caution. The importance of matching case-control studies for agonal factors and determining post-mortem markers of agonal status is underlined by the present findings.
Chapter 6:

STUDIES OF ALPHA SYNUCLEIN mRNA EXPRESSION IN PD

6.1 Introduction

6.1.1 Alpha synuclein protein

Synuclein protein was first demonstrated in the electric organ of the ray *Torpedo californica* (Maroteaux et al, 1988) and subsequently characterised in rat (Maroteaux et al, 1988), bovine (Nakajo et al, 1990) and avian (George et al, 1995; Clayton 1997) brain. Alpha synuclein was first identified in human brain as an SDS-insoluble component of Alzheimer disease (AD) plaques (Ueda et al, 1993). This 35 amino acid fragment termed non-amyloid component (NAC) was used to clone and sequence a precursor protein of 140 amino acids - non-amyloid component precursor (NACP), which was shown to be strongly expressed in the cytosolic fraction of homogenates from human cortex and cerebellum. The fragment in AD plaques corresponded to positions 61-96 of the full length protein. Subsequently, antibodies raised to paired helical filaments from AD brain were also used to identify two highly similar human proteins, termed alpha and beta synuclein (Jakes et al, 1994). Alpha synuclein was found to have close sequence homology with previously reported rat sequences (Maroteaux et al, 1998) and the human sequence identified as the NACP of amyloid (Ueda et al, 1993). The brain-specific bovine protein, originally termed phosphoneuroprotein 14-kDa (Nakajo et al, 1990), was similar to the beta synuclein sequence. More recently, additional synuclein sequences, closely similar to the *T. californica* sequence have been demonstrated in rat and human tissue and designated gamma synuclein (Clayton and George 1998).
6.1.2 Expression of alpha synuclein in human brain

All three synucleins are highly expressed in brain, and to a greater degree than in other tissue (Lavedan 1998). For instance, in adult rat brain, beta synuclein is reported to comprise as much as 0.1% of protein in brain homogenates (Shibayama-Imazu et al, 1993). *In situ* hybridization studies of human post-mortem brain demonstrated that alpha synuclein mRNA was strongly expressed in neurons throughout the brain. Abundant hybridization signal was seen in melanised (dopamine) neurons of the substantia nigra pars compacta, the deep layers of cortex, including the entorhinal cortex, in granule cells of the dentate gyrus of the hippocampal formation, hippocampal pyramidal neurons, the intralaminar nucleus of the thalamus, the locus ceruleus and pontine formation and in the granular layer of the cerebellum. Little signal was seen in the caudate and putamen and none in the medial or lateral globus pallidus or the subthalamic nulceus (Solano et al, 2000). In these studies, also, little hybridization signal was seen in white matter, supporting the view that alpha synuclein gene expression was predominantly neuronal.

Alpha synuclein may be strongly expressed during the early development of the brain. In fetal human brain, alpha synuclein immunoreactivity has been demonstrated in neuronal precursor cell bodies throughout the cortex; in contrast, in adult cortex, alpha synuclein immunoreactivity has been reported to be synaptic (Bayer et al, 1999) suggesting that the cellular distribution of alpha synuclein alters during neuronal differentiation from being largely cytosolic to becoming incorporated into synaptic structures.
6.1.3 Cellular distribution of alpha synuclein protein

There is much evidence that alpha synuclein is associated with synaptic membranes and synaptic vesicles. In rat hippocampus and cerebral cortex both alpha synuclein mRNA and protein have been shown to increase during development and, in adult rat, anti NACP immunostaining colocalised with the synaptic protein, synaptophysin in presynaptic terminals, evidence supporting a close association of alpha synuclein with the synapse (Petersen et al, 1999). Studies of cytosolic and particulate fractions from murine brain also showed the same change in subcellular distribution of alpha synuclein (Hsu et al, 1998). Fractionation of adult murine cerebral cortex demonstrated an association of alpha synuclein with vesicles. Cytosolic and vesicle fractions were prepared by centrifugation, following labelling with a biotinylated alpha synuclein probe. Labelled alpha synuclein was distributed mostly in the vesicle fraction, in which it had the same distribution as the synaptic vesicle-associated protein SNAP25, but with a smaller amount in the soluble component. In the synapse, therefore, alpha synuclein is distributed between a soluble and a vesicle-bound pool and is similar in this respect to other vesicle proteins, like SNAP25 and distinct from proteins tightly-associated with the synapse, such as synaptophysin and syntaxin. In rat brain, the expression of synaptophysin increased during development but the ratio alpha synuclein/synaptophysin was higher in immature animals than in adults, indicating that alpha synuclein expression preceded synaptic development (Petersen et al, 1999). Similarly, in differentiating cultures of fetal rat hippocampal neurons, synuclein protein expression was observed in terminal structures several days later than synapsin I, a protein associated with dendritic
or pre-synaptic specialisations and concentrated at presynaptic sites, suggesting that synuclein is not a constitutive protein of synapses and is unlikely to have a role in synaptogenesis (Withers et al, 1996).

Subfractionation of adult human cortical samples (Irizarry et al, 1996) and examination with an antibody to 15 residues (125-140) at the c-terminal end of the protein (i.e., not the moiety which is expressed in AD plaques) demonstrated that alpha synuclein immunoreactivity was highest in the soluble fraction of crude synaptosomes and was also strongly expressed in the crude synaptic vesicle fraction. Lower immunoreactivity was observed in synaptic plasma membrane but was nevertheless present. Using the same antibody against intact tissue, alpha synuclein immunoreactivity was observed in cerebral cortex in a pericellular pattern of neuropil staining and also in the apical dendrites of hippocampal pyramidal neurons, though not in neuronal cell bodies, supporting the findings from subcellular fractionation studies. Taken together, these observations suggest that alpha synuclein is highly expressed during human development, predominantly in fetal neuronal soma but becomes incorporated into terminals as neuronal pathways develop.

In primary cultures of cortical neurons, examined by fluorescence resonance energy transfer (FRET) techniques, transfected alpha synuclein was found to be closely associated with cellular membranes, through both its n- and c-terminal regions (McLean et al, 2000). Early studies of synuclein protein (gamma synuclein) in the electric organ of T. californica indicated that it was located in
pre-synaptic terminals. Subsequently, the pre-synaptic localisation of alpha and beta synucleins was confirmed in a number of species, including humans (for review see Lavedan 1998). Ultrastructural analysis of anti NACP immunostaining in rat brain showed that this was associated with the synaptic vesicles (Petersen et al, 1999). In rat optic nerve (Jensen et al, 1998), alpha synuclein migrated partly in the fast axonal transport component, a characteristic of proteins associated with vesicles. However, more recent studies of cultured neurons indicate that alpha synuclein travels in both fast and slow rate components (Jensen et al, 1999).

### 6.1.4 Structure and function of alpha synuclein

Synuclein proteins are natively unfolded and have a random structure in solution. However, more than half the sequence of the synucleins is taken up by imperfect repeats of the amino acid sequence KTKEGV. There are six of these domains in alpha and gamma synuclein, five in beta synuclein; in all three proteins the third domain (KTKEGV) is identical in all three genes and in all species studied. These repeated motifs in the n-terminal region predict an amphipathic helical structure, with paired lysine residues on either side of the helix marking a polar/apolar interface, a conformation which is typical of class A2 apolipoprotein helices (Segrest et al, 1992). These characteristics indicate that, like the apolipoproteins, the synucleins have a exchangeable lipid-binding capacity. It has been proposed that its structure allows the apolar side of the alpha synuclein molecule to sink into membrane lipids. The exchange between ordered lipid- or protein-bound and unfolded structure suggests that alpha
synuclein could act as a dynamic modulator of protein-protein or protein-lipid interactions.

Alpha-synuclein has been shown to bind to synthetic vesicles containing acidic phospholipids and this binding appears both to increase the degree of helix formation and to stabilise it (Davidson et al, 1998). Vesicle binding activity of alpha synuclein has been shown to depend on the first four KTKEGV repeats. Biotinylated alpha synuclein lacking the first two KTKEGV repeats (amino acids 30-140) or lacking the first four repeats (amino acids 55-140) were incubated with a crude vesicle fraction from murine brain and their distribution examined by centrifugation. Binding of full length alpha synuclein was largely to the vesicle fraction. Binding of alpha synuclein lacking the first two repeats was significantly reduced in the vesicle fraction, with much higher amounts appearing in the soluble fraction; binding of the 55-140 protein was completely abolished.

The third repeat domain KTKEGV, which is common to all three synucleins, is typical of the structure of the rho family of GTP-binding proteins and, although the function of these domains in alpha-synuclein is unknown, indicate that it may be involved in receptor-mediated intra-cellular signalling (Maroteaux et al, 1988). The rho family of proteins activate phospholipase D which catalyses the conversion of phosphatidylcholine to phosphatidic acid. Phosphatidic acid is thought to be involved in the generation of secretory vesicles (Frohman et al, 1996) and is an important component of the second messenger intracellular
signalling cascade, giving rise to diacylglycerol. Diacylglycerol is thought to potentiate neurotransmission by increasing the size of the readily releasable pool of neurotransmitter via the activation of protein kinase C (Frohman et al, 1996, Jenco et al, 1998). The two isoforms of phospholipase D are differentially regulated; PLD1 is activated by the rho family of proteins, ADP ribosylation factors (ARF) and protein kinase C and has low catalytic activity in brain extracts in the absence of these factors. By contrast, PLD2 has high catalytic activity in brain extracts and appears insensitive to activation factors for PLD1, but is strongly and selectively inhibited by alpha- and beta-synuclein in vitro (Jenco et al, 1998). This inhibition is modified by the presence of phospholipids, suggesting that inhibition of PLD2 may occur at the membrane surface.

Alpha synuclein shares sequence homology with the 14-3-3 proteins (Ostrerova et al, 1999), a class of chaperone proteins which interact with a number of molecules involved in receptor-mediated intra-cellular signalling. Alpha synuclein is co-precipitated with the 14-3-3 proteins both from brain extracts and from transfected cell lines. It similarly co-precipitates with proteins known to bind to 14-3-3 proteins, including protein kinase Cs (PKC), BAD (a Bcl-2 homologue which regulates cell death) and extra-cellular regulated kinase (ERK). The biochemical properties of the two proteins are not precisely analogous; 14-3-3 proteins bind Raf1, which does not bind to alpha synuclein, both proteins inhibit all three PKC isoforms and both bind to BAD, alpha synuclein associating with non-phosphorylated BAD and 14-3-3 proteins with the phosphorylated epitope. This suggests that binding of these regulatory
proteins to alpha synuclein and 14-3-3 are independent of one another. Nevertheless, the sequence and functional similarities between the two make it likely that alpha synuclein, like the 14-3-3 proteins is also a protein chaperone involved in the regulation of intra-cellular signalling pathways and it has been proposed (Ostrerova et al, 1999) that the synucleins may be part of the 14-3-3 superfamily of proteins.

The role of alpha synuclein in normal human brain function is not understood at present. However, its structure suggests that it is able to form a close association with membranes, and it has been demonstrated in synaptic vesicles. It has structural and functional similarities with the 14-3-3 and the rho family of proteins, both important regulators of the intracellular signalling cascade. Alpha synuclein inhibits the activity of PLD2, another important modulator of intracellular signalling. Taken together, these properties strongly suggest that alpha synuclein is involved in the regulation of cell signalling and the control of synaptic activity. This view is supported by findings in knockout mice (Abeliovich et al, 2000). Abeliovich and colleagues studied alpha synuclein knockout (Syn -/-) mice, with particular attention to the nigra-striatal dopamine system. Syn/- mice developed and behaved normally and showed no disorders of movement. No alteration was seen in the structure of the nigro-striatal projection and numbers of tyrosine hydroxylase-positive midbrain neurons were unchanged between Syn/- and wild-type mice. Dopamine transmission was studied in striatal slices. The time course of Ca2+ -stimulated release of dopamine and its re-uptake into terminals followed a normal pattern, although
striatal dopamine levels in Syn-/- mice was shown to be lower than in wild-type mice. However, expression of alpha synuclein modulated some aspects of dopamine transmission. Paired stimulation of wild-type striatal slices typically demonstrated a delay in recovery of peak dopamine release rates following stimuli, a phenomenon known as paired stimulus depression. Paired stimulus depression was reduced in Syn -/- mice, in which recovery from stimuli was significantly more rapid than in their wild-type siblings; this change demonstrated that expression of alpha synuclein could modulate dopamine release and the authors concluded that alpha synuclein was a negative regulator of dopamine transmission, probably through the action of PLD2 on the generation of both phosphatidic acid and diacylglycerol.

6.1.5 Alpha-synuclein and the neuropathology of PD

The possibility that alpha synuclein might play an important role in neurodegeneration was recognised after it was demonstrated that a peptide fragment of alpha synuclein was a component of amyloid plaques in Alzheimer’s disease (Ueda et al, 1993). Subsequent studies of the expression of alpha synuclein in PD brain showed that alpha synuclein was an important, and perhaps the major, filamentous component of Lewy bodies. Thus, alpha synuclein is important to the pathological processes in sporadic PD in addition to the rarer familial disease (see above, Chapter 1). Alpha synuclein has been identified in nigral Lewy bodies and in dystrophic neurites in neuropathologically-confirmed cases of idiopathic (sporadic) Parkinson’s disease using antibodies to residues 116-131(Spillantini et al, 1997) and to 129-
140 (Mezey et al, 1998) of the alpha synuclein molecule. Nigral Lewy bodies also reacted with an antibody which recognised residues 11-34 of the molecule, indicating that full-length alpha synuclein might be present (Spillantini et al, 1997). Alpha synuclein immunoreactivity was also seen in Lewy bodies in the frontal cortex (cingulate gyrus), as well as in the nigra, in a case of dementia with Lewy bodies (DLB) (Spillantini et al, 1997) and were present in the amygdala in a case of diffuse Lewy body disease (DLBD) (Mezey et al, 1998). Recently, studies of cortical Lewy bodies in PD has shown that these are also immunoreactive for alpha synuclein (Mattila et al, 1998).

6.1.6 Role of alpha synuclein in Lewy body formation

Since the discovery that alpha synuclein is a constituent of Lewy bodies, considerable attention has been focussed on physico-chemical properties which might underlie the formation of neuronal inclusions. Alpha synuclein is the precursor of the non-\(A\beta\) component (NAC) of Alzheimer’s disease amyloid protein and has been reported to co-purify with \(A\beta\) in the SDS-insoluble fraction of AD brain. This moiety of alpha synuclein shares a number of properties with \(A\beta\) protein which may be of relevance to the formation both of senile plaques and Lewy bodies. Alpha synuclein is a natively unfolded protein but in appropriate conditions will form fibrils and high molecular weight aggregates in similar conditions to \(A\beta\) protein and with similar reaction kinetics. NAC is amyloidogenic with the capacity to form beta sheets, and the binding of NAC to \(A\beta\) promotes the aggregation and fibrillation of \(A\beta\) (Hashimoto et al, 1998; Iwai et al, 1995; Jensen et al, 1997; Paik et al, 1998). The formation of fibrils by
alpha synuclein in vitro follows a characteristic nucleation-dependent time-course, consisting of a lag phase and an elongation phase, followed by equilibrium. This process is time and concentration-dependent; aggregated alpha synuclein seeds the process by acting as nuclei. In this respect alpha synuclein fibril formation resembles that of beta-amyloid fibres and paired helical filaments in AD (Conway et al, 2000). The formation of nuclei is thought to be the rate-limiting step also for LB formation (Wood et al, 1999).

The behaviour of the mutant protein offers some insight into the process. The Ala53T mutation of familial PD results in increased fibrillisation rates (Conway et al, 2000). However, the critical concentrations for mutant and wild-type alpha synuclein to form fibrils, are very similar so accelerated formation of mutant fibrils may be due to different nucleation rates (Wood et al, 1999) rather than alterations in the solubility of the protein or increased stability of fibrils. It has been proposed that the Ala53T mutation lowers the activation energy of critical regions of alpha-synuclein to convert from random coil to beta-sheet formation, causing rate-limiting nuclei to form. In support of this hypothesis, seeding of wild-type proteins by the mutant form shortens the lag phase of fibril formation (Conway et al, 2000).

Alpha synuclein mutations might also change its intracellular processing or distribution, resulting in the formation of LB. Studies of the association of wild-type and mutated alpha synuclein with synaptic components of fractionated rat cerebral cortex (see above, Petersen et al, 1999) indicated that alpha synuclein
with the Ala30P mutation was devoid of significant vesicle binding activity. The authors suggested that a major deleterious effect of the A30P mutation might be to prevent alpha synuclein moving in the fast rate component of axonal transport, thus leading to a build-up of protein over time, which in turn may stimulate LB formation (Jensen et al, 1998). In contrast, in cortical primary cultures, transient transfection of neurons with alpha synuclein showed that the protein was widespread in the cell and that the missense mutations had no effect on distribution. The strong association of both n- and c-terminal regions of alpha synuclein with cell membranes demonstrated using FRET techniques were unaffected by the presence of either synuclein mutation (McLean et al, 2000). These data suggest that in intact cells, alpha synuclein interacts very strongly with the membrane. Aggregation of the protein might therefore require dissociation from membranes.

Lewy body formation is the pathological end point of altered synuclein processing both in familial and sporadic cases. The presence of the mis-sense mutations associated with familial PD may dispose the protein to abnormal expression but other mechanisms must be involved in Lewy body formation in idiopathic PD which may be linked to other cellular abnormalities in the sporadic form of the disease. For instance, aggregation of alpha synuclein was induced by incubation in the presence of cytochrome C/hydrogen peroxide or with hemin and hydrogen peroxide and this aggregation was blocked by antioxidants or iron chelators respectively (Hashimoto et al, 1999 a,b). This suggests a possible mechanism for formation of aggregates, i.e. an iron-catalysed reaction
mediated by cytochrome C (which also appears to be incorporated into LB) and hydrogen peroxide, which might be linked both to oxidative stress and mitochondrial dysfunction in dopamine neurons.

Recently, a novel protein which interacts with alpha synuclein in vivo and is colocalised with alpha synuclein in cultured neurons was identified, termed synphilin-1. When this protein was co-transfected with portions of alpha-synuclein, eosinophilic cytoplasmic inclusions, resembling LB were formed in transfected cells. It is possible that synphilin-1 may modulate alpha synuclein interactions in the cell and may be involved in alpha synuclein aggregation in PD (Engelender et al, 1999).

6.1.7 Alpha synuclein expression in PD brain

In addition to the in situ hybridization studies of alpha synuclein mRNA distribution in human brain (described above), alpha synuclein mRNA expression in human brain was studied using a ribonuclease protection assay (Neystat et al, 2000). This demonstrated the presence of two splice variants of the gene, corresponding to transcripts SYN112 and SYN140, predicted by the studies of Ueda and colleagues (Ueda et al, 1993). There was no significant difference in the relative abundance of these two transcripts in the substantia nigra of control brains, although SYN112 was relatively more abundant in homogenates from frontal cortex. In PD cases alpha synuclein mRNA was reduced in homogenates of the substantia nigra to about 15-20% of the control value; no corresponding alteration of mRNA expression was seen in frontal
cortex. To control for cell loss in PD substantia nigra, mRNA values were standardised to the expression of vesicular monoamine transporter 2 (VMAT2), a marker for monoaminergic neurons but the reduction in alpha synuclein mRNA expression was not affected by adjustment for cell loss. The authors concluded that the reduction of nigral alpha synuclein mRNA represented reduced transcription at the cellular level and did not result simply from loss of dopaminergic nigral neurons.

6.2 Study rationale and design

The aim of these experiments was to examine the regional and cellular distribution of alpha synuclein mRNA in normal human brain using *in situ* hybridization. Semi-quantitative comparisons of alpha synuclein mRNA expression in PD and control cases were made in regions of predilection for Lewy body deposition in PD, the substantia nigra and the temporal and frontal regions of the cortex, to test the possibility that Lewy body formation might be associated with relative over-expression of the alpha synuclein gene.

6.3 Materials and methods

6.3.1 Alpha synuclein probe and ISHH

A 30-base oligonucleotide probe, TGGGAGCAAGATTTTCTTAGGCTTCAGG, complementary to bases 43-72 of exon 6 of the human alpha synuclein sequence (Genbank database accession number U46901) was used for these studies. Sequence homology with other genes was examined using the Genbank basic local alignment search
tool (BLAST) programme (Altschul et al, 1990). The probe sequence was homologous only with published human alpha synuclein sequences and had a 90% homology with the murine alpha synuclein sequence. This probe was not examined by northern analysis.

ISHH was carried out as described above. Specificity of hybridization signal was assessed in parallel hybridizations carried out in the presence of a one hundred-fold excess of unlabelled probe; neuronal hybridization signal was not significantly above tissue background in these hybridizations. Parallel hybridizations were set up for each group in the cortical and nigral hybridization sets.

6.3.2 Examination of alpha synuclein mRNA distribution

Using control tissue, alpha synuclein mRNA expression was examined in representative cryostat sections from basal ganglia, including the midbrain (substantia nigra) and striatum, in the temporal and frontal cortex and locus ceruleus, all regions affected by pathological change in PD. Midbrain sections were prepared as described in Chapter 3. In addition, occipital cortex and cerebellar folia were also hybridized.

6.3.3. Quantitative studies of substantia nigra

Substantia nigra alpha synuclein mRNA expression was examined in 8 PD and 4 control cases. Clinical and post-mortem details are shown in Table 6.1. Duplicate sections were hybridized to compensate for the variable loss of
neurons in PD nigra. The nigra was identified on midbrain tissue sections under low power and neurons from the delineated region were examined using the criteria and protocols described in Chapter 3. Care was taken to exclude neurons of the A10 or A8 regions. Neuromelanin was used as a marker for dopaminergic phenotype and only melanised neurons were assayed. Measurements were made over non-melanised cytoplasm. A total of 25 neurons, in a minimum of 5 separate fields, were examined in control cases on each of duplicate sections (total 50 cells). The largest number possible were examined in PD tissue sections (between 8 and 35 neurons per slide).

6.3.4. Quantitative studies of cortex

Cortical expression was examined in temporal cortex (parahippocampal gyrus) and frontal cortex (cingulate gyrus) in neurons from the deep layers of cortex (layers V and VI) where the majority of LB are found in these regions. Sections were cut from the temporal cortex at the level of the hippocampal formation to include parahippocampal, medial and superior temporal gyrus. Frontal cortex sections were cut at the level of the fornix and included the trunk of the corpus callosum, the cingulate gyrus and superior frontal gyrus. Neurons in the superior temporal gyrus and superior frontal gyrus respectively, were assayed to test the specificity of possible changes. For cortical studies, 6 PD cases and 5 control cases were used. Frontal cortex was examined in 3 control cases and all 5 PD cases; temporal cortex was examined in 4 control cases and all PD cases.
Table 6.1
Clinical details of cases examined for alpha synuclein expression

<table>
<thead>
<tr>
<th>Region</th>
<th>Cases</th>
<th>Sex</th>
<th>Age (years)</th>
<th>pm delay (hours)</th>
<th>tissue pH (pH)</th>
<th>duration (years)</th>
<th>L-DOPA (mg/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>7 PD</td>
<td>5F/2M</td>
<td>77.1 (72-82)</td>
<td>23.2 (9.3-56)</td>
<td>6.44 (6.23-6.63)</td>
<td>21.1 (9-40)</td>
<td>383 (200-500)</td>
</tr>
<tr>
<td></td>
<td>4 CON</td>
<td>3F/1M</td>
<td>73.8 (53-86)</td>
<td>33 (22-53)</td>
<td>6.5 (6.25-6.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>8 PD</td>
<td>4F/4M</td>
<td>76.4 (67-84)</td>
<td>24.4 (10.6-40)</td>
<td>6.5 (6.31-6.76)</td>
<td>11.1 (7-21)</td>
<td>472 (100-1100)</td>
</tr>
<tr>
<td></td>
<td>4 CON</td>
<td>3F/1M</td>
<td>73.8 (53-86)</td>
<td>33 (22-53)</td>
<td>6.5 (6.25-6.65)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Clinical variables for case-control groups. There were no significant differences between control and PD groups for any variable (Student’s *t*-test). The difference in disease duration between cortex and nigra groups in PD cases is not significant (*p* = 0.07)
(see Table 6.1 for details). Identification of neurons for assay was carried out systematically, using the same approach as for nigral neurons. The region to be examined, cortical layers V and VI, close to the white matter border, was identified under low power using anatomical markers and outlined on the slide. Neurons were assayed under a x40 objective, moving this in one direction along the long axis of the region. All neurons encountered were assayed, up to 30 neurons per case.

6.4 Results

6.4.1. Distribution of mRNA signal in brain

Alpha synuclein mRNA was strongly expressed in neurons of the pontine reticulum and to a lesser degree in melanised neurons of the locus ceruleus. Hybridization signal was not observed in the medulla or inferior olive. Melanised neurons of the substantia nigra expressed alpha synuclein mRNA strongly (Figure 6.1A, B) but no other labelled populations were seen in the midbrain except a few non-melanised neurons in the substantia nigra. In the cerebellum, neurons of the granular layer were lightly labelled; no labelling was seen in Purkinje neurons or in neurons of the deep cerebellar nuclei. Neurons of the frontal cortex, in the cingulate gyrus (Figure 6.1 C) and the superior temporal gyrus expressed alpha synuclein mRNA, the deep layer neurons being the more heavily labelled in both regions. In temporal cortex, neurons of the parahippocampal gyrus were positive for mRNA signal, again with deep neurons expressing the strongest signal, whereas neurons of the superior temporal gyrus appeared unlabelled. In hippocampus, hybridization signal was seen over
Figure 6.1
Alpha synuclein hybridization in nigra and cortex

A: Alpha synuclein mRNA expression in control substantia nigra, showing hybridization in both melanised and non-melanised neurons. Scale bar, 25 µm.

B: Alpha synuclein mRNA expression in melanised neurons in PD nigra. Scale bar, 25 µm.

C: Alpha synuclein mRNA expression in neurons of the cingulate gyrus. Scale bar, 25 µm.
granule neurons of the dentate gyrus, in hippocampal pyramidal neurons and in neurons of the subiculum. Hippocampal CA neurons were also labelled, CA 4 neurons only lightly, CA1 neurons most strongly.

In striatal sections, no labelling was seen in putaminal neurons but occasional neurons of the caudate nucleus were lightly labelled. Light hybridization signal was also seen in large cholinergic neurons of the basal forebrain, in fusiform neurons of the clastrum and in neurons of the medial medullary lamina. No labelling was seen in glia in any of these regions.

6.4.2 Quantitative analysis of substantia nigra

In controls the grain density value per cell varied between 0.054 and 0.165. In PD cases, grain density per cell was between 0.032 and 0.092. The mean value for control and PD groups was 0.098 and 0.049 respectively; these values were significantly different (p<0.005). In control nigra the background value was 0.062; 73% of cells were above this value. In PD cases, the background value was 0.072; only 20% of neurons were above this value. Pooled data histograms (Figure 6.2A) confirmed the reduction in hybridization signal in PD; grain density values were normally distributed, those in the PD group were strongly shifted to the left and were closer to background values. The cumulative frequency distribution (Figure 6.2B) in control and PD groups was also significantly different (p<0.001. Kolmogorov-Smirnov test).

The grain density value per cell was used to assess the effect of disease duration
Grain density frequency histograms (A) and cumulative frequency curves (B) of hybridization signal in melanised substantia nigra neurons (pooled data from 234 PD and 200 control neurons). Grain density values were between 0.005 and 0.215 in controls and 0.005 and 0.149 in IPD cases (see text). Analysis of cumulative frequency curves shows that the difference between control and PD values is significant, p < 0.001 (Kolmogorov-Smirnov test).
Clinical variables and nigral alpha synuclein mRNA expression

A.

Grain density values per cell and disease duration; there was a negative correlation between disease duration and alpha synuclein mRNA expression (R = 0.7)

B. Grain density values per cell and L-DOPA dosage (mg/24 hours) recorded in the final months of life. There appeared to be no relationship between L-DOPA dosage and mRNA expression (R = 0.4)
and L-DOPA dosage (see Figure 6.3). There was a small negative effect of
disease duration on alpha synuclein expression in melanised neurons ($r = 0.7$);
no effect of L-DOPA dosage levels could be distinguished.

**6.4.3 Quantitative analysis of temporal cortex**

Grain density values per cell in neurons of the parahippocampal gyrus were
between 0.055 and 0.22. The mean values for the control and PD groups were
similar, 0.145 and 0.132 respectively. The background value for the
parahippocampal gyrus, measured over cortical neurons hybridized in the
presence of a 100-fold excess of unlabelled probe, was 0.013. The majority of
neurons (> 90%) in control and PD cases were above this value. Frequency
histogram and cumulative frequencies derived from pooled data are shown in
Figure 6.4. Grain density values in the parahippocampal gyrus were normally
distributed. The grain density value histogram from PD cases was slightly
shifted to the left but this reduction did not reach significance ($p>0.05$
Kolmogorov-Smirnov test).

In the superior temporal gyrus neurons grain density values per cell were
between 0.01 and 0.17 in controls and 0.02 and 0.14 in PD cases. Grain density
frequency histograms (not shown) were strongly shifted to the left in both
groups, indicating that neurons were lightly labelled. The background value in
the superior temporal gyrus was 0.009. Around 18% of control neurons were
below this value, with a slightly higher proportion in PD cases (19%). The PD
cases had slightly fewer labelled cells than the controls; the cumulative
frequency of grain density in PD cases was significantly different from controls (p< 0.05 Kolmogorov-Smirnov test).

6.4.4 Quantitative analysis of frontal cortex

Grain density values per cell in the cingulate gyrus were between 0.044 and 0.203 in controls and 0.06 and 0.203 in PD cases. The background value for cingulate gyrus was 0.027; 95% of control and PD neurons were above this value. The mean value for control and PD groups were 0.142 and 0.112 respectively. Grain density values did not appear to be normally distributed in cingulate gyrus neurons (Figure 6.5A) and frequency histograms showed that in PD cases there was a decline in hybridization signal with fewer higher-expressing neurons present. The difference in cumulative frequency between the two groups (Figure 6.5B) was significant (p> 0.05 Kolmogorov-Smirnov test).

A similar pattern of expression was observed in the superior frontal gyrus. Grain density values per cell were between 0.072 and 0.224 in control cases and 0.047 and 0.244 in PD cases. The background value for superior frontal gyrus was 0.035. More than 95% of neurons in both groups were above this value. Grain density values did not appear to be normally distributed in these neurons and there was a reduction in the numbers of higher-expressing neurons seen in the PD cases, as in cingulate gyrus (Figure 6.6A). Analysis of cumulative frequency histograms (Figure 6.6B) showed that this difference was significant p< 0.05, Kolmogorov-Smirnov test).
Grain density frequency histograms (A) and cumulative frequency curves (B) of hybridization signal in deep neurons of the parahippocampal gyrus (pooled data from 203 PD and 89 control neurons. Grain density values were between 0.01 and 0.352 in controls and 0.014 and 0.299 in IPD cases. Values per cell and values for groups are shown in the text. Analysis of cumulative frequency curves showed that there was no significant difference between PD and control values (p > 0.05, Kolmogorov-Smirnov test).
Grain density frequency histograms (A) and cumulative frequency curves (B) of hybridization signal in deep neurons of the cingulate gyrus (pooled data from 210 PD and 90 control neurons). Grain density values were between 0.005 and 0.310 in controls and 0.015 and 0.312 in IPD cases. Values per cell and values for groups are shown in the text. Analysis of cumulative frequency curves showed that difference between PD and control values was significant, (p < 0.001, Kolmogorov-Smirnov test).
Figure 6.6
Alpha synuclein mRNA expression in superior frontal gyrus

A.

Grain density frequency histograms (A) and cumulative frequency curves from hybridization signal in deep neurons of the superior frontal gyrus (pooled data from 210 PD and 90 control neurons. Grain density values were between 0.027 and 0.310 in controls and 0.013 and 0.314 in IPD cases. Values per cell and values for groups are shown in the text. Analysis of cumulative frequency curves showed that the difference between PD and control values was significant, (p < 0.001 Kolmogorov-Smirnov test).
6.5 Discussion

6.5.1 Distribution of alpha synuclein mRNA

Alpha synuclein mRNA was widely expressed in the brain, and hybridization signal was observed both in cortical regions in which Lewy deposition is common in PD and in those which do not appear to be affected by the disease (occipital cortex). The results of this study are highly consistent with those of Solano and colleagues (Solano et al, 2000), where distribution of hybridization signal in the basal ganglia was strongest in melanised neurons of the substantia nigra, but with little or no labelling in the striatum. Except for scattered neurons in the caudate nucleus and in adjacent structures such as the claustrum and basal forebrain, the same pattern of expression was obtained in the present study. In contrast to Solano’s study, however, some labelled non-melanised neurons were observed in the substantia nigra. \textit{In situ} hybridization studies of tyrosine hydroxylase mRNA expression in nigral neurons (see above, Chapter 3) showed that a proportion of these non-melanised neurons were TH positive and therefore catecholaminergic. Their numbers were low in the substantia nigra (0-12%) which may explain the difference between the present and the earlier \textit{in situ} hybridization findings and it is possible that alpha synuclein expression in the midbrain may be confined to dopaminergic neurons, as has been suggested, although combined labelling with TH and alpha synuclein would be necessary to confirm this. In the hippocampus, the same distribution of mRNA signal was found in this study as previously reported, the highest signal being observed in CA3 neurons and the lowest in CA 4 with strong signal also seen in granule cells of the dentate gyrus. Alpha synuclein mRNA was also expressed in neurons of
the subiculum and parahippocampal gyrus, consistent with previous findings (Solano et al, 2000).

Expression of alpha synuclein mRNA was found to be exclusively neuronal in the conditions used in this study. No signal was observed in glia in any of the regions examined, although low levels of mRNA transcription cannot be ruled out. It appeared though, that alpha synuclein mRNA was widely and variably expressed in different neuronal populations and brain regions. The non-normal distribution of grain density values seen in the frontal cortex seems likely to reflect the heterogeneity of the cortical neuronal population, in contrast to the normal distribution of values in nigral neurons. The finding of strong expression in cortical neurons is congruent with the findings of Irizarry and colleagues, who demonstrated immunostaining consistent with the presence of alpha synuclein in the apical dendrites of cortical neurons (Irizarry et al, 1996).

6.5.2 Alteration in alpha synuclein mRNA expression

In PD cases, alpha synuclein mRNA expression was reduced in some brain regions susceptible to neuropathological changes. In melanised substantia nigra neurons, alpha synuclein mRNA expression was reduced by about 50% at the cellular level and confirms the decline of total nigral mRNA reported by Neystat and colleagues (Neystat et al, 1999). These authors standardised their RNA quantitation to expression of VMAT2, a marker for dopamine neurons and concluded that reduced mRNA expression was due to a decline in transcription in individual dopamine neurons rather than to nigral cell loss in PD. The results
of the present study support this conclusion. However, the change measured here by *in situ* hybridization is much smaller than that measured by the earlier study, which employed RNAase protection assays. This difference may result from the different assay protocols, or from different sampling methods; Neystat and colleagues measured homogenates of punch biopsy samples from substantia nigra, rather than sampling neurons from an entire cross-section. It is also possible that some differences might be due to ante-mortem degradation of mRNA in the earlier study, in which groups were not matched for tissue pH.

In contrast to the findings of the RNAase protection studies, where no change was seen in cortical samples, alpha synuclein mRNA expression was lower in frontal cortex in PD cases than in controls in the present study. The higher-expressing cells evident in frontal cortex showed markedly reduced abundance in PD cases indicating that alpha synuclein transcription was downregulated in some neurons. This change was seen both in cingulate gyrus, in which LB are commonly deposited in PD and also in superior frontal gyrus, in which they are not common. The probe used in these studies was designed to recognise both alpha synuclein transcripts which have been reported in human cortex (Ueda et al, 1993, Neystat et al, 1999) so the difference between PD and control brain found in the present study is unlikely to be related to differential expression of these transcripts. It would be of interest, however, to determine whether differential expression occurs in different cortical populations; such a study would require probes which would distinguish between the two.
The difference between this finding and the earlier study (Neystat et al, 1999) in which no change was seen, is likely to be due to sampling methods. In the present study, only neurons from the deep cortical layers were examined, although alpha synuclein mRNA was expressed in more superficial cortical neurons as well. Different levels of neuronal expression in different cortical layers might mask any change in a subgroup of the total population which might then be undetectable in homogenates of cortex.

The parahippocampal gyrus, which is also an area of predilection for LB formation, appeared not be affected in the same way as frontal cortex and no significant difference in cumulative frequency values was found in parahippocampal gyrus neurons. There was a small shift to the left in the PD curve, suggesting that a larger number of PD neurons expressed lower levels of this mRNA but this change did not reach statistical significance. The small but significant change seen in PD neurons in the superior temporal gyrus suggests that there is some downregulation of alpha synuclein expression in this region but the numbers of labelled neurons were very small and this finding is therefore difficult to interpret.

The significance of the reduction in alpha synuclein mRNA expression in nigral and cortical populations is not clear. If alpha synuclein plays a critical role in intracellular signalling, then it is likely that this function is affected in nigral and cortical neurons in which alpha synuclein mRNA expression is altered. The findings from the knock-out mice study (Abeliovich et al, 2000) raise the
possibility that downregulation of alpha synuclein expression might serve to increase dopamine transmission in nigral and some cortical populations but it is also likely that reduced expression of alpha synuclein in nigral neurons reflects on-going degeneration of this population. Hybridization signal was not measured over obviously degenerating neurons but it is possible that some neurons were, nevertheless, moribund. The finding that alpha synuclein mRNA expression was negatively correlated with disease duration would support this view. However, the reduction in alpha synuclein mRNA expression in cortical neurons, which have not been shown to degenerate in PD, indicates that the change seen in substantia nigra may also be the consequence of some other, more widespread, process.

6.5.3  Alpha synuclein mRNA expression and neurodegeneration

The presence of alpha synuclein mRNA was not a marker for Lewy body formation and hybridization signal was seen in this study in regions of the brain not thought to be associated with Lewy body pathology or cell loss in Parkinson’s disease. The cerebellum and occipital cortex both had labelled neurons. In addition, the down-regulation of mRNA expression in neurons of the frontal cortex, a region prone to Lewy body formation, indicate that the presence of these inclusion bodies is not related to high levels of alpha synuclein peptide, although quantitative studies of alpha synuclein peptide expression would be necessary to clarify this issue.

Studies of cultured neurons suggest that over-expression of human alpha
synuclein protein may be cytotoxic. Ostrerova and colleagues (Ostrerova et al, 1999) demonstrated increased rates of alpha synuclein expression in serum-deprived human embryonic kidney (HEK) cells, and suggested that alpha synuclein expression might be up-regulated by cell stress. Increased morbidity, measured as reduced expression of the activity of transfected luciferase, was demonstrated in serum-deprived HEK and SK-N-SH human neuroblastoma cells transfected with wild type, A53T and A30P human alpha synuclein gene. Trypan blue staining of cells confirmed increased rates of cell death in the presence of transfected alpha synuclein, the A53T mutation being more toxic than the wild type. Wild-type alpha synuclein also increased the toxicity of BAD protein, a homologue of Bcl2 which regulates cell death. Neither mutant form had this effect but the finding nevertheless suggests that the cell death pathway involved an association between BAD and alpha synuclein. Similarly, transfected alpha synuclein has been shown to reduce survival of cultured mouse nodose ganglion (sensory) neurons and superior cervical sympathetic ganglion neurons (Saha et al, 2000). However, apoptosis induced in developing nigral neurons by injury to their striatal target was not associated with increased alpha synuclein expression (Kholodilov et al, 1999) and in these studies alpha synuclein mRNA and protein expression were associated with normal neurons, rather than apoptotic cells. In the studies presented here, brain regions vulnerable to pathological change showed a decline in alpha synuclein abundance, which was correlated with disease duration in substantia nigra, suggesting that over-expression of alpha synuclein is not a factor in cell death or Lewy body formation in PD.
The findings of Neystat and colleagues demonstrated that in nigral dopaminergic neurons, reduction of alpha synuclein mRNA appeared to precede reduction of VMAT2 expression, leading the authors to suggest that downregulation of alpha synuclein mRNA expression might be an early event in nigral pathophysiology in PD (Neystat et al, 1999). Although studies from this laboratory and its collaborators have shown that VMAT2 and dopamine transporter are both reduced in surviving nigral neurons in PD (Harrington et al, 1996), the results presented here would support this conclusion and also raise the possibility that a widespread change in alpha synuclein transcription may be an early event in Parkinson's disease. It would be of interest therefore to establish whether brain regions in which LB pathology is not seen nevertheless also express lower levels of alpha synuclein mRNA in PD and whether such a change is affected by disease duration.

6.6 Summary

Alpha synuclein mRNA was widely expressed in brain and was seen in regions which appear to be unaffected in PD in addition to those which are prone to pathological change. High levels of alpha synuclein signal were not a marker for LB formation in PD. Alpha synuclein mRNA expression was reduced in nigral neurons in PD and in neurons of the frontal cortex, in cortical layers in which LB are most commonly seen, indicating that over-expression of alpha synuclein is not likely to be a cause of LB formation. In nigral neurons, alpha synuclein hybridization signal declined with duration of PD.
Chapter 7  GENERAL DISCUSSION AND CONCLUSIONS

7.1 Value of human tissue for molecular studies

The studies described above demonstrate the suitability of human post-mortem brain for *in situ* hybridization and other molecular biological techniques. Using the simplified protocols developed for use with flash-frozen tissue, *in situ* hybridization has been used to identify a number of mRNA species in neurons and to make quantitative comparisons between gene expression in neurologically-normal and PD brain. The issue of individual variation, one of the major concerns for such human studies, has been addressed in part by matching case-control studies for ante-mortem hypoxia, using tissue pH as an indicator. This factor appears to be a major determinant of mRNA expression in human post-mortem brain (Harrison et al, 1991; Burke et al, 1991; Barton et al, 1993), while many studies, including those reported here, demonstrate that for many mRNA species, post-mortem delay does not appear to be as important a factor in post-mortem preservation and not a major source of individual variation (e.g. Harrison et al, 1995). Nevertheless, in the absence of a specific assessment for each mRNA examined, case-control series in these studies have been matched closely for post-mortem delay.

The qualitative and quantitative results obtained from ISHH studies using this approach were reliable and consistent. The morphological integrity of tissue processed for ISHH and immunohistochemistry was excellent and the quality of autoradiographic ISHH results consequently very good, enabling reliable
identification of labelled cells and measurement of grain densities. Although the experiments directed towards understanding the storage characteristics of mRNA were only preliminary, nevertheless they indicate that mRNA species studied may be stable post-mortem for periods of many years at low temperatures. The mean grain density per cell in nigral neurons expressing TH, aldolase C, PFKFB3 and NDUFV1 mRNA, all nuclear-encoded species, plotted against months in storage showed no negative correlation with storage time (Figure 2.20, 2.21). Similarly, the integrity of mitochondrially-encoded ND1 mRNA did not appear to be preferentially affected by longer storage times.

Taken together, these findings show that ISHH can be routinely applied to the study of human brain and that reliable quantitative results can be obtained. ISHH is the method of choice for the identification of neuronal phenotype but is also an important approach to studying and comparing cellular activity in neurologically normal brains and in disease. It also seems likely that, as knowledge of the human genome advances and a larger number of candidate genes are identified which may play a role in neurodegenerative disease, ISHH studies will offer one of the best means of studying the distribution and expression of these genes and may provide information vital to understanding their role in pathological processes.

Excellent results were also obtained for immunohistochemistry and TUNEL histology, using rapidly-frozen tissue which had been stored for long periods at -70 °C, demonstrating the value of this tissue for a number of research
approaches. Rapidly frozen human tissue, therefore offers a versatile and stable resource for research into neurodegenerative disease and brain banking of flash-frozen tissue for study of neurodegenerative disease is likely to prove useful for the foreseeable future.

7.2 L-DOPA and nigral neurodegeneration

There appeared to be no relationship between L-DOPA dosage and TH mRNA expression in melanised nigral neurons (Figure 3.3) and, similarly, no correlation was observed in any of the other quantitative studies described above. Expression of mRNA encoding, aldolase C, PFKFB3, NDUFV1 (not shown) and ND1 was unrelated to recorded L-DOPA dosage, indicating that the values obtained for mRNA expression were not likely to be influenced by therapeutic L-DOPA administration. This finding was supported by the absence of an effect of disease duration on mRNA expression, indicating that increased exposure to L-DOPA did not affect mRNA expression for these genes. Only alpha synuclein mRNA expression in the nigra appeared to be correlated with disease duration and showed a decline with increasing duration. Although rather few values were available for regression analysis and the result may not be reliable, it does not appear from these studies that alpha synuclein mRNA expression was influenced by therapeutic L-DOPA administration. It seems likely, however, that alpha synuclein expression is affected by the disease process, given its presence in brain stem LB, and the duration-related decline in mRNA expression may indicate a disease-induced change.
The possibility that L-DOPA therapy might contribute directly to neurodegeneration in PD has recently attracted great interest, not least because of the development of synthetic dopamine agonists which could, theoretically, pre-empt neurotoxicity. L-DOPA therapy is associated with a number of clinical complications, which develop with length of exposure to the drug, approximately half of all patients experiencing them after around five years of treatment (Marsden and Parkes 1977). These complications include fluctuations in the motor response to the drug, dyskinesias, and variable “wearing off” effects at the end of the dose. In addition, some patients experience confusion and hallucinations. It has been observed that motor complications develop very rapidly in humans and primates intoxicated with MPTP but that complications develop more slowly in human subjects with disease of recent onset. Thus it is thought likely that some of the symptoms observed are not solely due to L-DOPA and that the on-going degeneration of the nigra-striatal projection interacts with drug effects to contribute to their development (Obeso et al, 2000).

It has also been proposed, however, that L-DOPA therapy might of itself induce continuing degeneration of nigral neurons and the nigro-striatal projections, through toxic mechanisms, such as increased generation of free radicals from the metabolism of dopamine (Olanov 1993). Experimental evidence for the cytotoxicity of L-DOPA comes chiefly from studies of catecholaminergic cells in culture. L-DOPA has been shown to induce cell death in rat primary mesencephalic cultures (Pardo et al, 1995a; Nakao et al, 1997; Alexander et al,
1997), in PC12 cells (Walkinshaw and Waters 1995; Basma et al, 1995; Kim-Han and Sun 1998) and catecholamine-enriched neuroblastoma cells (Pardo et al, 1995b; Lai et al, 1997). It is generally accepted that L-DOPA toxicity to cultured neural cells is mediated by oxidative stress. In studies of rat mesencephalic neurons the levels of toxic quinones generated by auto-oxidation of dopamine correlated well with the severity of cell death and death could be partially prevented by antioxidants (Pardo et al, 1995), suggesting the involvement of oxidative stress due to exposure to toxic metabolites. The cytotoxicity of L-DOPA to PC12 cells was thought to be due auto-oxidation of L-DOPA itself rather than to toxic metabolites of dopamine, since inhibition of the conversion to dopamine by carbidopa did not prevent cell death (Basma et al, 1995). In contrast, in studies of mesencephalic neurons, L-DOPA was found to be less toxic than dopamine, suggesting that, in this system, L-DOPA-induced cytotoxicity might depend on its conversion to dopamine (Alexander et al, 1997). L-DOPA cytotoxicity in cultures of PC12 cells could be partially prevented by antioxidants such as ascorbic acid (Walkinshaw and Waters 1995), or by expression of the synthetic enzyme for reduced glutathione, glutathione peroxidase (Kim-Han and Sun 1998), and in neuroblastoma cells by ascorbic acid, glutathione and N-acetyl-L-cysteine (Lai and Yu 1997), supporting the view that an oxidative stress mechanism was involved in cell death. Some studies suggested that metabolic activity in cultured cells might also be affected by the generation of reactive oxygen species. L-DOPA was also shown to inhibit mitochondrial complex IV in neuroblastoma cells, an effect which could also be prevented by ascorbic acid (Pardo et al, 1995b), while the complex I inhibitor,
rotenone, enhanced the toxicity of L-DOPA towards mesencephalic neurons (Nakao et al, 1997).

L-DOPA has also been shown to be toxic to cultures of striatal neurons (Cheng et al, 1996; Maeda et al, 1997) in which there is evidence that L-DOPA induces cell death by facilitating glutamate release. The L-DOPA-induced death of cultured rat striatal neurons was blocked by addition of Mg$^{2+}$ to the culture medium or the removal of Ca$^{2+}$, or the application of tetrodotoxin, all of which would inhibit glutamate release. In these cultures, L-DOPA treatment increased glutamate concentration in the culture medium, suggesting that in this neuronal population, the toxicity of L-DOPA was mediated by increased glutamate and excitotoxic mechanisms (Maeda et al, 1997). In contrast, in mesencephalic cultures, L-DOPA toxicity could not be prevented by blockade of NMDA or non-NMDA glutamate receptors, making it unlikely that excitotoxic mechanisms were involved.

It is proposed that in vivo, in human subjects, L-DOPA therapy may cause further degeneration of nigral neurons through an oxidative stress mechanism or of striatal neurons through glutamate-mediated excitotoxicity, acting on neurons already compromised by disease. However, results from in vivo experiments have given less clear-cut results than cell culture studies. L-DOPA has been demonstrated to increase nigral production of hydroxyl radicals in rats in vivo (Smith et al, 1994) and to compromise the survival of dopaminergic midbrain neurons of the ventral tegmental area in rats previously exposed to the
neurotoxin, 6OHDA (Blunt et al, 1993), although substantia nigra neurons were unaffected. One study of normal rats exposed to L-DOPA for a period of six weeks reported a reversible decrease of complex I activity in the nigra and striatum of these animals (Przedborski et al, 1993). In contrast, recent studies of administration of L-DOPA to rats with unilateral 6OHDA lesion of the nigrostriatal projection did not demonstrate any evidence of increased hydroxyl radical formation (Camp et al, 2000) and prolonged treatment of 6OHDA-lesioned rats with L-DOPA did not induce the death of TH-positive cells either in lesioned or sham-operated ventral midbrain (Dziewczapolski et al, 1997), suggesting that in vivo L-DOPA was not toxic either to healthy or compromised neurons.

There is little clinical evidence, moreover, for L-DOPA toxicity in humans (Calne 1992). Clinical studies have shown that large cumulative doses of L-DOPA over protracted periods do not cause de novo parkinsonian symptoms, dyskinesias or nigral cell loss or accelerate existing nigro-striatal disease. One individual who received large doses of L-DOPA over a four years showed no signs of nigral degeneration at autopsy (Quinn et al, 1986) and in another group of people treated with large doses of L-DOPA over long periods (between 9 and 26 years) for non-progressive parkinsonism (one case), essential tremor, 3 cases, dystonia (one case) showed no detectable worsening of the disease process and no development of parkinsonian symptoms. In two subjects from this group, examined at autopsy, no cell loss could be distinguished in the nigra (Rajput et al, 1997). A post-mortem study of complex I activity in putamen in L-DOPA-
treated PD patients showed no change in complex I activity or of other enzymes of the mitochondria and respiratory chain (Cooper et al, 1995), in contrast to the findings in experimental animals. It seems possible that in vivo, in human subjects, L-DOPA does not have the same effects as those that can be demonstrated in culture and that its potential to generate oxidative stress may be modulated by other factors. For instance, it has been suggested that nigral glia may prevent the development of toxic effects on the nigra in vivo. Evidence for this comes from culture studies. Fetal mesencephalic neuronal cultures treated with conditioned medium from cultured mesencephalic glia were protected from the cytotoxic effects of L-DOPA (Mena et al, 1996), particularly by a small molecular size fraction of the medium, which contained free radical scavenger molecules, while a large molecular size fraction, which contained neurotrophic molecules was also effective (Mena et al, 1996, 1997). Glial conditioned medium has also been shown to protect cultured midbrain dopaminergic neurons from the toxic effects of MPP⁺ (Mena et al, 1999). These findings suggest a role for nigral glia in protection of nigral neurons from toxic molecules in vivo and, possibly, in mitigating the effects of disease.

The absence of any correlation between L-DOPA dosage and hybridization signal in the studies described above shows that mRNA expression is not modified by L-DOPA and suggests that it may have no effect on gene expression in the nigra. There was no evidence in these studies to support the view that toxic metabolites of L-DOPA might damage neuronal DNA or RNA in vivo.
7.3 Nigral gene expression

In the quantitative studies described above, cellular expression of several mRNAs were unchanged in PD, in particular, the dopamine synthetic enzyme, TH. This finding is at variance with other ISHH studies of TH mRNA expression in nigral neurons which have demonstrated reduced transcription of TH (Javoy-Agid et al, 1990; Kastner et al, 1993). Other mRNAs have been shown to be reduced in the nigra in PD, including the dopamine transporter (DAT) (Uhl et al, 1994; Harrington et al, 1996; Joyce et al 1997) and neurofilament (Hill et al, 1993). This suggests that important functions of neurons may be compromised by reduced mRNA levels. In the case of TH where close correlation between mRNA and peptide expression in the nigra has been demonstrated (Kastner et al, 1993), reduced expression of mRNA would indicate reduced synthesis of the encoded peptide, with effects on dopamine synthesis and transmission in surviving neurons. It does not appear from the present results, however, that there is any alteration of dopamine synthesis in surviving melanised neurons. The absence of any change also indicates that compensatory regulation of TH in PD does not take place at the level of gene expression. Further, there was no evidence in this study to support the view that up-regulation of dopamine synthesis could contribute to ongoing degeneration in nigral neurons as a response to increased exposure to oxidative stress.

Several other mRNA species concerned with metabolism (aldolase C, PFKFB3 and NDUVF1), or with cell structure (β-tubulin) were unaltered in PD in this study. The findings with respect to the metabolic and structural mRNAs are
congruent with the lack of effect of the disease on TH mRNA and suggest that surviving melanised neurons in PD are capable of normal rates of transcription of some genes and may have some important functions preserved. If these neurons are not moribund, then pharmacological intervention to support them may prove to be an effective therapeutic strategy.

7.4 Up-regulation of TH mRNA expression in non-melanised neurons
A significant increase in TH mRNA expression was seen in non-melanised neurons, suggesting that compensatory up-regulation of TH expression, and potentially dopamine transmission, could take place at the level of gene expression in this sub-population. This finding is supported by that of a later study which demonstrated a larger increase in TH mRNA expression in non-melanised neurons of the VTA, which are more numerous than those of the nigra (Tong et al, 2000). The proportions of non-melanised to melanised neurons in the nigra and VTA were similar in PD and control midbrain, indicating that non-melanised neurons are equally affected by the disease. Nevertheless, altered gene expression in VTA neurons may represent compensatory changes which underlie the greater resistance of this region to neurodegeneration and the late appearance or absence of cognitive changes in PD.

7.5 Nigral neuronal heterogeneity and patterns of neuronal loss
The striking heterogeneity of mRNA expression observed in these studies in nigral neurons is intriguing. This phenomenon was seen in control as well as PD
nigra and was not, therefore, a feature of the disease and may have physiological significance. The studies of Damier and colleagues (Damier et al, 1999a) have shown that midbrain calbindin immunoreactivity can be used to delineate nigral subregions in control and PD nigra in a manner independent of the disposition of nigral neurons. Nigral neurons of the ventral midbrain were classified depending upon whether they were embedded in the matrix of calbindin-positive fibres or whether they were located in calbindin-poor regions (nigrosomes). Five separate nigrosomes were identified, orientated approximately rostro-caudally (Damier et al, 1999a). The organisation of these calbindin subregions was preserved in the nigra in PD, enabling quantitative studies of nigral cell loss to be related to an anatomical framework which could be reliably identified, despite the loss of TH immunoreactivity. Five PD cases were studied using this approach (Damier et al, 1999b). There was an overall loss of around 64% of dopamine neurons in the midbrain. Cell loss was maximal in the substantia nigra with high variation in the degree of loss between midbrain populations e.g. the VTA, as has been shown in other studies (e.g. Fearnley and Lees 1991; Gibb and Lees 1991). Within the calbindin-defined nigral compartments, greater loss of neurons was observed in the nigrosomes than the matrix. The different nigrosomal subregions also showed differing rates of loss, which were reproducible from one case to another and showed a clear relationship to disease duration. No gradient was observed in the nigrosomes and neuronal loss was similar in each nigrosome at all rostrocaudal levels. In contrast, cell loss in the matrix followed a caudo-rostral gradient, as has been described. The overall pattern of cell death in the nigra showed that loss was greater in the ventral region than the dorsal or
medial regions and greater in this region in the nigrosomes than in the matrix. These findings strongly suggest the existence in the ventral nigral region of different neuronal compartments, which are differentially vulnerable to PD, perhaps with different patterns of gene expression able to modulate the response of neuronal sub-populations to the disease (Damier et al, 1999b).

The findings from this study, that TH mRNA expression is unchanged in nigral neurons in PD and that ND1 mRNA expression is reduced are in contrast to findings from other studies which have reported a decrease (Javoy-Agid et al, 1990; Kastner et al, 1993) or an increase (Joyce et al, 1997) in TH mRNA expression and an increase in ND1 mRNA expression (Ruberg et al, 1997). This variability between studies is likely to be due at least in part to technical factors, such as individual variation or the size of the sample group. The other studies cited here did not match case-control groups for tissue pH, and thus for individual variability in mRNA preservation and in some instances, only small numbers of cases were examined. In the carefully-matched, large series of individuals examined here, nigral TH mRNA expression in the population as a whole was comparable in control and PD neurons. However, it is still possible that changes in mRNA expression may be localised to sub-populations of nigral neurons, especially in the light of the findings of Damier and colleagues. Differential changes of expression midbrain sub-populations of neurons could possibly account for some of the variability between the results of different studies.
In the light of the heterogeneity of expression of TH, ND1 and alpha synuclein mRNA observed in the present study, it would be of interest to examine mRNA expression in control nigra, using the pattern calbindin-positive innervation to identify individual sub-populations, to determine whether subregional variations of mRNA expression could be demonstrated. If neurons in the matrix and nigrosome compartments have different phenotypes, than this might prove a powerful approach to the study of the genes involved in nigral cell death (Damier et al, 1999a).

7.6 Nigral metabolic activity and PD

Altered activity of respiratory chain and mitochondrial enzymes seems to be intimately linked with a number of neurodegenerative diseases in addition to PD. Deficits of mitochondrially-encoded complex IV (cytochrome oxidase) and alpha-ketoglutarate dehydrogenase, a key Kreb’s cycle enzyme have been demonstrated in pathologically-affected regions of cortex in Alzheimer’s disease (Gibson et al, 1998) and are also seen in Friedreich’s ataxia (Sheu and Blass, 1999). Alpha-ketoglutarate dehydrogenase activity is also compromised in frontal cortex in progressive supranuclear palsy (Albers et al, 2000). In vivo, abnormalities of glucose utilization have been demonstrated, using PET, in individuals with Alzheimer’s dementia.

Compromise of metabolic function and reduced ATP synthesis may initiate neurodegenerative changes by a number of mechanisms. Cybrid cells, formed by fusion of mtDNA from Alzheimer’s disease platelets with cells depleted of
mitochondrial DNA show transmitted deficits of cytochrome oxidase with accompanying compromise of Ca$^{2+}$ buffering and reduced uptake of Ca$^{2+}$ into mitochondria. Since the activity of mitochondria is strongly linked to calcium fluxes, this may further affect mitochondrial function. These cybrid cells also had increased levels of free radicals and signs of oxidative stress, including increased malondialdehyde and protein carbonyl levels. Thus altered Ca$^{2+}$ regulation and increased exposure to toxic molecules result from the deficit in complex IV and lead to neuronal degeneration (Sheehan et al, 1997; Swerdlow et al, 1997). In cultured chick retina neurons, inhibitors of oxidative phosphorylation result in a disruption of the resting potential of the cells, leading to lifting of the Mg$^{2+}$ blockade of glutamate receptors and persistent activation of these receptors, allowing glutamate to become toxic. These changes could be prevented by antagonist blockade of NMDA receptors (Flint Beal 1992). The deficit in complex I activity in nigral neurons in PD, may therefore render them susceptible to changes similar to those described in Alzheimer’s disease and in experimental studies, i.e. the generation of toxic reactive oxygen species, further deficits in mitochondrial function and possible excitotoxicity from the glutamatergic innervation of the nigra.

Studies of threshold effects of inhibition of mitochondrial ATP synthesis in isolated, non-synaptic mitochondria, using rotenone to inhibit mitochondrial complex I, showed that the threshold at which energy metabolism became compromised was at 72% inhibition of complex I activity. As inhibition of complex I activity increased up to 72%, ATP synthesis and oxygen consumption
remained at close to normal levels in but fell rapidly thereafter (Davey and Clark 1996). Complexes III and IV had similar threshold values of 70% and 60% respectively. The results of the study implied that threshold effects would permit non-synaptic mitochondria to maintain near-normal levels of oxidative phosphorylation, despite significant inhibition of respiratory chain enzymes. In synaptic mitochondria, however, the threshold at which ATP synthesis was reduced by inhibition of complex I was 25%, a much smaller value than that observed in non-synaptic mitochondria while threshold values for complexes III and IV were 80% and 70% (Davey et al, 1998). This finding showed that in the synapse, complex I played an important role in determining the rate of oxidative phosphorylation. The authors commented that the reported decrease in mitochondrial complex I activity in PD would extrapolate to a damaging inhibition of ATP synthesis in the synapse (Davey et al, 1998). Although cultured astrocytes are able to survive anaerobic conditions using energy derived from glycolysis, it was thought unlikely that neuronal glycolysis would be able to compensate for loss of mitochondrial ATP synthesis. In the studies described here, expression of ND1 mRNA was reduced in the cell body by around 25%. If ND1 enzyme activity is closely related to ND1 transcription and this reduction is consistent throughout the cell, then the change would represent a more serious metabolic deficit in synapses and one large enough to compromise energy production while the cell body might remain close to normal respiratory rates. There is no evidence of compromised complex I activity in dopamine terminals in the striatum; normal complex I activities have been observed in the caudate nucleus (Schapira et al, 1990b) and in striatal homogenates (Cooper et al, 1995)
in PD but it is possible that dendritic terminals in the nigra might be affected.

Glycolysis is essential to the survival of brain tissue in conditions of metabolic stress. Study of electrical activity in rat hippocampal slices in anaerobic conditions showed that in the absence of glycolysis, ion transport and recovery from hypoxia were compromised (Roberts 1993). The anoxia tolerance of some fish and amphibian species is based on the ability of the brain to reduce transmission and to mobilise brain glycogen stores in order to generate ATP and maintain ion gradients (Lutz et al, 1996). In guinea pig hippocampal slices electrical activity in the granule cell layer fell following glucose deprivation and was extinguished after 30 minutes. Replacement of glucose with mannose or fructose resulted in recovery of electrical activity after a period of about 20 minutes. If lactate or pyruvate were present, then recovery proceeded rapidly and electrical activity was maintained. There was also no reduction of ATP in the presence of these substrates, showing that glycolysis was essential for maintaining the neural activity of tissue (Yamane et al, 2000). Similar results have been obtained with rat brain synaptosomes which indicate that glycolytic ATP synthesis may have an important role to play in maintaining neural cells, although its relationship to ion permeability is complex. In rat vesicle preparations in anoxic conditions, basal lactate production and intracellular Ca\textsuperscript{2+} and Na\textsuperscript{+} increased, while ATP content fell rapidly. This effect could be potentiated by veratridine, which increased the influx of sodium. Nevertheless, glycolysis was sufficient to cover energy demand and maintain normal levels of ATP in vesicles in which had been preincubated in Na\textsuperscript{+} channel blockers (Gleitz
et al, 1996). In rat synaptosomal preparations in anoxic conditions, lactate synthesis rose to cover the energetic demand and was able to generate 80% of the ATP synthesised in the presence of oxygen. In synaptosomes stimulated by veratridine, anaerobic glycolysis was reduced in a concentration-dependent manner. This inhibitory effect could be blocked by tetrodotoxin indicating that Na+ influx inhibits anaerobic generation of ATP and that ATP generation by glycolysis is probably under physiological control.

In primary cultures of rat astrocytes in anoxic conditions, intracellular glucose levels fell rapidly, in parallel with a decrease in glycogen stores, showing that ATP generation by glycolytic pathways was essential for cell survival under these conditions. This was supported by the finding that the replacement of glucose with 2-deoxyglucose resulted in a fall of ATP and cell death. Anaerobic generation of ATP was dependent on mobilization of glycogen stores and could be diminished by addition of cycloheximide, suggesting that de novo protein synthesis and gene expression were involved. This was confirmed by northern analysis which demonstrated that induction of genes for aldolase and for D-hexose-6-phosphotransferase took place in hypoxic cultures (Niitsu et al, 1999).

It is possible, therefore, that de novo synthesis of glycolytic enzymes might take place in neurons exposed to a deficit in ATP synthesis through inhibition of mitochondrial energy production, although it seems unlikely that this might be sufficient to protect the cell against long-term compromise of mitochondrial respiration. However, no change was seen in the expression of aldolase C or
PKF3 mRNAs in nigral neurons, showing that induction of these genes did not take place in nigral neurons in PD. Unlike other neurons of the brain stem examined in the present studies, nigral melanised neurons expressed relatively low levels of aldolase C mRNA and PKF3 mRNA, which remained unaltered in the PD cases studied. It is possible that the higher rates of mRNA expression for aldolase C, observed in other neuronal populations, e.g. pontine neurons, represent a greater capacity for glycolysis, which is not shared by the nigral population. It is tempting to speculate that energy metabolism in nigral neurons is unlike that of other large neurons, represented by the very high levels of expression of the ND1 subunit of complex I and the very low levels of aldolase C and PKF3 mRNA and that the metabolic characteristics of nigral neurons may underlie their vulnerability to disease process in PD.

There is some evidence that dopaminergic nigral neurons have intrinsic metabolic differences from other neuronal types. Marey-Semper and colleagues studied the effects of rotenone on dopamine, GABA and 5HT uptake in mouse synaptosomes and mesencephalic cultures as an index of energy metabolism, since impairment of ATP production would disrupt membrane gradients. Dopamine uptake was more sensitive to the effects of rotenone in both synaptosomes and mesencephalic cells than 5HT or GABA. In synaptosomes derived from the nucleus accumbens, dopamine uptake had a lower sensitivity to rotenone than synaptosomes from the dorsal striatum, suggesting a constitutive difference in energy metabolism in striatonigral neurons, compared to neurons of the VTA. The authors observed that MPP+ destroys only a fraction of the
neural cells on which it acts and that its selective toxicity for the nigrostriatal pathway cannot be accounted for entirely by specificity of uptake into striatal terminals and may be due to a constitutive metabolic deficit in these neurons (Marey-Semper et al, 1993). A number of possible explanations were put forward to explain this difference: i) mitochondrial oxidative phosphorylation in nigral neurons was weaker than in other populations, for which evidence could be provided by the lower levels of staining for cytochrome oxidase in the nigrostriatal pathway, ii) that the energetic demand of dopamine neurons is higher than others or iii) that auto-oxidation of dopamine could impose an increased energy demand in nigral neurons. In the studies presented here substantia nigra neurons appeared to be much more sensitive to terminal hypoxia than, other neurons of the brain stem. In the studies of TUNEL histology and apoptotic signs in the nigra described above (Chapter 5) the incidence of fragmented DNA in nigral neurons was higher than that observed in other neuronal groups in either control or PD midbrain, in cases which had low tissue pH. In a subsequent study of cerebellar tissue, from cases with severe ante-mortem hypoxia and low tissue pH, no signs of DNA fragmentation were observed in either granule of Purkinje neurons (unpublished observations). It seems possible then, that some intrinsic metabolic characteristic of these neurons renders them selectively sensitive to anoxia or inhibition of metabolism by neurotoxins, perhaps resulting in increased exposure to oxidative stress and consequent neurodegeneration.

7.7 Alpha-synuclein and the induction of apoptosis

The role played by alpha synuclein or abnormal forms of this protein in the
formation of LB and in neurodegeneration in PD is not clear, but alpha synuclein has been shown to be toxic when over-expressed in some neuronal cultures. Micro-injection of human alpha synuclein into cultured mouse sensory neurons (nodose ganglion) substantially reduced (around 80% at 24 hours) survival of these neurons in culture, while γ-synuclein had no effect. A similar effect was noted on superior cervical sympathetic ganglion neurons in culture. When examined by fluorescent TUNEL labelling, many cells expressed strong nuclear fluorescence, suggesting that the mode of cell death induced by expression of alpha synuclein was apoptotic (Saha et al, 2000). Similar results were obtained with the mutant forms (A53T and A30P) of the human gene. Cell death could not be prevented in these cultures by expression of Akt/PKB, a constituent of the PI3 kinase pathway, which is concerned with the transduction of survival signals, although Akt/PKB is known to promote cell survival by inhibiting the release of cytochrome C from mitochondria and preventing alterations in the mitochondrial membrane potential (Kennedy et al, 1999). In contrast, apoptotic cell death could be prevented by expression of the anti-apoptotic protein BclX₁. This suggested that synuclein-induced apoptosis was mediated by the apoptotic cascade downstream from the PI3 kinase pathway and cytochrome C release and through interaction with proteins of the Bcl family. It seems possible also, that mitochondrial changes may be critical to the apoptotic effects of synuclein in cultured neurons, since these can be prevented by factors which are thought to be downstream from the release of cytochrome C. Alpha synuclein has been reported to interact with BAD, a pro-apoptotic member of the Bcl2 family of proteins and may function as a chaperone protein for PKC and BAD (Ostrerova
et al, 1999) and the two appear to be regulated together. Thus, the binding of these two proteins is inversely correlated; in cell culture or tissue preparations, agents which stimulate PKC activity reduce the formation of the BAD-synuclein complex. In nodose ganglion neurons, expression of the pro-apoptotic genes Bax and BAD both caused cell death, but this effect was potentiated by co-expression of these two genes with alpha synuclein (Saha et al, 2000). The association between alpha synuclein and BAD, therefore, may be integral to the functioning of the apoptotic pathways described in cultured neurons.

7.8 Alpha synuclein and proteasome activity

In a study of an alpha synuclein-inducible cell line, proteasome activity and mitochondrial membrane were both modified by alpha synuclein. PC12 cells were induced to express either wild-type or A30P alpha synuclein, using a tetracycllin-inducible gene expression system. In cells induced to express either form of the gene by the presence of doxycyclin, proteasome activity was decreased but greater reduction was seen in cells expressing the mutation. In the presence of the proteasome inhibitor, lactacystin, cells induced to express either form underwent apoptotic cell death, demonstrated by nuclear staining but, again, the effect was greater in the population expressing the A30P mutation and caspase 9 activation was also increased in cells expressing the mutant protein, in comparison with the un-induced baseline measures. Mitochondrial depolarisation was examined using a carbocyanin dye which responds to mitochondrial membrane potential. There was no difference in membrane potential between mutant and wild-type cultures but in lactacystin-treated
cultures depolarisation of the mitochondrial membrane was demonstrated, with a greater effect in cultures expressing the mutant form. These findings indicated that alpha synuclein when over-expressed in PC12 cells was able to inhibit proteasomal function and increased the sensitivity of these cells to apoptotic cell death (Tanaka et al, 2001), especially in the presence of the A30P mutation, which was able to activate caspase 9. The mechanism by which alpha synuclein was able to inhibit the proteasome is not clear but it has been reported recently that alpha synuclein interacts with a regulatory sub-unit of the proteasome (Ghee et al, 2000). In addition, cells expressing mutant alpha synuclein showed a selective increase in apoptotic cell death, associated with mitochondrial depolarisation. This could be partly prevented by cyclosporin A, an inhibitor of mitochondrial permeability transition. Apoptotic cell death induced by mutant alpha synuclein has also been demonstrated in rat primary mesencephalic cultures and rat dopaminergic cell lines (Zhou et al, 2000) and alpha synuclein has also been shown to promote mitochondrial deficit and oxidative stress in a neuronal cell line (Hsu et al, 2000). Thus, there appear to be a number of cellular mechanisms whereby alpha synuclein expression, particularly of mutant forms associated with the development of familial PD, could be involved in inducing mitochondria-dependent, apoptotic cell death in neurons. In addition, the possible failure of protein degradation in the formation of LB, suggests that the relationship between alpha synuclein and proteasomal function may be an important factor in the pathophysiology of PD.
7.9 Downregulation of alpha synuclein mRNA

The finding of these studies, that alpha synuclein expression was greatly reduced in nigral neurons was therefore, surprising. It is possible that the cells encountered and analysed in these experiments were in a terminal condition and that reduced alpha synuclein mRNA expression reflected this. In support of this possibility, alpha synuclein mRNA expression was negatively correlated with disease duration, suggesting an ongoing neuronal degeneration involving alpha synuclein. Nevertheless, evidence for the involvement of alpha synuclein in induction of apoptotic cell death comes from experimental studies of cultured cells and there is little evidence to clarify whether the pathways which have been described in culture operate in vivo in intact systems. For instance, the findings of Kholodilov and colleagues indicate that up-regulation of alpha synuclein is associated with neuronal survival in a rat target injury model of apoptotic cell death in nigral (Kholodilov et al, 1999). In this model, alpha synuclein was up-regulated and exclusively expressed in normal, as compared to apoptotic, neurons. It seems possible therefore that alpha synuclein expression may also be associated with normal neuronal function in nigral neurons and reduced levels of alpha synuclein may be deleterious to nigral neurons.

It is also possible that the down-regulation of alpha synuclein is a reflection of reduced protein synthesis in response to loss of striatal dendrites and it has been suggested that nigral neurodegeneration may begin in the striatum (Damier et al, 1999b) and that the degenerative process could affect nigral neurons retrogradely. Oxidative stress induced by dopamine and excitotoxicity from
glutamate input are likely to have their effects in terminal fields and cell death signals in PD could originate at remote synapses. Cortical synaptosomes from rat brain have been demonstrated to respond to staurosporin and oxidative insults induced by Fe^{2+} by loss of membrane symmetry, increase in caspase activation and increase in mitochondrial Ca^{2+} uptake. In synaptic mitochondria, the concentration of reactive oxygen species was also increased and changes in mitochondrial membrane potential were demonstrated (Mattson et al, 1998). These results showed that a number of markers of the apoptotic pathway could be demonstrated in synaptosomes exposed to apoptotic or oxidative stress. In addition, synaptosomes were shown to generate soluble factors which induced apoptosis in PC12 cells, as shown by changes in nuclear morphology, visualised with propidium iodide. Similar effects were obtained in the dendrites of hippocampal neurons in culture exposed to apoptotic stimuli, including the accumulation of activated caspase 3 and changes in mitochondrial membrane polarisation (Mattson et al, 1998). Thus apoptosis-related processes have been shown to occur locally in synapses and dendrites of neurons independently of the nucleus, suggesting that it may be possible for apoptotic loss of synapses to occur in the nigro-striatal pathways, resulting in a reduced demand for synaptic proteins and perhaps, reduced synthesis of alpha synuclein. It would therefore be of interest to determine whether mRNA encoding other synaptic proteins, or proteins associated with axonal transport are reduced in nigral neurons in PD.

7.10 Is Parkinson’s disease a disease of the substantia nigra?

Alpha synuclein expression may be related to disorders of axonal transport
throughout the brain. The studies presented in this thesis have concentrated in
gene expression in nigral neurons, as the most active site of neurodegeneration
in PD. Nevertheless, neuropathological studies of PD have shown that changes
are widespread and that neurodegeneration occurs in many other sites. Alpha
synuclein immunochemistry shows that a number of forebrain sites and, in
particular, the frontal and temporal cortex have a heavy burden of
neurodegeneration, including the formation of Lewy neurites and deposition of
numerous Lewy bodies. These pathological changes must have serious
implications for the functioning of affected brain areas, even in the absence of
extensive cell loss seen in the nigra and locus ceruleus. For instance, studies of
cortical Lewy body numbers in PD has indicated that high numbers of cortical
Lewy bodies are associated with dementia (Mattila et al, 1998). It has therefore
been proposed (Braak and Braak 2000) that PD is a generalised disorder of
axonal transport affecting many regions of the brain and arising from a
widespread degenerative stimulus, which results in neuropathological changes in
numerous regions and massive cell death in the nigra.

In the studies presented here, alterations in gene expression in PD were not
confined to the nigra. Alpha synuclein mRNA expression was decreased in the
nigra but also in the frontal cortex, indicating that a similar degenerative process
was involved in the two populations. In frontal cortex, both cingulate gyrus and
superior frontal gyrus were affected although the cingulate gyrus is a region
prone to LB formation while few LB are seen in the superior frontal gyrus. This
suggests that if downregulation of alpha synuclein mRNA is a sign of
degeneration, then this is not confined to regions in which widespread neuropathological change is seen. Similarly, mitochondrially-encoded ND1 mRNA expression was reduced in the nigra but alteration of this mRNA was also seen in Purkinje neurons of the cerebellum. In PD the increase in complex I mRNA expression was paralleled by change in aldolase C mRNA expression, strongly suggesting that metabolic activity in these neurons was increased by the disease process. Recent collaborative studies of this laboratory have also shown reduced activity of a Kreb’s cycle enzyme, alpha-ketoglutarate dehydrogenase, in the cerebellum in PD (Gibson et al, submitted for publication). These findings show that change in metabolic enzyme activity is not confined to the nigra, although reduction of complex I activity may be specific to this nucleus. The sum of what is known about the neuropathology and biochemistry of PD demonstrates the global nature of this disorder and suggests that the striking loss of cells from the nigra and locus ceruleus is only part of a wider pattern of devastation of the brain and may not be the primary focus of the disease.

7.11 Conclusions
In spite of the variation observed between individuals in many studies of brain gene expression, human post-mortem brain is an invaluable tool for such studies and measurement of brain pH offers a simple means to match groups for post-mortem studies. Flash-freezing of brain tissue, rather than formalin fixation, preserves its structure and permits application of in situ hybridization and other histological protocols.

No change was seen in surviving melanised neurons in PD nigra for a number of important genes, in particular mRNA encoding TH was unaffected, indicating that dopamine synthesis in these neurons was unaltered in PD. Nevertheless,
upregulation of TH mRNA in non-melanised neurons suggests that some compensatory changes may take place at the levels of gene expression. Alpha synuclein mRNA expression and that of the mitochondrially-encoded ND1 sub-unit of complex I were both reduced in melanised nigral neurons in PD. This suggests that the deficit in complex I activity observed in nigral homogenates in PD may result in part from altered mitochondrial gene transcription. No effect of disease duration on transcription was observed here, suggesting that a change in mitochondrial DNA might be involved, as is suggested by studies of cybrid cell lines. The absence of a change in expression of the NDUFV1 subunit implies that nuclear-encoded mitochondrial subunits may be unaffected but a much larger study would be necessary to determine whether reduced expression is specific to mitochondrial complex I or the ND1 subunit. Patterns of metabolic gene expression may be differently regulated melanised nigral neurons, which expressed lower levels of glycolytic enzymes, perhaps lowering their threshold for metabolic insult. A potential metabolic deficit has been proposed to explain the selective vulnerability of this population and would be consistent with the finding that melanised neurons were prone undergo to DNA fragmentation in hypoxic conditions.

Downregulation of alpha synuclein gene transcription was observed in the nigra in PD in this study, implying that over-expression of this gene was unlikely to be the cause either of nigra neuronal death or the formation of LB. This change was not specific to the nigra; reduced expression of alpha synuclein mRNA was also demonstrated in frontal cortex, consistent with the view that PD is a global brain disease, perhaps associated with alteration of axonal transport or synaptic activity and not one focussed on the substantia nigra.

7.1.2 Suggestions for future studies

An important issue raised by the findings in this study is whether downregulation of
alpha synuclein mRNA is an early event in PD and what its relationship is to neurodegeneration. Semi-quantitative studies of other regions, both those in which neurodegeneration occurs, e.g. basal forebrain and regions thought to be unaffected, such as the occipital cortex, using cases with a range of disease duration, would provide useful information. Parallel studies of alpha synuclein peptide could be carried out to examine the relationship between this and mRNA expression. To address the possibility that downregulation of alpha synuclein mRNA reflects alteration of axonal transport in PD (Braak and Braak, 2000) or destruction of synapses, it would be interesting to study mRNA and peptide expression of genes involved in axonal transport (e.g. tau) and synapse formation both in the nigra and other affected brain regions.

Further insight into the involvement of metabolic factors in the death of nigral neurons could be gained from comparison of mitochondrial and nuclear metabolic gene expression between the substantia nigra and neurons of the VTA, which were not examined in this study. Extension of the studies of ND1 mRNA to other brain regions could establish whether reduced ND1 mRNA expression accompanies neurodegeneration in PD, e.g. in frontal cortex or basal forebrain, or whether it is confined to the substantia nigra, as suggested by the findings on enzyme activity. The cellular basis for lowered ND1 mRNA expression could be explored by examination of other mitochondrially-encoded sub-units of complex I, combined with analysis of mtDNA to determine the copy number in control and PD nigra. These studies might clarify whether reduced ND1 mRNA expression is the result of reduced mitochondrial numbers and a lowered number of templates for transcription or whether it is related to other factors such as mRNA stability.
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Appendix 1

Publications arising from this thesis

1. Papers in refereed journals


Kingsbury AE, Marsden CD, Foster OJF (1999) The vulnerability of nigral neurons to Parkinson’s disease is unrelated to their intrinsic capacity for dopamine synthesis: an in situ hybridization study. Movement Disorders 14 (2) 206-218


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